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Antibody response to a delayed booster dose of anthrax vaccine and botulinum toxoid

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Abstract

We evaluated the prevalence and concentration of serum antibodies 18–24 months after primary inoculation with anthrax and botulinum vaccines, and assessed the reactogenicity and immunogenicity of a significantly delayed booster dose of these vaccines. Five hundred and eight male active-duty military personnel received one, two or three inoculations with anthrax vaccine and/or botulinum toxoid in 1990/1991 in preparation for Operations Desert Shield/Desert Storm. Subjects were vaccinated with the licensed anthrax vaccine, adsorbed (AVA) and pentavalent (ABCDE) botulinum toxoid (PBT) BB-IND 3723. Anthrax protective antigen (PA) IgG antibody was measured in serum using an immunocapture enzyme-linked immunosorbent assay (ELISA). A mouse neutralization test was used to determine the titer of *Clostridium botulinum* type A antitoxin in serum samples. The prevalence of anti-PA IgG was 30% in individuals 18–24 months after priming with one, two or three doses of AVA. After boosting, 99% of volunteers had detectable anti-PA IgG; only two individuals failed to respond. The prevalence of antibodies against botulinum toxin type A was 28% 18–24 months after initial priming. Following boosting, 99% of volunteers had serum titers >0.02 IU/ml, and 97% responded with titers ≥0.25 IU/ml.

Systemic reactions to booster vaccinations could not be specifically ascribed to one or the other vaccine, but were generally mild and of brief duration. Forty-five percent of volunteers reported one or more systemic reactions over the course of 7 days. Injection site reactions of any kind occurred in 25% of AVA recipients and in 16% of PBT recipients; persistence of local reactions beyond 7 days was infrequent.

While the kinetics and durability of immune responses must be studied, these findings suggest that booster doses of anthrax vaccine and botulinum toxoid sufficient to stimulate a robust anamnestic response may be given at times distant from receipt of the primary inoculations.

Keywords: Botulinum toxoid; Pentavalent; Immune response

1. Introduction

Bacillus anthracis and neurotoxins produced by *Clostridium botulinum* are widely regarded as premier biological warfare agents [1]. At the onset of Operation Desert Shield, the government of Iraq possessed numerous types of ordinance, including weaponized forms of anthrax spores and botulinum toxins [1–3]. Allied coalition forces, confronted with the prospect of possible biological or chemical warfare, undertook extensive preparations in the form of physical (e.g. protective mask and suit) and pharmacological (e.g. vaccines, antibiotics, and antidotes) countermeasures.

Vaccines developed against anthrax and botulism before the Gulf War afforded the opportunity to consider prophylaxis as a viable option in countering these threats. Because these products—anthrax vaccine, adsorbed (AVA) and the pentavalent (ABCDE) botulinum toxoid (PBT)—were considered to be safe, immunogenic, and efficacious when administered prophylactically [4–11], inoculation of select Department of Defense (DoD) personnel against anthrax and botulinum toxins was undertaken.

By the conclusion of the conflict in May 1991, approximately 150,000 US service members had received at least one injection with AVA, and approximately 8000 had received at least one inoculation with PBT. Due to the cessation of hostilities and removal of the threat, completion of the recommended full vaccination series for both vaccines was considered unwarranted, and additional vaccine doses were not administered. We sought to determine if antibodies presumably induced by AVA and PBT through vaccination at the time of the Gulf War could be detected 18–24 months

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later. More importantly, we wished to examine the antibody response to a booster dose of AVA, or AVA plus PBT, in a cohort of vaccinees to gauge the efficiency of immune recall to these priming antigens.

2. Methods

2.1. Vaccines

AVA is derived from an attenuated, non-encapsulated strain of *B. anthracis* [12]. *B. anthracis* protective antigen (PA), the major component of the vaccine, is adsorbed onto an aluminum hydroxide adjuvant. AVA has been licensed in the United States for protection against anthrax since 1970. The package insert requires the vaccine to be administered subcutaneously (SQ) in 0.5 ml doses at weeks 0–2–4, and months 6–12–18. Annual AVA booster doses (0.5 ml SQ) are recommended as long as the individual remains at risk [12].

PBT is an investigational product containing a combination of aluminum phosphate-adsorbed toxoids derived from formalin-inactivated, partially purified types A, B, C, D and E botulinum toxins. The vaccination schedule currently recommended by the Food and Drug Administration for IND 161 requires that PBT be administered as 0.5 ml SQ in three doses over 12 weeks (0–2–12 weeks) and a booster dose of 0.5 ml SQ at 12 months. Additional booster doses are recommended at 2 year intervals based upon serum antibody titer. PBT was administered during the Gulf War and in the present study under BB-IND-3723.

