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Humoral and Cell-Mediated Immune Responses to Alternate Booster Schedules of Anthrax Vaccine Adsorbed in Humans

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Protective antigen (PA)-specific antibody and cell-mediated immune (CMI) responses to annual and alternate booster schedules of anthrax vaccine adsorbed (AVA; BioThrax) were characterized in humans over 43 months. Study participants received 1 of 6 vaccination schedules: a 3-dose intramuscular (IM) priming series (0, 1, and 6 months) with a single booster at 42 months (4-IM); 3-dose IM priming with boosters at 18 and 42 months (5-IM); 3-dose IM priming with boosters at 12, 18, 30, and 42 months (7-IM); the 1970 licensed priming series of 6 doses (0, 0.5, 1, 6, 12, and 18 months) and two annual boosters (30 and 42 months) administered either subcutaneously (SQ) (8-SQ) or IM (8-IM); or saline placebo control at all eight time points. Antibody response profiles included serum anti-PA IgG levels, subclass distributions, avidity, and lethal toxin neutralization activity (TNA). CMI profiles included frequencies of gamma interferon (IFN-γ)- and interleukin 4 (IL-4)-secreting cells and memory B cells (MBCs), lymphocyte stimulation indices (SI), and induction of IFN-γ, IL-2, IL-4, IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) mRNA. All active schedules elicited high-avidity PA-specific IgG, TNA, MBCs, and T cell responses with a mixed Th1-Th2 profile and Th2 dominance. Anti-PA IgG and TNA were highly correlated (e.g., month 7, $r^2 = 0.86$, $P < 0.0001$, $\log_{10}$ transformed) and declined in the absence of boosters. Boosters administered IM generated the highest antibody responses. Increasing time intervals between boosters generated antibody responses that were faster than and superior to those obtained with the final month 42 vaccination. CMI responses to the 3-dose IM priming remained elevated up to 43 months. (This study has been registered at ClinicalTrials.gov under registration no. NCT00119067.)

Anthrax vaccine adsorbed (AVA; BioThrax; Emergent BioSolutions Inc., Lansing, MI) is the only Food and Drug Administration (FDA)-approved vaccine in the United States for prevention of anthrax in humans. The primary immunogen in AVA is anthrax toxin protective antigen (PA). Serum anti-PA antibody levels are accurate immune correlates of protection in nonhuman primate (NHP) models of inhalation anthrax and for predicted probability of survival in humans (1–3). There is a significant lack of data in humans regarding the onset, duration, quantitative analysis, and functional activity of humoral antibody and cell-mediated immunity (CMI) responses following priming and boosting with AVA.

In 2012, the preexposure schedule for AVA was approved as a priming series of three 0.5-ml intramuscular (IM) injections at 0, 1, and 6 months (3-IM) with subsequent boosters at 12 and 18 months and annually thereafter for those at continued risk of infection (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm304758.htm; http://www.fda.gov/downloads/BiologicsBloodVaccines/BloodBloodProducts/ApprovedProducts/LicensedProductsBLAs/UCM074923.pdf). In 2013, AVA received market approval in the European Union (EU) using a 3-IM priming series and 3-yearly booster schedule (http://emergentbiosolutions.com/sites/default/files/BioThrax_Germany.pdf).

These recent changes in the FDA-approved priming schedule and route of administration for AVA and EU approval of an alternate schedule warranted detailed characterization of their immunological impact. Serological noninferiority analyses for peak anti-PA IgG and lethal toxin neutralization activity (TNA) in response to the 3-IM priming schedule and alternative booster schedules were reported previously, and the safety profile of AVA administered IM in humans was confirmed to be similar to that for other alum-containing vaccines (4–6). Less frequent AVA injection doses resulted in a reduction in some injection site adverse events (AEs), and IM administration resulted in reduced frequency, duration, and severity of AEs (5–11). The potential for increasing the intervals between booster doses requires an assessment of sustained antibody functional activity, CMI, and the ability to develop rapid protective anamnestic responses (5, 6, 12).

In a rhesus macaque model of inhalation anthrax, the AVA

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TABLE 1 Human clinical trial vaccination and booster schedules[a]

Group designation[a]	Primary series[b] (mos)	Booster Schedule (mos)	Total no. of AVA doses	Route	Mo 0	Mo 0.5	Mo 1	Mo 6	Mo 12	Mo 18	Mo 30	Mo 42
8-SQ	0, 0.5, 1, 6	12, 18, 30, 42	8	SQ	AVA	AVA	AVA	AVA	AVA	AVA	AVA	AVA
8-IM	0, 0.5, 1, 6	12, 18, 30, 42	8	IM	AVA	AVA	AVA	AVA	AVA	AVA	AVA	AVA
7-IM	0, 1, 6	12, 18, 30, 42	7	IM	AVA	Saline	AVA	AVA	AVA	AVA	AVA	AVA
5-IM	0, 1, 6	18, 42	5	IM	AVA	Saline	AVA	AVA	Saline	AVA	Saline	AVA
4-IM	0, 1, 6	42	4	IM	AVA	Saline	AVA	AVA	Saline	Saline	Saline	AVA
Placebo	NA[c]	NA	0	IM/SQ	Saline	Saline	Saline	Saline	Saline	Saline	Saline	Saline

[a] Reprinted from Vaccine, vol 32, J. G. Wright et al., Effect of reduced dose schedules and intramuscular injection of anthrax vaccine adsorbed on immunological response and safety profile: a randomized trial, p 1019–1028, Copyright 2014, with permission from Elsevier.
[b] Human priming and booster schedule designations based on the approved schedule as of 17 May 2012. The 0, 1, 6 month series constitutes 3-IM priming (http://www.fda.gov/BiologicsBloodVaccines/Vaccines/ApprovedProducts/ucm304758.htm).
[c] NA, not applicable.

3-IM priming series diluted up to 1/10 with no additional boosters provided significant levels of protection (60 to 100%) for up to 4 years after the first vaccination (13). The immunological characteristics of these long-term protective responses in NHPs have been reported previously, and anti-PA IgG was identified as the most accurate immune correlate of protection (COP) (1). Serum antibody levels decline in humans and NHPs in the absence of boosters. However, a COP cross-walk analysis between NHPs and humans receiving only the 3-IM priming series estimated that even the lowest levels of anti-PA IgG provided significant probability of survival in humans (86.8% to 95.8%) in a combined model for two alternate booster schedules (3).

In the present COP substudy of the CDC Anthrax Vaccine Research Program (AVRP) human clinical trial, we conducted the first detailed evaluation in humans of the earliest onset, magnitude, and duration of PA-specific humoral and CMI profiles analogous to those providing long-term protection in NHPs (13). The objectives were to evaluate the potential immunological effects of route of vaccine administration and the feasibility of adopting alternate booster schedules. Characterization of the humoral antibody responses to AVA included serum anti-PA IgG levels and subclass distributions, antibody avidity, and TNA. Assessment of CMI in peripheral blood mononuclear cells (PBMCs) included lymphocyte stimulation indices (SI), frequencies of gamma interferon (IFN-γ)- and interleukin 4 (IL-4)-secreting cells and memory B cells (MBCs), induction of IFN-γ, IL-2, IL-4, IL-6, IL-1β, and tumor necrosis factor alpha (TNF-α) gene transcription (mRNA), and overall Th1-Th2 disposition. The kinetics of anamnestic anti-PA IgG responses to the boosters at months 6 and 42 were determined in a subset of study participants.

MATERIALS AND METHODS

The study design for the CDC AVRP human clinical trial has been reported in detail previously (6). Laboratory methods and statistical analyses of humoral and CMI responses in humans were by design similar or equivalent to those reported previously for the analogous responses in rhesus macaques (13). Modifications for species-specific conditions and reagents are described below.

Human study cohort. A subset of 359 participants (23.0%) of the main AVRP study (n = 1,563) consented separately to participate in the COP substudy. Additional details are provided in the supplemental material. The study was sponsored by the CDC under an Investigational New Drug application and was approved by the human investigations committees at participating clinical sites and at the CDC (www.clinicaltrials.gov, registry number NCT00119067).

Vaccination and control group schedules are as previously described and provided in Table 1 (6). Study participants were randomized into one of six study groups to receive a 3-dose intramuscular (IM) priming schedule (0, 1, and 6 months; 3-IM) with a single booster at 42 months (4-IM); 3-IM priming with boosters at 18 and 42 months (5-IM); 3-IM priming with boosters at 12, 18, 30, and 42 months (7-IM); the 1970 licensed series of 6 doses (0, 0.5, 1, 6, 12, and 18 months) and two annual boosters (30 and 42 months) administered either subcutaneously (SQ) (8-SQ) or IM (8-IM); or saline control at all eight time points. All study participants that were according to protocol (ATP) for immunogenicity received eight injections within specific periods; saline injections were given at time points when AVA was omitted (Table 1). Placebo injections were saline (0.9%, wt/vol, NaCl; Abbott Laboratories, Chicago, IL). All available samples from COP substudy participants ATP were analyzed. Group sizes for COP substudy analyses are provided in Tables S1 and S2 in the supplemental material. All immunological testing was blinded until the end of the study.

Antibody response kinetics. Additional samples for serum antibody kinetics analyses were drawn across all study groups after the month 6, 30, and 42 injections at days 3 to 6, 7 to 10, and/or 11 to 15, respectively. To reduce the burden on study participants, each person was randomized to one of the kinetics periods after each of the relevant injections. The actual number of days postinjection was recorded. Group sizes for kinetics analyses are provided in Tables S3, S4, and S5 in the supplemental material for months 6, 30, and 42, respectively. Groups 4-IM, 5-IM, and 7-IM were combined for the month 6 analyses, as all participants had received the same 3-IM priming series (4, 6). The anti-PA IgG rates of response between treatment groups at each of month 6, month 30, and month 42 for days 4 to 9 after vaccination with AVA were analyzed using weighted regression and analysis of variance (ANOVA) with the significance level set to a P value of <0.05.

Protective antigen and standard reference sera. Recombinant PA (rPA) and lethal factor (rLF) were obtained from BEI Resources (Manassas, VA). The preparation and performance characteristics of human reference standards AVR414 and AVR801 used in the anti-PA total IgG and subclass serology assays are described elsewhere (14).

Anti-PA IgG ELISA. The quantitative anti-PA IgG enzyme-linked immunosorbent assay (ELISA) was performed according to the method of Semenova et al. (15). The assay empirical lower limits of detection (LOD) and quantification (LLOQ) were 1.7 μg/ml and 3.7 μg/ml of anti-PA IgG, respectively (15).

Anti-PA IgG subclass analyses. As reported previously for analogous studies with NHPs, assays for all four IgG subclasses were run when the total anti-PA IgG was ≥5 μg/ml (13). Relative subclass proportions were determined in a sample when the total anti-PA IgG was ≥12.5 μg/ml, all four subclasses were evaluated, and at least one IgG subclass was detected. Detection and quantification of each of the anti-PA IgG subclasses were done in separate assays using anti-human specific monoclonal antibodies for each of the four 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]). The conjugate horseradish peroxidase (HRP)-labeled sheep anti-mouse, gamma chain IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA; 515-035-062), was used for IgG1, and HRP-labeled donkey anti-mouse, gamma chain IgG (Jackson ImmunoResearch Laboratories, Inc.; 715-035-150), was used for IgG2, -3, and -4. The anti-PA IgG subclass concentrations were calculated in nanograms per milliliter by interpolation to an AVR414 calibration curve using a four-parameter logistic-log model (4-PL) and SoftMax Pro software (version 4.3) (16). The LOD for IgG subclasses were 0.019, 0.081, 0.003, and 0.026 μg/ml for IgG1, IgG2, IgG3, and IgG4, respectively.

Anti-PA IgG avidity. Serum samples with ≥5 μg/ml of total anti-PA IgG were evaluated for avidity, an indirect assessment of polyclonal antibody affinity, immune response maturation, and a surrogate for MBC persistence (13, 17). The avidity indices (AIs) 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 percent maximum detected signal versus NH_4SCN concentration. The AI was reported as the concentration of NH_4SCN required to elute 50% of bound anti-PA IgG (13).

Lethal toxin neutralization activity. In addition to quantifying the anti-PA IgG responses by ELISA, samples from all COP substudy participants and a 30% random selection of full-study serum samples (total of 48% of all samples) were evaluated for functional activity using an *in vitro* TNA assay according to the method of Li et al. (6, 18). The murine monocyte/macrophage cell line J774A.1 (TIB-67) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). Reportable values were the reciprocal serum sample dilution effecting 50% neutralization of anthrax lethal toxin (ED_{50}). Endpoints were calculated using SAS version 9.0 (SAS Institute Inc. Cary, NC). The assay LOD and LLOQ using reference serum AVR801 were ED_{50} titers 11 and 36, respectively (6, 18).

Frequencies of IFN-γ- and IL-4-secreting cells. PBMCs were prepared as described previously (19). IFN-γ- and IL-4-producing cells were enumerated by enzyme-linked immunosorbent spot assay (ELISpot) assay following *in vitro* restimulation with rPA (24 h for IFN-γ assays and 36 h for IL-4 assays). Staphylococcal enterotoxin B (SEB) 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 mean rPA-specific IFN-γ or IL-4 SFU/10^6 PBMCs ± standard error of the mean (SE).

Lymphocyte stimulation indices. PBMCs 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 of 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 scintillation counter (Packard, Meriden, CT). SIs were calculated as follows: mean counts per minute of stimulated cells ÷ mean counts per minute of unstimulated cells (13).

Cytokine mRNA level analysis. PA-specific cytokine mRNA induction in PBMCs was evaluated by real-time quantitative reverse transcription-PCR (RT-PCR) using a PE Applied BioSystems Prism 7700 (Applied BioSystems Inc., Foster City, CA). Assays were performed in duplicate and mean results reported. Induction of genes for IFN-γ and IL-2 were interpreted as representing Th1-type responses; induction of IL-4 and IL-6, an important accessory cell cytokine, was interpreted as representing Th2-type responses; and induction of acute-phase cytokines IL-1β and TNF-α was indicative of T cell activation (20). Additional details are provided in the supplemental material.

Frequency of PA-specific memory B cells. A detailed procedure has been described elsewhere (19, 21). Briefly, PBMC were plated in a 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 of CpG ODN-2006, and 1/10,000 *Staphylococcus aureus*, Cowan strain (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; MAHA N4510) 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 1 μg/ml of mouse anti-human pan-Ig Fc biotin-conjugated antibody (Hybridoma Reagent Laboratory, Baldwin, MD). Data were represented as the frequency (mean percent ± SE) of rPA-specific anti-PA IgG-secreting cells versus the total IgG-secreting cells in PBMCs. The LOD was 0.002 antigen-specific IgG-secreting cells per 10^6 PBMCs. Sample sizes were designed to focus on PA-specific B cell frequencies at months 6, 18, and 42 (see Table S2 in the supplemental material).

Statistical analyses. Statistical analyses are described in detail in the supplemental material. To enhance analytical resolution, all reportable values were included in the statistical analysis. All valid results were included in the analysis. No attempt was made to impute missing values. The five active vaccination study groups were analyzed separately except for some analyses as stated when the data for the IM-administered vaccine groups receiving the same schedule were combined (4-IM, 5-IM, and 7-IM up to and including month 12; 4-IM and 5-IM for months 13 and 18 [Table 1]) (4, 6).

For the TNA and anti-PA IgG ELISAs, values less than or equal to the LLOQ were assigned one-half the LLOQ (22). ANOVA models were used to compare the average response within each AVA study group to the saline control group and to evaluate dose-response trends using SAS version 9.3.1 GLM, MIXED, PROBIT, and LOGISTIC procedures. Anti-PA IgG and TNA correlation models were fitted using the SAS version 9.1.3 MIXED procedure. A significance level of 0.05 was used for all analyses, with adjustments for multiple comparisons where necessary.

RESULTS

The anti-PA IgG sex-related immunogenicity, the noninferiority peak response analyses for the AVRP human study participants at months 2, 7, and 43 and the linear regression correlations between anti-PA IgG and TNA at those specific time points have been reported previously and are not addressed in this report (4, 6).

The key points for the present immunological analyses were the onset, magnitude, trough values, and persistence of the anti-PA IgG and TNA, antibody avidity, IgG subclass distribution, antibody response kinetics, and CMI profiles in AVA-vaccinated humans. The sample sizes and ATP status of study participants evaluated for humoral and CMI analyses are provided in Tables S1 and S2, respectively, in the supplemental material.

Anti-PA IgG responses. The individual group anti-PA IgG data are presented in Fig. S1A and Table S6 in the supplemental material. The onset of a statistically significant anti-PA IgG response was detectable at 1 month after the first vaccination and sustained at significant levels for all AVA dose schedules for the duration of the 43-month study.

As previously reported for month 7, 4 weeks after administration of the month 6 dose, the anti-PA IgG levels were statistically noninferior and not significantly different in any of the vaccination groups (range, 196.2 to 241.1 μg/ml) (6). These data indicate that priming was complete and equivalent for both the 3-dose (4-IM, 5-IM, and 7-IM) and 4-dose (8-SQ and 8-IM) priming schedules. Anti-PA IgG levels subsequently declined in all groups,

with no significant differences at month 12 (range, 35.2 to 44.1 μg/ml) until receipt of the next booster (see Table S6). The month 18 levels were the lowest responses for the 5-IM group during the study: 15.1 μg/ml (95% confidence interval [CI], 12.4, 18.4). At month 18, all groups except 4-IM received a booster.