2.2. Study population and design

This was a prospective open-label study involving a cohort of DoD personnel who had received one, two or three doses of AVA alone, or both AVA and PBT, 18–24 months previously during Operations Desert Shield and Desert Storm (ODSDS) in 1990/1991. The study protocol was reviewed and approved by institutional review boards at the US Army Medical Research Institute of Infectious Diseases (USAMRIID), and the Human Subjects Research Review Board of the US Army Surgeon General (HSRRB).

Prospective volunteers, recruited from military units receiving AVA or AVA/PBT during ODSDS that had remained more or less intact upon return to their home stations, were briefed on the nature of the study and provided written informed consent before participation. Vaccinations administered during ODSDS were documented in individual health records, in hard-copy unit rosters, and in automated spreadsheets at the time of initial vaccinations. Data from individual and unit records were transcribed onto study clinical record forms.

Volunteers had blood drawn to measure antibodies against AVA and PBT that had been administered 18–24 months previously. Individuals then received a booster of the respective vaccines; both AVA and PBT were administered subcutaneously in the deltoid region. AVA was always injected into the right arm, and PBT always injected into the left arm. Volunteers were evaluated 30 min after vaccine boost; on days 1, 2, 3, and 7; and once between days 24 and 36. Volunteers were queried for symptoms and examined for injection site reactions by a clinician at each of these visits. A positive reaction at any visit was considered a reaction. Those with persistent complaints were followed for longer periods. Approximately 1 month after inoculation, volunteers were asked to provide an additional serum specimen for measurement of antibodies.

Five hundred and eight individuals (490 in 1992, 18 in 1994) enrolled in the study. Thirteen volunteers were dropped for various reasons: five had received only PBT; two had no reaction records; three who were given AVA/PBT were determined to have inadequate documentation of initial dosing; and three did not receive a boost. Thus, 495 subjects were analyzed for safety evaluations after boosts (483 subjects from 1992 and 12 from 1994). A total of 452 received AVA/PBT (440 in 1992 and all 12 in 1994). The 43 volunteers who received AVA only were boosted in 1992. Because of the difference in time interval, the 1994 subjects (only 12 volunteers) were not analyzed for antibody response. Two hundred seventy-nine subjects had anthrax anti-PA antibody measured (259 AVA/PBT and 20 AVA alone). For PBT, 326 had boost titers for botulinum toxin type A performed. For subjects who had titers determined, the mean times between the initial one, two or three doses and the AVA boost were 570, 602 and 695 days for the AVA only group. For the AVA/PBT group, the mean times between the initial AVA dose and the boost were 595, 603 and 724 days, respectively. The mean times between initial doses of PBT and the PBT booster dose were 582, 600 and 719 days for the one, two and three initial dose groups.

2.3. Antibody assays

2.3.1. Immunocapture ELISA for anthrax protective antigen

Antibodies against anthrax PA were measured using an immunocapture enzyme-linked immunosorbent assay (ELISA). The capture ELISA using baculovirus-derived recombinant PA (rPA) was described in detail previously [13]. This rPA, when used as a vaccine, protected guinea pigs against a parenteral challenge with *B. anthracis* [14]. ELISA results were expressed as antibody titer, defined as the reciprocal of the last dilution equal to or greater than an optical density value of 0.2. Sera were coded and tested beginning at a 1:100 dilution. Serum dilutions were carried to 1:6400. Titers of 1:100 or more indicated an immune response. For statistical evaluation, titers <1:100 were considered to be 1:50 and titers >1:6400 were considered to be 1:10,000.

2.3.2. Botulinum toxin neutralization bioassay

The mouse protection bioassay is the standard assay for detecting and measuring antibody responses to botulinum toxoids [15]. The ability of increasing dilutions of serum

to inactivate the lethality of a standard test dose of the neurotoxin is measured. Mouse protective end points are determined for a standard antitoxin of known concentration (expressed as International Units (IU)/ml) and for the test serum that is assayed simultaneously. One International Unit is defined as the amount of antibody neutralizing 10,000 mouse intraperitoneal 50% lethal doses (LD50). In this study, we measured antibodies against type A botulinum toxin. The procedure was described previously [15,16]. Following the primary series, a neutralizing antibody concentration against type A of 0.02 IU/ml was considered indicative of seroconversion, according to the FDA IND 161. An antibody concentration of 0.25 IU/ml (approximate titer of 1:16) is considered satisfactory for deferring a booster for two more years. We evaluated the postboost response using both levels. The antibody response to type A may not be representative of antibody response to toxoids BCDE contained in this pentavalent vaccine.