Month 30 anti-PA IgG levels were the lowest (trough) values for annual booster groups 7-IM, 8-IM, and 8-SQ: 7-IM, 30.3 μg/ml (95% CI, 26.4, 34.9); 8-IM, 34.2 μg/ml (95% CI, 30.0, 38.9); and 8-SQ, 28.7 μg/ml (95% CI, 25.4, 32.4). At month 30, all vaccination groups except 4-IM and 5-IM received a booster. Anti-PA IgG levels in all booster groups increased significantly by month 31. Responses were highest in the 8-IM group and were the maximum point estimate for this group during the entire study: 8-IM (359.2 μg/ml [95% CI, 322.4, 400.3]). The other two booster group responses, in order of magnitude, were 309.6 μg/ml (95% CI, 276.8, 346.3) for 7-IM and 250.9 μg/ml (95% CI, 224.0, 281.0) for 8-SQ. The 8-IM group response was statistically superior to the 8-SQ group response but not statistically significantly different from the 7-IM group response. Responses in the unboosted groups remained relatively stable in the same interval; 4-IM month 30, 8.6 μg/ml (95% CI 7.1, 10.4), and month 31, 5.6 μg/ml (95% CI, 4.6, 6.8), and 5-IM month 30, 35.8 μg/ml (95% CI, 30.2, 42.5), and month 31, 33.8 μg/ml (95% CI, 28.5, 40.2).

Month 42 anti-PA IgG levels were the trough values for the 4-IM group that had received no boosters since completion of priming at month 6 (see Table S6 in the supplemental material). All groups received their final booster at month 42, and final responses were determined at month 43 (6). The interval between boosters was 36 months for the 4-IM group, 24 months for the 5-IM group, and 12 months for each of the 7-IM, 8-IM, and 8-SQ groups. As previously reported, the responses at month 43 were significantly higher in all of the IM groups than for the 8-SQ group (219.2 μg/ml [95% CI, 195.3, 246.0]) (6). The 4-IM group, with a 36-month interval in vaccinations, had the highest anti-PA IgG response: 438.6 μg/ml (95% CI, 373.5, 514.9), which was more than twice the response in the comparator 8-SQ group. The other group responses, in order of increasing magnitude, were 298.6 μg/ml (95% CI, 263.1, 339.0) for 7-IM, 314.7 μg/ml (95% CI, 274.1, 361.3) for 5-IM, and 340.7 μg/ml (95% CI, 306.5, 378.8) for 8-IM. Responses were not significantly different between the 5-IM, 7-IM, and 8-IM groups (6). The 4-IM response was significantly (more than 2-fold) higher than those for all other groups and statistically superior. At the completion of the study, there was an anti-PA IgG response hierarchy of 4-IM > 5-IM > 7-IM and 8-IM > 8-SQ; fewer boosters with greater intervals between injections generated higher anti-PA IgG levels.

Lethal toxin neutralization activity. As reported by Wright et al., 48% of all available human serum samples were evaluated for TNA, and the month 7 responses were statistically noninferior between groups (6). Group sizes and changes in TNA titers over time are presented in Table S7 and Fig. S1B in the supplemental material.

Postpriming, the serum TNA declined in all groups, with no significant differences at month 12 (ED_{50} range, 225.0 to 283.9). Study groups receiving a month 12 dose (7-IM, 8-IM, and 8-SQ) demonstrated an anamnestic response at month 13, at which time the response of the 8-IM group was significantly higher than that of the 8-SQ group: 8-IM, ED_{50} = 1,932.7 (95% CI, 1,672.9, 2,232.8); 8-SQ, ED_{50} = 1,355.5 (95% CI, 11,549.6, 1,584.5); and 7-IM, ED_{50} = 1,778.9 (95% CI, 1,510.9, 2,094.4). At the same time point (month 13), the TNA in the nonboosted 4-IM and 5-IM groups had declined to ED_{50}s of 165.5 (95% CI, 125.6, 218.0) and 197.1 (95% CI, 149.5, 259.8), respectively, and continued to decline to ED_{50}s of 88.7 (95% CI, 65.5, 120.0) and 96.3 (95% CI, 69.9, 132.7) by month 18. As with anti-PA IgG, these were the lowest TNA postpriming response levels for the 5-IM group during the study. Responses in the month 12 booster groups also declined significantly by month 18; 7-IM, ED_{50} = 320.4 (95% CI, 248.6, 413.0); 8-IM, ED_{50} = 325.1 (95% CI, 262.7, 402.3); and 8-SQ, ED_{50} = 273.0 (95% CI, 222.5, 334.8). These values were not significantly different from each other but were significantly higher than in the groups that did not receive a booster.

At month 18, all groups except 4-IM received a booster. TNA levels in all booster groups rose significantly by month 19. Responses were highest in the 5-IM group that, to this point in the study, received only one booster with an interval of 12 months; 5-IM, ED_{50} = 1,963.8 (95% CI, 1,625.8, 2,372.0); 7-IM, ED_{50} = 1,343.2 (95% CI, 1,120.8, 1,609.8); 8-IM, ED_{50} = 1,446.5 (95% CI, 1,203.1, 1,739.0); and 8-SQ, ED_{50} = 1,137.0 (95% CI, 945.6, 1,367.0). Among the groups that received a booster, the 5-IM group responses were significantly higher than those for 8-SQ and 7-IM.

Month 30 TNA levels were the trough values for annual booster groups 7-IM, 8-IM, and 8-SQ; 7-IM, ED_{50} = 201.0 (95% CI, 158.8, 254.5); 8-IM, ED_{50} = 206.9 (95% CI, 171.2, 250.2); and 8-SQ, ED_{50} = 171.7 (95% CI, 140.0, 210.6). At month 30, all groups except 4-IM and 5-IM received a booster. TNA levels in all booster groups increased significantly by month 31. Responses were highest in the 8-IM group and were the maximum point estimate for this group during the entire study: ED_{50} = 1,743.5 (95% CI, [1,446.7, 2,101.2]). The other two booster group responses, in order of magnitude, were an ED_{50} of 1,116.9 (95% CI, 936.2, 1,332.6) for the 8-SQ group and an ED_{50} of 1,544.4 (95% CI, 1,257.5, 1,896.7) for the 7-IM group. The value for the 8-IM group was significantly higher than for the 8-SQ group. Responses in the unboosted groups remained relatively stable in the same interval, with only small declines; 4-IM month 30, ED_{50} = 56.2 (95% CI, 40.0, 79.0), and month 31, ED_{50} = 52.4 (95% CI, 38.5, 71.5); 5-IM month 30, ED_{50} = 300.4 (95% CI, 242.8, 371.6), and month 31, ED_{50} = 207.4 (95% CI, 155.2, 277.0).

Month 42 TNA levels were the trough values for the 4-IM group that had received no boosters since completion of priming at month 6. In this group at this time point, 81.0% of participants (57/70) had quantifiable TNA (≥LLOQ), with an $ED50_{50}$ of 36.35 (95% CI, 27.1, 48.8). The other group responses, in order of increasing magnitude, were an ED_{50} of 168.5 (95% CI, 131.8, 215.3) for 5-IM, an ED_{50} of 213.6 (95% CI, 167.1, 273.0) for 8-SQ, an ED_{50} of 217.6 (95% CI, 169.2, 279.9) for 7-IM, and an ED_{50} of 298.9 (95% CI, 245.7, 363.6) for 8-IM. Responses were not significantly different between the 7-IM, 8-IM, and 8-SQ groups. The 8-IM group response was significantly higher than the 5-IM group response. The 4-IM responses were significantly lower than for all other groups.

All groups received their final booster at month 42, and final responses were determined at month 43 (6). The intervals between boosters were 36 months for the 4-IM group, 24 months for 5-IM, and 12 months for each of 7-IM, 8-IM, and 8-SQ. The 8-SQ group responses (ED_{50} = 1,005.68 [95% CI, 822.38, 1,229.84]) at month 43 were significantly lower than for the 4-IM and 5-IM groups. The 4-IM group, with a 36-month interval between vac-

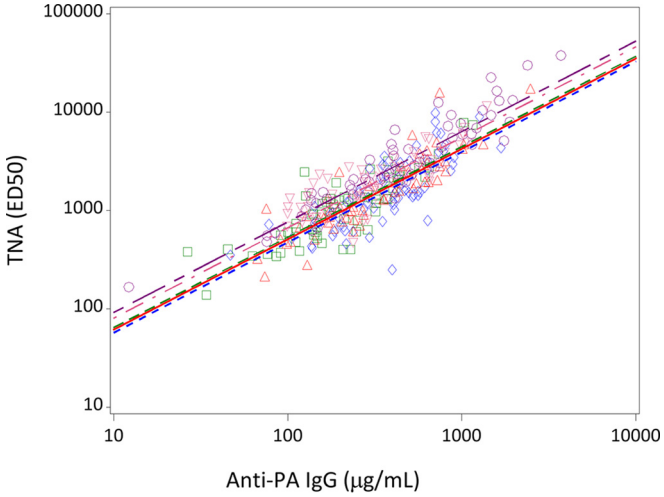

FIG 1 Correlation of TNA and anti-PA IgG antibody in humans vaccinated with AVA. Shown is a linear regression analysis of TNA (ED_{50}) versus anti-PA IgG in humans vaccinated with AVA postpriming at 43 months ($n = 328$ observations). The final regression model was $\log(Y_{ijk}) = \beta_0 + \alpha_i + \beta_1 \times \log(x_{ijk})$. The group-specific intercept shown is the sum of $\beta_0 + \alpha_i$ as specified in the model. For example, in the 8-IM study group at month 43, the log TNA ED_{50} was predicted from log total anti-PA IgG as $0.8 + 0.9 \times \log(IgG)$. There was a significant correlation between anti-PA IgG and ED_{50} at every sample collection time, and the model r^2 was high (>0.80) from month 7 through month 31. Statistically significant differences in the intercepts among the study groups were present at months 2, 6, 13, 19, 30, 42, and 43 (shown above). Although differences were small, the direction of the shifts and study groups that differed suggest that the booster TNA response per unit of anti-PA antibody was greater for subjects who had increased intervals between booster vaccinations. Slope and intercept analysis are provided in Tables 2 and 3. Green squares and line, 8-SQ; blue diamonds and line, 8-IM; red triangle and line, 7-IM; pink inverted triangles and line, 5-IM; purple circles and line, 4-IM.

cinations, had the highest TNA response: an ED_{50} of 2,853.7 (95% CI, 2,203.7, 3,695.3). The other group responses, in order of increasing magnitude, were an ED_{50} of 1,469.5 (95% CI, 1,167.8, 1,849.1) for 7-IM, an ED_{50} of 1,540.3 (95% CI, 1,74.6, 1,861.4) for 8-IM, and an ED_{50} of 1,918.7 (95% CI, 1,640.3, 2,244.4) for 5-IM. Responses were not significantly different between the 5-IM, 7-IM, and 8-IM groups. Except for 7-IM, all IM group responses were significantly higher than for the 8-SQ group. The 4-IM response was significantly higher than for all other groups. At the completion of the study, there was a TNA response hierarchy of 4-IM > 5-IM > 8-IM ≥ 7-IM > 8-SQ. Fewer boosters with greater intervals between injections generated higher TNA levels at month 42 (see Table S7 in the supplemental material).

Correlation of anti-PA IgG levels with TNA. A previous report provided specific correlations of anti-PA IgG and TNA at only months 2, 7, and 43 using linear regression (4, 6). The present analysis incorporated all time points into a mixed model to evaluate the effects of time point and study group; both effects were significant. The final regression model was $\log(Y_{ijk}) = \beta_0 + \alpha_i + \beta_1 \times \log(x_{ijk})$. The study group-specific intercept was the sum of $\beta_0 + \alpha_i$ as specified in the model so that, for example, in the 8-IM study group at month 43, the $\log_{10}$ TNA ED_{50} was predicted from $\log_{10}$ total anti-PA IgG as $0.8 + 0.9 \times \log(IgG)$. Because the models were fitted separately at each time point, the random subject effect was not required. There was a significant correlation between anti-PA IgG and ED_{50} at every sample collection time, and

TABLE 2 Parameter estimates for correlation of TNA and anti-PA IgG at month 43[a]

Parameter	Study group	Parameter estimate[b]	Study group effect (*P* value)	r^2
Study group specific intercept	8-SQ	0.9	<0.0001	0.7729
	8-IM	0.8		
	7-IM	0.9		
	5-IM	1.0		
	4-IM	1.0		
Slope	Combined	0.9	NA	

[a] The final regression model was $\log(Y_{ijk}) = \beta_0 + \alpha_i + \beta_1 \times \log(x_{ijk})$. Data are the parameter estimates and *P* values for tests of whether the individual estimates differ from zero, *P* values for tests of whether the study group-specific intercepts differ, and the r^2 for the model. The study group-specific intercept shown is the sum of $\beta_0 + \alpha_i$ as specified in the model, so that for the 8-IM study arm at month 43, the log TNA ED_{50} is predicted from log total anti-PA IgG measured by ELISA as $0.8 + 0.9 \times \log(IgG)$. Higher intercept values indicate increased TNA per unit of anti-PA IgG. NA, not applicable.

[b] The difference of each parameter estimate from 0 was statistically significant (<0.0001; a significant difference would be evident at a *P* value of <0.05).

the model r^2 was high (>0.80) from month 7 through month 31. Together, these findings indicate a very strong positive correlation between anti-PA IgG levels and *in vitro* functional activity. No differences in anti-PA IgG and ED_{50} correlations were noted between the 8-IM and 8-SQ study groups at any time point, indicating that IM versus SQ route of vaccination did not have a detectable effect on the relationship between anti-PA IgG concentration and TNA (6). In the present analytic approach, there were statistically significant differences detected in the *y* axis intercepts among the study groups at months 2, 6, 13, 19, 30, 42, and 43. Data for month 43 are provided as representative of the high level of correlation and an example of statistically different intercepts (Fig. 1; Table 2). The direction of the shifts between study groups indicated that the booster TNA response per unit of anti-PA antibody was greater for subjects who had increased intervals between booster vaccinations. For example, at month 43, the intercepts for the 4-IM and 5-IM study groups were significantly greater than those of the 8-SQ, 8-IM, and 7-IM study groups (Fig. 1; Table 3). The differences in intercepts were small and the clinical impact was unknown.

Anti-PA IgG response kinetics. Totals of 1,303, 1,003, and 880 participant observations were available across all groups, including controls for anti-PA IgG kinetics analysis at months 6, 30, and 42, respectively (see Tables S3 to S5 in the supplemental material). In general, at each of the kinetic time points the antibody levels

TABLE 3 Paired comparisons of TNA and anti-PA IgG correlations by study group at month 43

Study group	Pairwise comparison estimate (Tukey's adjusted *P* value)[a]			
	8-SQ	8-IM	7-IM	5-IM
8-IM	−0.1 (0.5241)			
7-IM	0.0 (0.9827)	0.0 (0.8567)		
5-IM	0.1 (0.0431*)	0.1 (<0.0001*)	0.1 (0.0079*)	
4-IM	0.2 (0.0002*)	0.2 (<0.0001*)	0.2 (<0.0001*)	0.1 (0.4057)

[a] Tukey's multiple-comparison procedure was used to compare the intercepts for each study group to the control arm and to the other study groups at an overall 0.05 level of significance within each set of comparisons for each parameter at each time point. *, statistically significant difference.