2.4. Reactogenicity assessment

Subjects were evaluated by a physician or nurse 30 min after the boost injection; on each of days 1, 2, 3, and 7; and once between days 24 and 36. Findings were recorded on a study clinical record form. Subjects with reactions were followed until resolution.

2.5. Statistical analysis

Antibody response rates for AVA and PBT were transformed to logarithms for analysis by Fisher exact test for responder rates and analysis of variance (ANOVA) for geometric mean comparisons. Some measurements were not taken to endpoint. The majority of such measurements were postboost anthrax titers that had an assay ceiling of 1:6400. Anti-botulinum neurotoxin type A antibody concentrations that were not taken to lower endpoint were discarded unless less than 0.06 IU/ml in which case the value 0.019 was substituted. These occurred only in the preboost data. Any postboost botulinum concentrations which were recorded as greater than some concentration were set to that concentration. These truncations reduced the sample variances and geometric means to levels less than their true values. The Cochran-Armitage exact test for binomial trend was used to assess significance of increasing rate of erythema and/or induration (E/I) for AVA/PBT and PBT with an increasing number of initial doses. All tests were at the 95% confidence level (two-tailed). Data analysis was conducted using Statistical Analysis System (SAS, Cary, NC) [17].

3. Results

Demographic characteristics of the 495 volunteers who received AVA or AVA/PBT boosters are provided in Table 1. All subjects were male; 91% were white, and 84% were between 20 and 40 years old (mean 33.9 years).

Table 1
Demographics of analyzable subjects receiving AVA only or AVA and PBT boosters

	AVA only (*N*)	AVA/PBT (*N*)	Total volunteers (*N*)
Gender			
Male	43	452	495
Female	0	0	0
Age (mean age 33.9)			
Unknown	1	0	1
20–29	19	91	110
30–39	21	287	308
40–49	2	73	75
50–59	0	1	1
Total	43	452	495
Ethnicity			
White	41	413	454
African–American	0	18	18
Asian	0	4	4
Hispanic	1	13	14
Pacific Island	0	2	2
Other	1	2	3
Total	43	452	495
Number of initial doses of AVA			
1	16	11	27
2	25	324	349
3	2	117	119
Total	43	452	495
Number of initial doses of PBT			
1	0	17	17
2	0	318	318
3	0	117	117
Total	0	452	452

3.1. Reactogenicity

Subjects were evaluated after administration of AVA or AVA/PBT boosters for systemic and injection site reactions (Tables 2 and 3). One or more systemic reactions were recorded for 11 (26%) vaccinees receiving AVA alone, and 205 (45%) receiving AVA and PBT during four query points (30 min, days 1, 2, 3 and 7) over the first 7 days following boosting. The most common systemic reaction, myalgia, was seen in 23% of AVA recipients and 31% of AVA/PBT recipients (Table 2). Reactions consisting of headache, malaise, or arthralgia were reported in 7–9% of AVA vaccinees and 13–20% of AVA/PBT recipients. Rash appeared in 17% of those receiving AVA and PBT, but in no one receiving AVA alone. Fever (temperature $\geq$100.5 °F as measured by the clinical staff) occurred in 13 volunteers (AVA group 1/40 (2.5%); AVA/PBT group 12/438 (2.7%). Low-grade temperature elevation (temperature 98.7 °F to 100.4 °F) occurred in AVA and AVA/PBT volunteers at a rate of 2/40 (5%) and 76/438 (17%), respectively.

Table 2
Systemic reactions recorded among volunteers within 7 days of receiving AVA or AVA/PBT boosters

Systemic reactions	Anthra × vaccine only		Anthrax and botulinum vaccines	
	Yes (%)	No (%)	Yes (%)	No (%)
Anorexia	0	43 (100)	17 (3.8)	435 (96.2)
Breathing difficulty	0	43 (100)	1 (0.2)	451 (99.8)
Fever ($T \geq 98.6$ °F)	3 (8.1)	37 (91.9)	88 (20.0)	350 (79.9)
Headache	4 (9.3)	39 (90.7)	75 (16.6)	377 (83.4)
Malaise	3 (7.0)	40 (93.0)	76 (16.8)	376 (83.2)
Arthralgia	3 (7.0)	40 (93.0)	59 (13.1)	393 (86.9)
Myalgia	10 (23.3)	33 (76.7)	140 (31.0)	312 (69.0)
Nausea	0	43 (100)	16 (3.5)	436 (96.5)
Rash	0	43 (100)	78 (17.3)	374 (82.7)
Overall	11 (25.6)	32 (74.4)	205 (45.4)	247 (54.6)

The denominator for fever for AVA group and AVA/PBT group was 40 and 438, respectively.