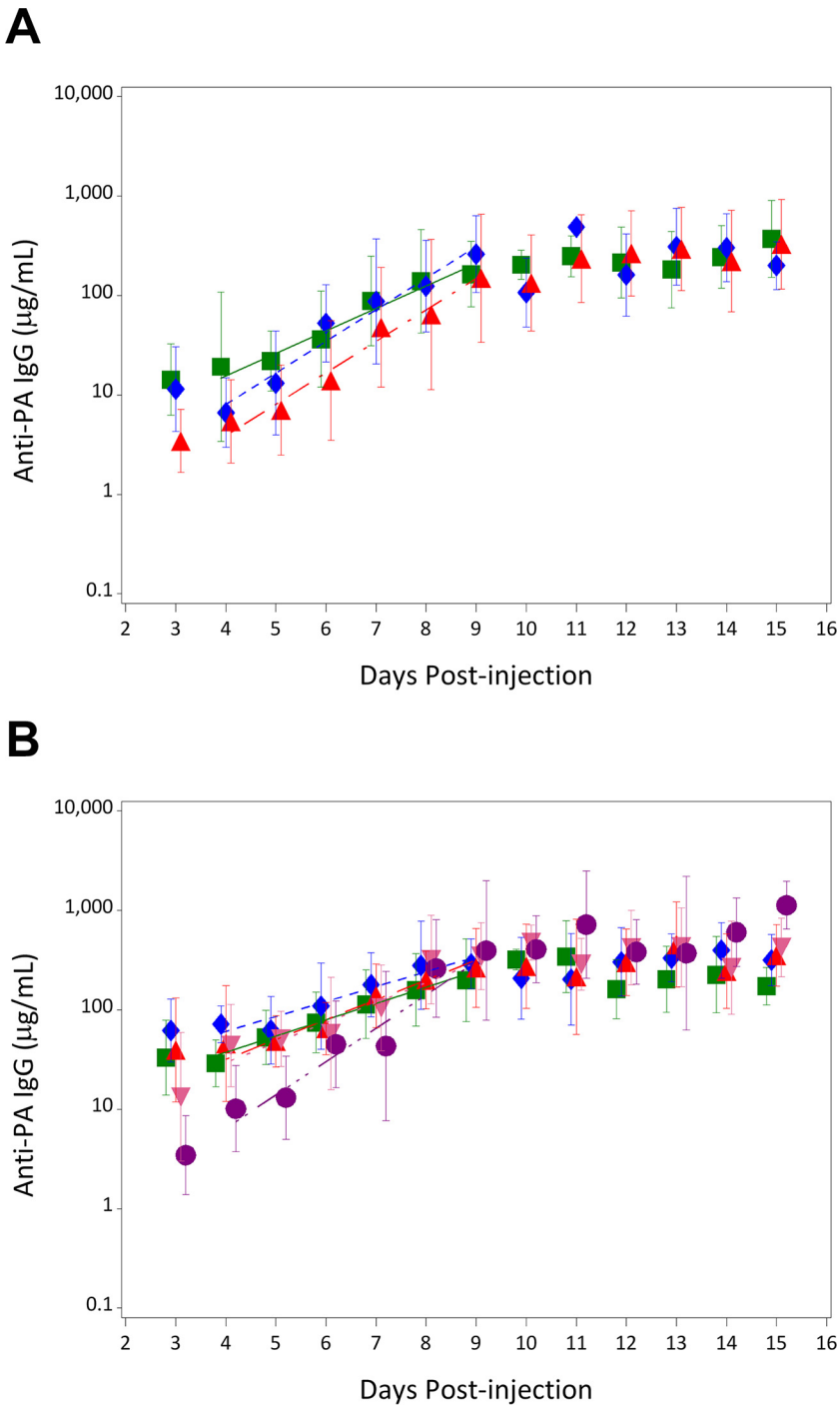

FIG 2 Antibody response kinetics in humans following vaccination at month 6 and month 42. Rates of anti-PA IgG antibody response between treatment groups were analyzed for days 4 to 9 after vaccination with AVA using weighted regression and analysis of variance (ANOVA) with the significance level set at a *P* value of <0.01. Error bars represent ± 1 standard deviation. The study groups have been offset slightly on the *x* axis to allow the error bars to be seen without overlapping. (A) At month 6, the $\log_{10}$ increase per day of anti-PA IgG was significantly different between the 8-SQ and 8-IM study groups (0.227 versus 0.318; $P = 0.0119$). The rate of response for the combined 754-IM group was significantly greater than that of the 8-SQ group (0.316 versus 0.227; $P = 0.0092$). Green squares and line, 8-SQ; blue diamonds and line, 8-IM; red triangles and line, 754-IM. (B) At month 42, the $\log_{10}$ increase per day of anti-PA IgG was not significantly different between the 8-SQ, 8-IM, 7-IM, and 5-IM groups (0.164, 0.150, 0.199, and 0.201, respectively; *P* values, 0.3211 to 0.6704). The 4-IM study group, however, had a significantly higher response rate (0.335) than did all other groups ($P < 0.0001$ to 0.0124). Antibody response levels post-day 9 are included for visual comparison. Green squares and line, 8-SQ; blue diamonds and line, 8-IM; red triangles and line, 7-IM; pink inverted triangles and line, 5-IM; purple circles and line, 4-IM.

rose linearly until day 9 postvaccination and then plateaued (Fig. 2). In response to the final priming dose at month 6, the $\log_{10}$ increase per day of anti-PA IgG was significantly different between the 8-SQ (0.227) and 8-IM (0.318) study groups ($P = 0.0119$). The rate of response at month 6 for the combined 7-, 5-, and 4-IM (754-IM) group (0.316), which did not receive a month 0.5 vaccination, was also significantly greater than that of the 8-SQ group ($P = 0.0092$) but not the 8-IM group ($P = 0.9678$) (Fig. 2A).

At month 30, the $\log_{10}$ increase per day of anti-PA IgG was not significantly different between the 8-SQ and 8-IM study groups (0.187 versus 0.247; $P = 0.0954$) or between the 8-SQ and 7-IM groups (0.187 versus 0.246; $P = 0.1096$), although both IM groups trended higher than the SQ group. The 5-IM and 4-IM groups were not compared at this time point because no vaccination was administered.

At month 42, the $\log_{10}$ increase per day of anti-PA IgG was not significantly different between the 8-SQ, 8-IM, 7-IM, and 5-IM groups, although the 7-IM and 5-IM groups trended higher (0.164, 0.150, 0.199, and 0.201, respectively; P values of 0.3211 to 0.6704). The 4-IM study group, however, had a significantly higher response rate than did all other groups, at 0.335 ($P <$ 0.0001 to 0.0124). Following the month 42 booster, the 5-IM and 4-IM group responses met or exceeded those of 7-IM by day 4 and day 8 postinjection, respectively (Fig. 2B).

Anti-PA IgG subclasses. Maturation of the immune response and Th modulation by AVA was assessed by inspection of the anti-PA IgG subclass distributions. Predominance of IgG2 and IgG3 was considered representative of Th1 responses; IgG1 and IgG4 were considered representative of Th2 responses (23). In general, anti-PA IgG1 and IgG2 were predominant, particularly at low total IgG levels (Fig. 3). Anti-PA IgG1 relative proportions were the highest for all groups, ranging from 0.5 to 0.8. In the 8-SQ, 8-IM, and 7-IM annual booster groups, there was an increased contribution from IgG4 from month 31 (injection 7) onwards. IgG4 levels for these groups increased approximately 3-fold such that IgG2 and IgG4 levels approached equivalence as IgG2 trended downward (relative proportion, 0.2). The emergence of IgG4 at later time points versus IgG2 was not evident in groups receiving the reduced booster schedules (4-IM and 5-IM). There was no apparent relationship between the emergence of an IgG4 response and vaccine reactogenicity (data not shown). Although the frequency of AEs in the 8-SQ group increased from 84.34% to 91.76% between injections 7 and 8, there was not a similar increase in the 8-IM group (6). The sustained presence of high proportions of PA-specific IgG1, a background of IgG2, and a later emergence of IgG4 in the high-frequency booster groups indicated a mixed Th1-Th2 response with Th2 dominance.

Anti-PA IgG avidity. Antibody avidity, a surrogate for maturation of the immune response and establishment of immunological memory, increased significantly following completion of the month 6 vaccination in all groups measured at month 7 (Fig. 4; see also Table S8 in the supplemental material). The AIs remained stable from months 7 through 42 (range of AIs = 0.3 to 0.4) for all study groups and were highest at month 43, after the month 42 vaccination (range of AIs = 0.45 to 0.55). There were small but statistically significant differences in AI between groups at months 7, 18, and 42.

At month 1, only the 8-SQ and 8-IM study groups (AVA vaccination at months 0 and 0.5) had sufficient levels of anti-PA IgG to determine avidity. At month 2, the AI was measurable in all study groups and there were no significant differences between groups. At month 7, AIs ranged from 0.33 to 0.39 across all groups. The 8-SQ and 8-IM study groups had similar AIs (0.37 and 0.39, respectively). The AI for the 8-IM group was significantly higher than for the 7-IM (AI = 0.33), 5-IM (AI = 0.34), and 4-IM (AI = 0.34) groups. At month 18, the AI for the 5-IM group (0.29) was significantly lower than for the 4-IM (0.41; $P = 0.0124$), 8-IM (AI = 0.39; $P = 0.0265$), and 8-SQ (AI = 0.39; $P = 0.0214$) groups. Avidity in the 7-IM group (AI = 0.35) was not significantly different from those in the other groups at this time point.

At month 42, when the antibody levels for group 4-IM were at their trough, the respective avidity was also lowest. Both the 4-IM (AI = 0.28; $n = 11$) and 5-IM (AI = 0.33; $n = 24$) groups had avidities significantly lower than for the 7-IM (AI = 0.48; $P =$ 0.0053 to 0.0134) and 8-IM (AI = 0.44; $P = 0.0260$) groups. Avidity in the 8-SQ group (AI = 0.39) was not significantly different from those in the other groups at this time point. AIs for all groups were highest at month 43, ranging from 0.45 to 0.55, and there were no significant differences between groups (see Table S8).

Frequencies of IFN-γ- and IL-4-secreting cells. Th1-Th2 disposition was evaluated by determining the onset and duration of PA-specific IFN-γ (Th1)- and IL-4 (Th2)-secreting cells. Antigen-specific IFN-γ- and IL-4-secreting cells were detectable (mean ± SE) in all vaccinated study groups at month 1, but only the 8-SQ (IFN-γ, 28.8 ± 4.1 SFU/10^6 cells; IL-4, 34.6 ± 4.3 SFU/10^6 cells) and 8-IM (IFN-γ, 38.2 ± 7.5 SFU/10^6 cells; IL-4, 39.8 ± 5.7 SFU/10^6 cells) groups had levels significantly above those of control groups (IFN-γ, 8.4 ± 3.0 SFU/10^6 cells; IL-4, 1.1 ± 0.2 SFU/10^6 cells). IFN-γ- and IL-4-secreting cells remained detectable through month 43 in all groups (Fig. 5). At month 7, following completion of the priming series at month 6, all study groups had significantly higher frequencies of IL-4-secreting cells (range, 42 to 48 SFU/10^6 cells) compared to the controls (2.8 ± 1.0 SFU/10^6 cells). In general, with the exception of IFN-γ-secreting cells in the 4-IM study group at month 31, IFN-γ- and IL-4-secreting cells were increased compared to those in the control group throughout the 43-month study and were highest at month 43 for all study groups (ranges: IFN-γ, 17 to 23 SFU/10^6 cells; IL-4, 85 to 189 SFU/10^6 cells). There were no clearly significant differences between study arms or schedules except at month 19, in response to the booster at month 18, when the 7-IM and 8-IM groups displayed significantly higher frequencies of IL-4-secreting cells than for the 4-IM group. The persistence of both IFN-γ- and IL-4-secreting cells was indicative of a mixed Th1-Th2 response. The higher frequency of IL-4-secreting cells was indicative of Th2 dominance (20).

Lymphocyte SIs. Stimulation indices (SIs) provide an assessment of antigen-specific immune cell competence and metabolic activity in the PBMC population. In contrast to the anti-PA IgG and TNA, SIs were detectable in all study groups by 0.5 month (response to a single vaccination) and statistically significantly different from control values for the 8-SQ (SI, 1.62 [95% CI, 1.30, 2.00]; $P = 0.0142$) and 7-IM (SI, 1.63 [95% CI, 1.32, 2.01]; $P =$ 0.0112) groups (Fig. 6). By month 1, in response to the second vaccination, the SI increased for 8-SQ (SI, 3.52 [95% CI, 2.71, 4.58]) and 8-IM (SI, 2.03 [95% CI, 1.49, 2.78]). The values for groups that had received only one vaccination prior to month 1 (7-IM, 5-IM, and 4-IM) were also statistically significantly different from the values for controls at that time point (SI range, 1.44 to 1.93). From month 2 onwards, there were no significant differences in the SI

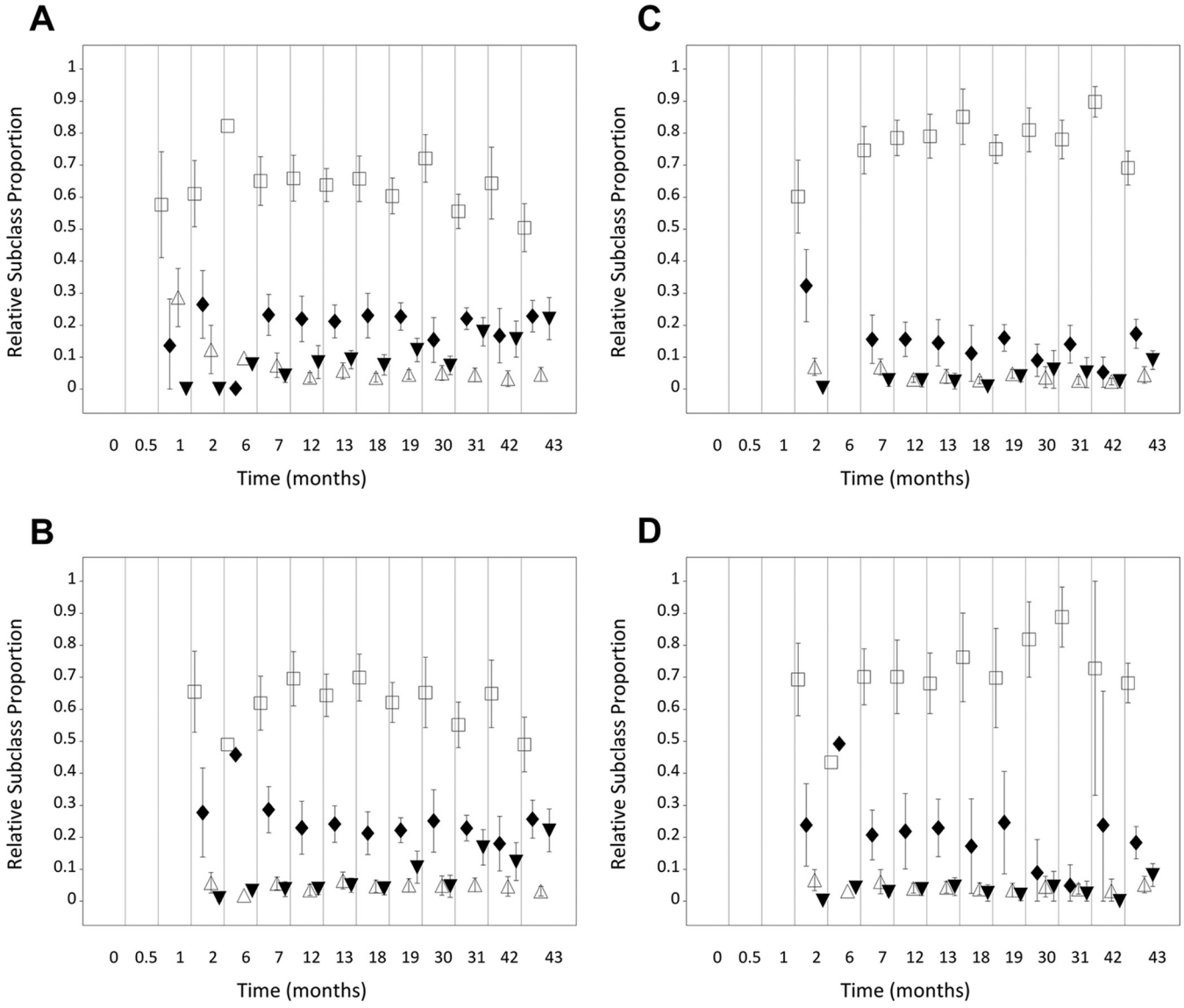

FIG 3 Anti-PA IgG subclass distributions in humans vaccinated with AVA. Assays for all four IgG subclasses were run when the total anti-PA IgG was ≥5 μg/ml. Relative subclass proportions were determined in a sample when the total anti-PA IgG was ≥12.5 μg/ml, all 4 subclasses were evaluated, and at least one IgG subclass was detected. Anti-PA IgG subclass distributions in the 8-SQ study group (A) and 7-IM (B) study group demonstrate the effect of SQ compared to IM administration. The effects of reduced booster schedules in groups receiving 3-IM priming are represented by the 5-IM (C) and 4-IM (D) boosters. Error bars represent 95% CI. □, IgG1; ◆, IgG2; △, IgG3; ▼, IgG4.

between the vaccine groups for the remainder of the 43-month study (SI range, 2.0 to 3.0), indicating persistent PA-specific lymphocyte competence in the absence of boosters (Fig. 6).

Cytokine mRNA levels. Cytokine mRNA fold increase was evaluated for selected time points up to month 19. Changes in the levels of mRNA for IFN-γ and IL-2 were interpreted as representing Th1-type responses; changes in IL-4 and IL-6, an important accessory cell cytokine, were interpreted as representing Th2-type responses; induction of acute-phase cytokines IL-1β and TNF-α was indicative of T cell activation. Time points at which there were significant increases in PA-induced PBMC cytokine mRNA levels are provided in Table S10 in the supplemental material.

In general, all cytokine mRNA targets trended upward relative to the control group over the period evaluated. IFN-γ mRNA levels were significantly increased in 7-IM at two time points, months 13 and 19, both 1 month postvaccination. IL-2 mRNA levels were significantly increased for all groups at month 7. At months 12, 18, and 19, all groups except one of the groups receiving eight vaccinations (8-SQ) showed a significant increase in IL-2 mRNA levels (see Fig. S2A to C in the supplemental material). 7-IM and 4-IM showed increased IL-2 mRNA levels from months 6 and 7, respectively. Increased levels of IL-4 were observed in three or more groups at months 1 and 13. The 7-IM study group showed increased mRNA levels from months 7 to 19. IL-1β mRNA levels were significantly increased for all groups at month 7. TNF-α mRNA levels were significantly increased for all groups at months 7 and 19. These data support the interpretation of a mixed Th1-Th2 response to AVA. There were no clear correla-