In the AVA only booster group, E/I of any degree was observed at the injection site in 33% of subjects who had previously received AVA alone during ODSDS. Local reactions were mild, with no subject developing E/I >120 mm in diameter (Table 3). Among those receiving both AVA and PBT, local reactions to AVA were seen in 26% overall. Three individuals (0.7%) developed E/I >120 mm in diameter. Local reactions to PBT were infrequent (16% overall) and relatively mild. A trend toward increased frequency of E/I was seen in those who had received increasing numbers of PBT injections during ODSDS ($P = 0.003$) but not for AVA/PBT ($P = 0.21$) (Table 3).

Frequency of injection site reactions (E/I) for both AVA and PBT peaked on postvaccination day 2 (Fig. 1). A small proportion of reactions started on the day of vaccination. Induration and erythema usually resolved within 2–3 days of onset. Reactions occurring or persisting after 7 days were uncommon. For those receiving AVA only, erythema was not noted beyond 7 days, but induration persisted beyond 7 days in 2.9% (1/34). Induration persisted for more than 7 days in 3.3% (13/386), and erythema in 0.2% (1/386) for AVA recipients in the AVA/PBT group. One volunteer who received AVA in the AVA/PBT group had evidence of minimal induration to postvaccination day 47. No volunteer who received PBT in the AVA/PBT group had a persistent injection site reaction on postvaccination day 36.

No correlation was observed between subjects who reported having had reactions to AVA and PBT during ODSDS and those who developed reactions to the boost doses of these vaccines (data not shown).

Table 3
Erythema/induration reaction incidence by size for subjects administered AVA and AVA/PBT

Number of initial vaccine doses	Erythema/induration reaction				Total
	No reaction *N* (%)	<50 mm *N* (%)	50–120 mm *N* (%)	>120 mm *N* (%)	
Anthrax vaccine					
1	11 (68.7)	5 (31.3)	0	0	16
2	17 (68.0)	7 (28.0)	1 (4.0)	0	25
3	1 (50.0)	0	1 (50.0)	0	2
Total	29 (67.4)	12 (27.9)	2 (4.7)	0	43
Anthrax and botulinum vaccines					
Anthrax arm					
1	8 (80.0)	1 (10.0)	1 (10.0)	0	10
2	238 (74.9)	47 (14.8)	30 (9.4)	3 (0.9)	318
3	78 (70.9)	22 (20.0)	10 (9.1)	0	110
Total	324 (74.0)	70 (16.0)	41 (9.3)	3 (0.7)	438
P-value					0.02
Botulinum arm					
1	15 (93.8)	1 (6.3)	0	0	16
2	271 (87.1)	31 (10.0)	9 (2.9)	0	311
3	84 (73.0)	28 (24.3)	3 (2.6)	0	115
Total	370 (83.7)	60 (13.6)	12 (2.7)	0	442
P-value					0.0003

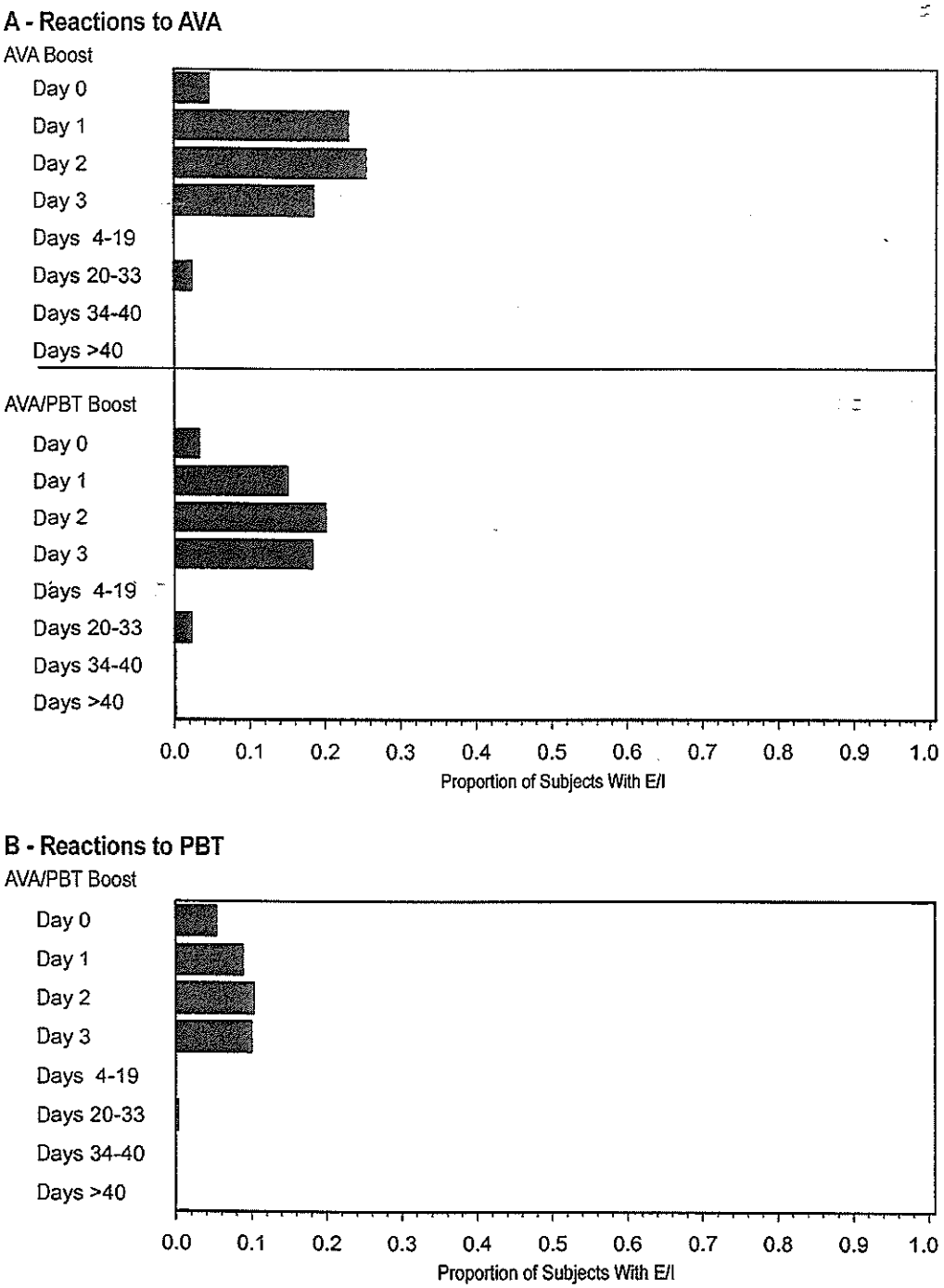

Fig. 1. Incidence of injection site reactions to AVA and AVA/PBT: (a) injection site reactions for the AVA only and for AVA in the AVA/PBT group; (b) injection site reactions for PBT.

3.2. Antibody response to AVA

3.2.1. Antibody 18–24 months after initial doses of AVA

Of the 483 members of the 1992 cohort receiving initial AVA doses during ODSDS, anthrax anti-PA antibody titers were measured in 279 before boosting. Twenty of these individuals had received AVA only, and 259 had received AVA/PBT. Overall, the prevalence of anti-PA antibody was 30% (85/279) (Table 4). Among those who had received one primary AVA vaccination, 3 of 13 (23%) had detectable antibody; of the 196 individuals who received two primary vaccinations, 47 (24%) had detectable antibody; and of the 70 who received three primary vaccinations, anti-PA antibody was found in 35 (50%) ($P < 0.001$ for difference in response rates). The highest preboost titer measured was 1:400; the overall GMT was 1:66. Antibody titer tended to

Table 4
Anti-PA IgG antibody in volunteers before and after AVA booster stratified by number of initial AVA doses received during ODSDS