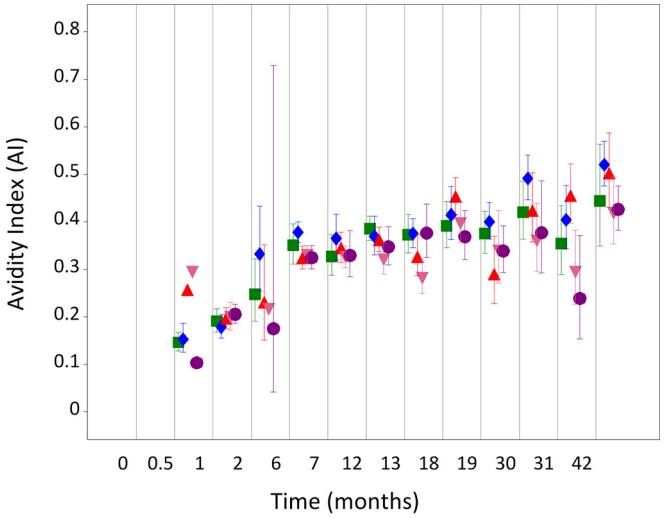

FIG 4 Anti-PA IgG avidity analyses in humans vaccinated with AVA. Shown are the avidity index (AI) and 95% CI for AVA vaccination groups. Avidity indices were determined by dissociation of antibody-antigen complexes. At month 1, subjects in the 8-SQ and 8-IM study groups (AVA vaccination at month 0 and 0.5) showed anti-PA antibodies with measurable avidity compared to the 7-IM, 5-IM, and 4-IM study groups (AVA vaccination at month 0). At month 2, the AI was measurable in all samples and there were no significant differences between the vaccination study groups. At month 7, AIs ranged from 0.33 to 0.39. The avidity indices remained stable from month 7 through 42 (approximate AI = 0.3 to 0.4), when the AIs for 4-IM and 5-IM were the lowest. At month 43, after the month 42 vaccination, the AI was highest for all study groups (range, 0.45 to 0.55). Error bars represent 95% CI. Green squares, 8-SQ; blue diamonds, 8-IM; red triangles, 7-IM; pink inverted triangles, 5-IM; purple circles, 4-IM.

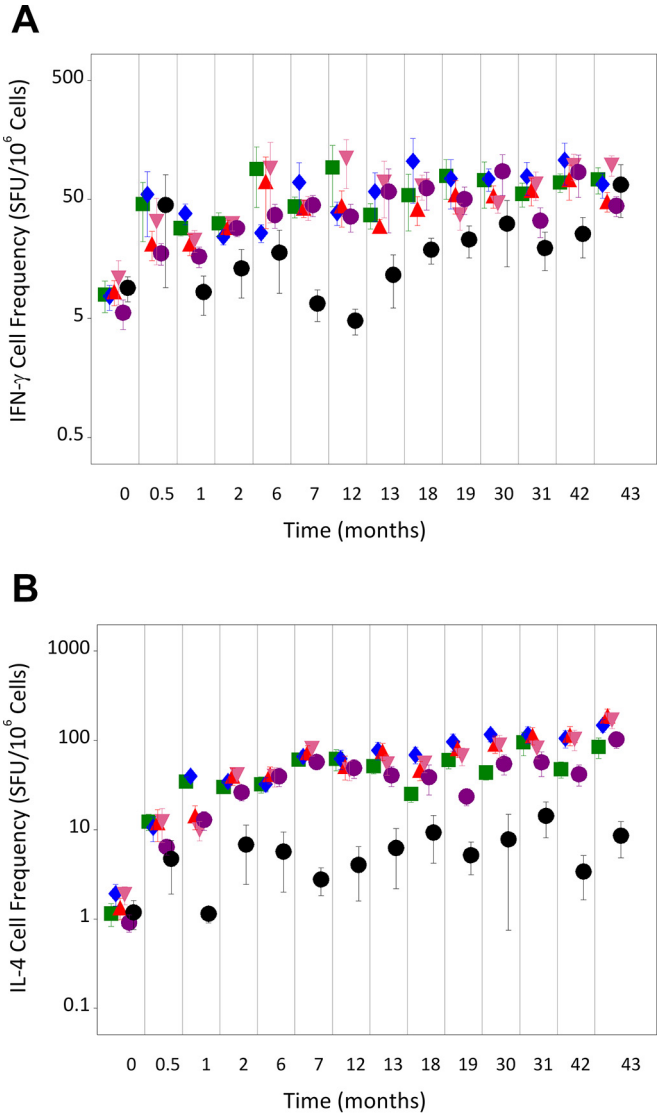

FIG 5 Frequencies of IFN-γ- and IL-4-secreting cells in humans vaccinated with AVA. (A) Frequencies of IFN-γ-secreting cells (mean SFU/10^6 PBMCs) determined by ELISpot analyses. At month 1, the 8-SQ and 8-IM groups showed a significant increase in the number of IFN-γ-secreting cells compared to the placebo controls; the 8-IM group showed a significant increase compared to 4-IM group. There was a significant difference in the number of SFU/10^6 cells between the various vaccination groups. Error bars indicate 1 standard error (SE). (B) Frequencies of IL-4-secreting cells (mean SFU/10^6 PBMCs) determined by ELISpot analyses. At month 1, significantly more IL-4 SFU/10^6 cells were detected in the 8-IM group than in the 8-SQ group at this early time point. Both the 8-IM and 8-SQ groups had more IL-4 SFU/10^6 cells than any of the reduced-schedule vaccination groups at month 1, indicating the impact of the month 0.5 vaccination on the rapidity of the immune response to PA. The frequency of circulating memory T cells capable of secreting IL-4 when stimulated with PA remained significantly higher for all vaccination groups than for the control arm for the duration of the study, 43 months. These data are consistent with AVA stimulating long-term memory T cells to PA. Error bars indicate 1 SE. Green squares, 8-SQ; blue diamonds, 8-IM; red triangles, 7-IM; pink inverted triangles, 5-IM; purple circles, 4-IM; black circles, control.

tions between mRNA levels and ELISpot or SI responses (data not shown).

Frequency of PA-specific MBCs. Memory B cell (MBC) frequencies (percent rPA-specific cells per total IgG-secreting cells) in PBMCs were analyzed with outliers excluded. In some groups at some time points, the total number of observations was small. Data from study groups 4-IM, 5-IM, and 7-IM were combined for analysis up to the month 12 time point (754-IM); data from study groups 4-IM and 5-IM were combined for analysis of the months 13 and 18 time points (54-IM) (see Table S11 in the supplemental material). For visual representation, groups with ≥5 data points were graphed (see Fig. S3). The first significant increase in MBC frequencies above the control study group was at month 6 in the 8-IM group (0.11%; $P = 0.0286$), prior to the month 6 vaccination. The month 7 MBC frequencies in all vaccination groups were significantly different from those in the controls. The group means ranged from 0.14 to 0.22% cells, with the highest responses in the 8-IM and 754-IM groups, which were also significantly different ($P = 0.019$). At time points following month 7, there were sporadic occurrences of vaccine study groups that had values that were significantly different from that for the control study group. For example, at month 12, the IM vaccination group values remained significantly higher than controls. At month 18, prior to the booster vaccination, all groups had significantly higher values, with the 8-IM group having the highest and the combined 54-IM group having the lowest (0.08% for 8-SQ, 0.13% for 8-IM, 0.08% for 7-IM, and 0.07% for 54-IM). Differences between groups were not significant. The 8-IM study group showed significantly increased mean frequencies at months 30 and 42 (0.25% and 0.21%, respectively) compared to the control study group (0.03% and 0.05%, respectively). There was variability within a study group such that there were large standard errors at some time points. In

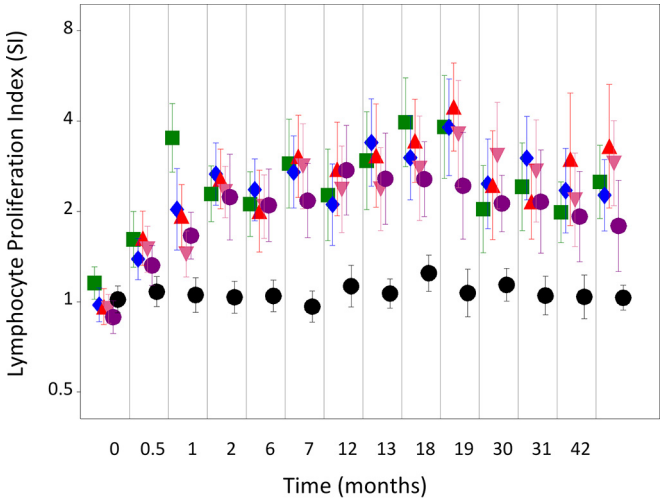

FIG 6 Lymphocyte stimulation indices (SI) in humans vaccinated with AVA. The lymphocyte (T cell) proliferation assay provides a very sensitive method for demonstrating the induction of long-term PA-specific memory lymphocytes in PMBCs. Significant SI responses were detected at month 1 in the 8-SQ and 8-IM groups. All of the values for the vaccine treatment groups were significantly higher than for the placebo controls by month 2. SI was sustained at significantly higher levels than controls in all groups for the duration of the study. Error bars indicate 95% CI. Green squares, 8-SQ; blue diamonds, 8-IM; red triangles, 7-IM; pink inverted triangles, 5-IM; purple circles, 4-IM; black circles, control.

general, for groups with >1 data point, the mean frequencies of MBCs increased in response to the priming series at month 7 and remained elevated for the 43 months examined (range, 0.03 to 0.35%; mean, 0.14%; median, 0.14%).