Number of initial vaccine doses	N	Subjects with detectable antibody (≥1:100)		Geometric mean titer (GMT)	
		Before booster N (%)	After booster N (%)	Before booster	After booster
AVA only					
1	9	2 (22)	8 (89)	58	4529
2	10	5 (50)	10 (100)	93	7621
3	1	1 (100)	1 (100)	100	10000
AVA/PBT					
1	4	1 (25)	4 (100)	59	10000
2	186	42 (23)	185 (99)	63	9268
3	69	34 (49)	69 (100)	75	10000
Total AVA					
1	13	3 (23)	12 (92)	59	5781
2	196	47 (24)	195 (99)	63	9183
3	70	35 (50)	70 (100)	75	10000
		$P < 0.001$*	$P = 0.154$*	$P = 0.036$[a]	$P = 0.002$[a]
Overall	279	85 (30)	277 (99)	66	9183

[a] *P*-value tests significance of GMT difference by priming dose number before and after boost.
* *P*-value tests significance of difference is anti-PA IgG antibody response rate by priming dose before and after boost.

increase with increasing numbers of primary doses ($P = 0.036$).

3.2.2. Antibody response to booster dose of AVA

The overall AVA booster antibody response rate was 99% (Table 4). No difference in boost response rate was observed between groups receiving AVA alone (19/20) and those receiving AVA/PBT (258/259) ($P > 0.05$). Two boost failures were seen: one in the AVA only group involving a subject who had received one initial dose of AVA, and one in the AVA/PBT group who had received two initial doses of AVA.

The booster antibody response was brisk and strong; overall GMT was higher than the maximum titer (1:6400) measured in the assay, with a significant trend toward higher titer associated with receiving two or three initial doses compared to only one dose of AVA ($P = 0.036$). The distribution of antibody titer before and after the booster dose of AVA is shown in Fig. 2.

3.3. Antibody response to PBT

3.3.1. Antibody response 18–24 months after initial doses of PBT

Antibodies against type A botulinum toxin were measured in 169 soldiers who had been vaccinated with PBT during ODSDS (Table 5). Overall, 47 (28%) had residual detectable antibody (≥0.02 IU/ml). The prevalence of antibodies, as well as antibody concentration, tended to increase with increasing numbers of PBT doses initially received.

3.3.2. Antibody response to booster dose of PBT

From among the 440 individuals boosted with PBT, anti-type A botulinum toxin concentrations were determined in 326 sera on day 24–36 after booster injection (Table 5). All but two of these subjects (99%) had antibody concentrations ≥0.02 IU/ml, and 315/326 (97%) had antibody concentrations ≥0.25 IU/ml. There was a significant trend

Table 5
Type A botulinum antibody in volunteers before and after PBT booster stratified by number of initial PBT doses received during ODSDS

No. of initial doses	N	Before booster		N	After booster (day 24)		
		Antibody ≥0.02 IU/ml, n (%)	Mean antibody concentration		Antibody ≥0.02 IU/ml, n (%)	Antibody ≥0.25 IU/ml, n (%)	Mean antibody concentration
1	4*	0	0.019	9	8 (88.9)	7 (77.8)	0.6
2	115	28 (24.4)	0.026	242	241 (99.6)	233 (96.3)	10.6
3	50	19 (38)	0.043	75	75 (100)	75 (100)	33
Overall	169	47 (28)	–	326	324 (99)	315 (97)	–
P-value		0.09[a]	0.0003[b]		0.055[a]	0.007[a]	<0.0001[b]

[a] *P*-value tests significance of the difference in seroconversion rate by priming dose number before and after boosting.
[b] *P*-value tests significance of the difference in mean antibody concentration by priming dose number before and after boosting.
* Omitted from *P*-values.

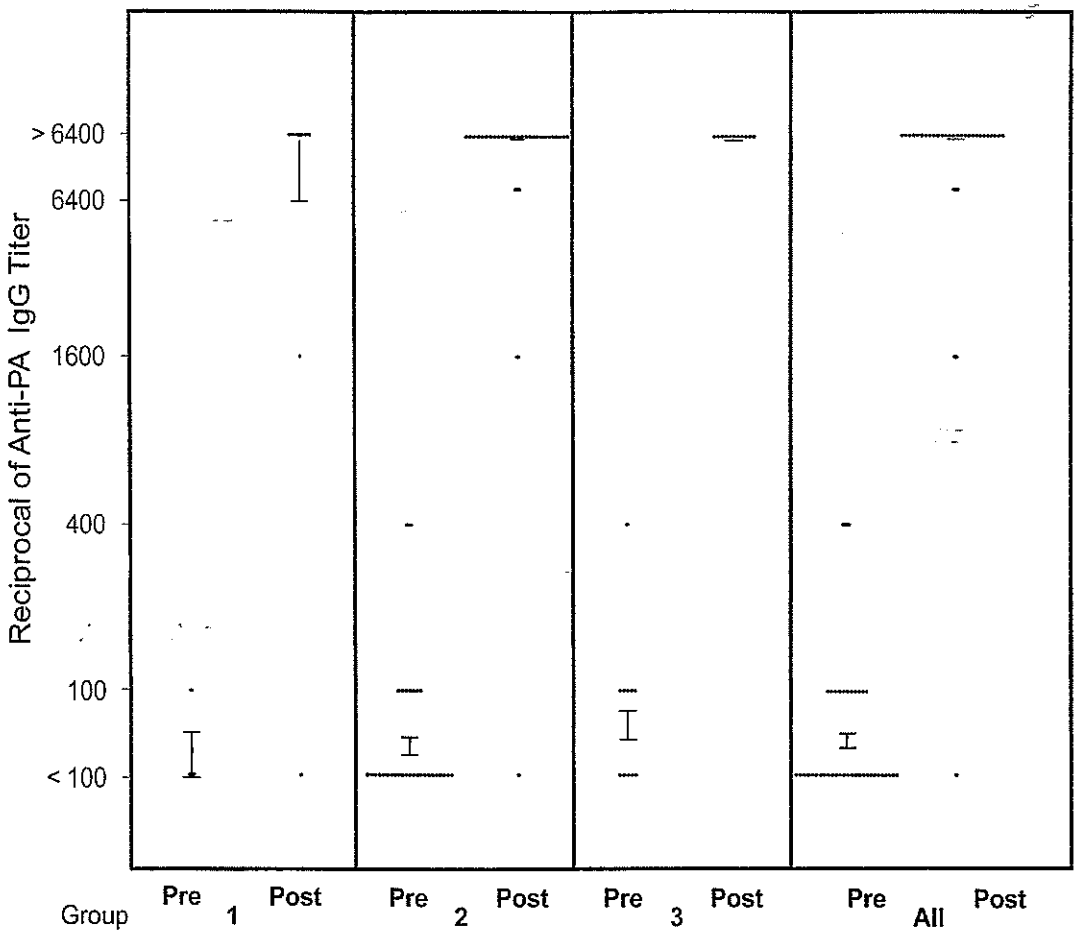

Fig. 2. Anti-PA IgG response to AVA before and following a delayed booster injection. The *x*-axis depicts anti-PA IgG titer pre and post a delayed boost dose of AVA stratified by initial AVA doses (one, two or three). Vertical bars represent geometric means with 95% confidence limits.

toward higher mean antibody concentrations with increasing numbers of PBT doses received 18–24 months previously ($P = 0.0001$). The distribution of antibody response to PBT before and after the booster is displayed in Fig. 3.

4. Discussion

Strategies for vaccination against biological weapons are complicated by logistical, cost, and risk-benefit issues. Options that provide for minimal initial dosing (priming) and later boosting in the face of imminent threat could alleviate many concerns of military and public health contingency planners.

This study demonstrates that antibodies against *B. anthracis* PA and at least one component (type A) of PBT persist in nearly a third of individuals up to 2 years after priming with one to three doses of vaccine. In addition, after boosting there was robust immune recall, even among the majority who had no detectable antibodies before boost administration. The ability to prime expectantly (e.g. during military basic training) and to boost at some distant time point (e.g. in anticipation of military deployment into hostile territory) has significant implications related to cost-savings, reducing unnecessary vaccine exposure, and general vaccine-related reactions.

Both AVA and PBT are considered to be effective in inducing protective immunity against natural disease [4–8,10]. Definitive proof of protection from possible higher aerosol challenge is problematic, however. Neither anthrax nor botulism challenge studies can be performed in humans for obvious ethical reasons. Vaccine effectiveness must therefore be inferred from in vitro experimental systems and surrogate non-human animal studies. While not perfect in replicating the human condition, non-human primates are generally considered to best model the effects of, and protection from, anthrax and botulism [6,8].

Studies in rhesus monkeys demonstrated that AVA is an effective prophylactic agent against inhalational anthrax [6]. Solid and long-lasting (up to 2 years) protection against high-dose aerosol challenges (>200 LD50 *B. anthracis*) has been shown [6].