DISCUSSION

We report the onset, magnitude, and duration of PA-specific humoral and CMI profiles and antibody response kinetics in AVA-vaccinated humans. Study groups received the 3-IM priming series and a range of alternate IM booster schedules compared to the original 1970-licensed SQ schedule. The data demonstrated that CMI and humoral antibody responses were detectable at 2 weeks and 4 weeks, respectively, after only one AVA injection. The 3-IM series elicited long-term production, for at least 3 1/2 years, of high-affinity PA-specific functional antibody and PA-specific peripheral MBCs and T cells. Human immunological responses to 3-IM priming with AVA were analogous to those providing long-term protection, of at least 4 years, after first vaccination against inhalation anthrax in rhesus macaques exposed to high doses of aerosolized *Bacillus anthracis* spores. Magnitudes of peak responses were lower in humans than in NHPs. For example, a 1/5 saline-diluted AVA dose in NHPs (minimum body weight, 2.6 kg) elicited magnitudes of anti-PA IgG similar to those elicited by a full dose in humans (13).

Systemic anthrax is an invasive infection with *B. anthracis*. It is associated with bacterial dissemination and toxin-mediated multiorgan dysfunction (24). A characteristic of the protective immune responses in NHPs was a mixed Th1-Th2 profile with Th2 dominance. A Th1-biased response is considered predominantly cell mediated, with a primary role in protecting against intracellular pathogens, including invasive bacteria, by activation of cytotoxic T lymphocytes and natural killer (NK) cells. IFN-γ secretion is a marker for Th1 cells. IFN-γ activates macrophages and induces the production of opsonizing antibodies by B cells. A Th2 response is characterized as a humoral antibody response mediating immunity against extracellular microbes and toxins. IL-4 secretion is a marker for Th2 cell activity, essential for the induction of B cell activity and promoting antibody production (25). The importance of a mixed Th1-Th2 profile with Th2 dominance may reside in its ability to both mediate extracellular neutralization of anthrax toxins and provide protection against intracellular germinating spores and vegetative cells of *B. anthracis*.

In humans, the 3-IM priming elicited long-lived plasma cells producing high-affinity PA-specific functional antibody for at least 3 1/2 years in the absence of boosters. Analogous to protective IgG profiles in NHP, the antibody responses indicated a mixed Th1-Th2 response with Th2 dominance. The Th2 bias was evident in the sustained presence of high proportions of PA-specific IgG1, a background of IgG2, and a later emergence of IgG4 in the high-frequency booster groups.

CMI and anamnestic antibody responses were indicative of long-lived immune effector cells and long-term immunological memory. PBMC lymphocyte SI and frequencies of IL-4- and IFN-γ-secreting cells indicated that T cell priming was stable by month 2 for all schedules. PA-specific MBCs and affinity maturation (AIs) developed during the 6-month priming period, were consolidated in response to the month 6 vaccination, and were sustained for the study duration. Frequencies of PA-specific MBCs were lower than previously reported for AVA recipients (19, 26), and variance between and within study groups was high.

Omission of the month 0.5 (week 2) vaccination from the priming series and use of the IM route of administration had no measurable detrimental effects on postpriming humoral or cellular immune responses. As previously reported, the magnitudes of anti-PA IgG responses at month 7 were not significantly different between 8-SQ and the combined 754-IM groups. The 8-IM group had the highest response at month 7 (6). The consolidation of anti-PA IgG levels and functional activity (AI and TNA) at month 7 indicated that immunological priming was complete following the month 6 vaccination. This is consistent with long-term protective responses reported for NHPs (13). This interpretation is also in agreement with available data on anthrax vaccine effectiveness in humans showing that there were no anthrax cases identified in persons that had completed the month 6 vaccination series, including those receiving no additional boosters (27, 28).

Postpriming, the serum anti-PA IgG levels declined in the absence of frequent boosters. However, antibody responses to vaccination at 1-year intervals, 1- and 2-year intervals, and a 3-year interval were rapid and high. From month 7 onwards, the IM route of administration consistently provided the highest peak antibody responses. The magnitude of the IM responses increased with greater intervals between boosters. Anti-PA IgG levels were lowest at month 42 in the 4-IM group (no boosters). As previously reported by Wright et al. (6), even at the lowest reported antibody levels at month 42, the majority (66%) of 4-IM study group participants demonstrated quantifiable anti-PA IgG. At month 43, the frequency of responders in the 4-IM group was 100%, with 99.4% being ≥4-fold higher than the assay LLOQ (6). The high response frequencies demonstrated that antibody levels below the assay limits of detection at month 42 did not indicate waning immunity.

Linear models of correlation between TNA ED_{50} anti-PA IgG

concentrations demonstrated that antibody quantity and neutralizing power were highly correlated when data were $\log_{10}$ transformed. Specific TNA was not affected by route of vaccine administration. There were indications that reducing the frequency and increasing the interval between boosters resulted in increased TNA per unit anti-PA IgG at later time points. However, these differences were small, and their clinical significance is unknown.

Antibody avidity increased throughout the priming series, stabilized on completion of priming at month 6, and remained high in all groups for the study duration. The route of AVA administration had no detectable impact on AIs. There were no indications of AI impairment from receiving annual boosters. AIs in both the 4-IM and 5-IM groups remained high but dropped below those of the annual booster groups at specific time points. The 4-IM group had the lowest AI value at month 42, concurrent with the lowest antibody levels for that schedule and just prior to the final vaccination. The lowest 5-IM AIs were at months 18 and 42. All groups demonstrated high AIs at month 43 in response to the final booster. These data indicate that the 6-month priming series established long-lived MBC populations capable of rapid differentiation and expansion into high-affinity antibody-secreting cells. However, a booster interval of 3 years may be a temporal limit for maintaining the highest affinity plasma cell populations (29).

A previously reported cross-species COP bridging between NHPs and humans estimated that at the lowest levels of serum antibody at month 42, the 4-IM group had a predicted minimum probability of survival from 86.8% to 90.2%, compared to 95.8 to 98.0% in the 5-IM group and 98.1 to 99.5% in the 7-IM annual booster group (1, 3). Of additional importance, therefore, is whether the anamnestic response kinetics in the reduced booster schedules are sufficient to meet or exceed those of the annual boosters and to provide protection against acute infection. The clinical course of untreated inhalation anthrax has previously been described as biphasic, with a symptomatic prodromal phase followed by a fulminant phase (30, 31). The prodromal phase is preceded by the incubation period between exposure and symptom onset. Empirical data on the minimum incubation period and time course of disease progression for inhalation anthrax are limited due to the low prevalence of the disease and the nonspecific nature of early symptoms (31–33). In a recent meta-analysis by Toth et al. of published human and NHP exposure and infection data, it was estimated that the median time from exposure to onset of symptoms in unvaccinated humans may be 9.9 days (34). This estimate was derived from an exposure scenario of 11,000 inhaled *B. anthracis* spores, a dose proposed to cause infection in 50% of susceptible nonvaccinated individuals (ID_{50}). Lower estimated exposure doses had associated longer periods of incubation (34). Historically, the estimated exposure levels in high-risk industrial environments were estimated at 21 to 2,100 infectious particles per 8-h day (35, 36), significantly lower than those proposed by Toth et al. A systematic review and analysis of 82 inhalation anthrax cases by Holty et al. indicated that postincubation, the mean time from symptom onset to death was 4.8 days (31). These studies indicated that the overall period from exposure to fulminant disease may be approximately 14 days. In the 2001 anthrax letter events, using the postmark date as the exposure time point, the median number of days to onset of illness in the first case cluster was 10 (range, 4 to 13 days), and in the second cluster, it was 7 (range, 5 to 13). In that outbreak, 11 of 22 confirmed cases were inhalation anthrax with an incubation period estimated at 4.5 days (range, 4 to 17). Those who survived inhalation anthrax started antimicrobial treatment, on average, at 4.7 days after symptom onset. The overall estimated time frame from exposure to effective intervention in susceptible individuals was thus approximately 9 days (37). In the present study, the kinetics of the anti-PA IgG response to the month 6 final priming vaccination were statistically significantly more rapid in groups vaccinated IM, irrespective of omission of the 0.5-month injection in the combined 3-IM groups (754-IM). In addition, the response kinetics following IM administration trended higher than for SQ administration at months 30 and 42, though not significantly different. At month 42, the response rate in the 4-IM group was significantly higher. Antibody responses stimulated by month 42 vaccination in both the 8-SQ and 7-IM groups were met or exceeded by those in the 5-IM and 4-IM reduced booster groups by day 4 and day 8 postinjection, respectively. The response rates for both the 5-IM and 4-IM groups were therefore within the estimated incubation period for systemic anthrax. These data indicate the feasibility of adopting alternate reduced AVA booster schedules.

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