Similarly, macaques vaccinated with PBT and challenged with small-particle aerosols of botulinum neurotoxin type

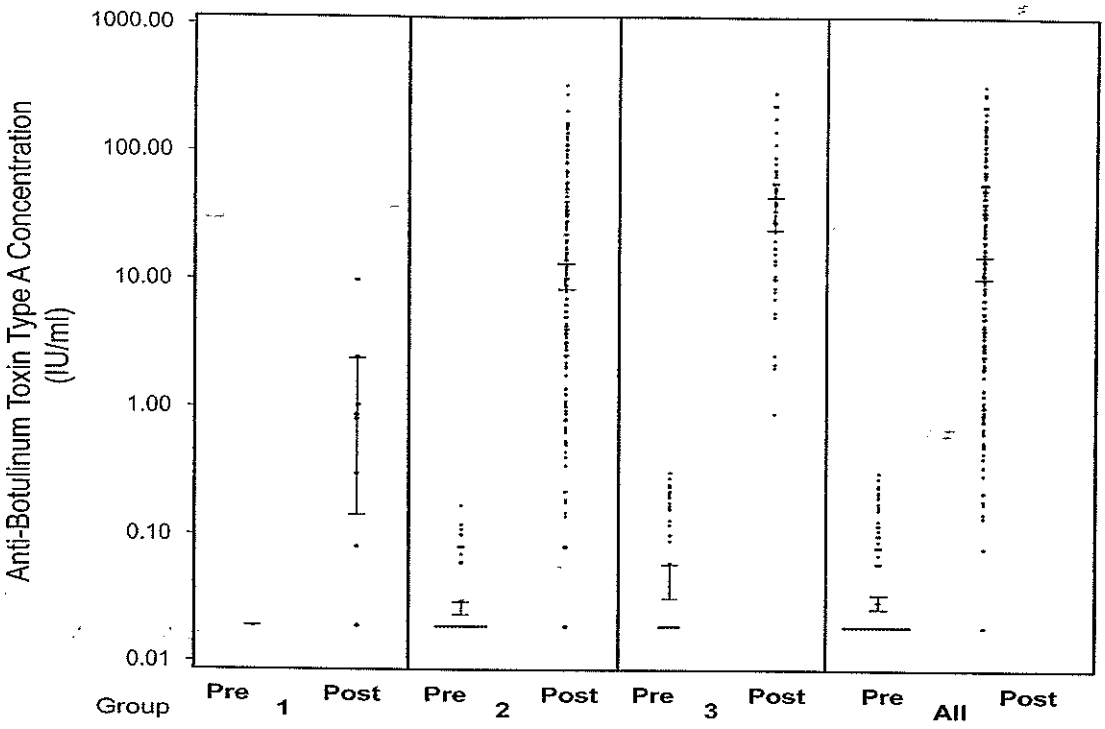

Fig. 3. Anti-botulinum neurotoxin type A antibody concentration before and following a delayed boost dose of PBT. The *x*-axis shows anti-botulinum neurotoxin type A antibody concentration pre and post, a delayed boost dose of PBT stratified by initial PBT doses (one, two or three). Vertical bars represent geometric means with 95% confidence limits.

A were protected [8,9]. Circulating antibody is clearly the critical determinant of protection against botulism; antitoxin administered prophylactically or therapeutically protected macaques against botulinum neurotoxin type A [10]. While anti-PA antibody correlates with immunity in some experimental models, ongoing experiments are attempting to define more precisely the relative contributions of humoral and cellular immunity to protection [18,19]. Among such studies will be attempts to relate the levels of anti-PA antibody that correspond to protection from aerosol challenge in model systems to antibody titers in humans.

Reactogenicity is a major determinant of vaccine acceptability. Our daily monitoring for systemic and local reactions during the first 3 days and on day 7 led to the capture of essentially any clinical symptom, including those not necessarily related to vaccination. One or more systemic complaints were recorded in 44% of recipients of AVA and/or PBT within the first 7 days after the boost. These symptoms ranged from myalgias (31%) and "fever" (20%) to rash, headaches, malaise, and arthralgias (13–17%). Other symptoms (nausea, anorexia and breathing difficulty) occurred much less commonly. Given the nature of the population vaccinated (highly active servicemen who performed strenuous exercises daily) and the fact that there was no placebo control group, these systemic complaints were difficult to attribute unequivocally to the vaccines. Moreover, attributing reactions to one or the other vaccine was generally impossible, as most volunteers (91%) received both AVA and PBT. Of significance, only 2.5% of the systemic complaints were designated by volunteers as severe enough to be noticeable, and no one stopped work because of them.

The majority (73–84%) of vaccine recipients in this study experienced no measurable injection site reaction. Those reactions that occurred were more frequent for AVA (27%) than for PBT (16%). Among those seen, most (64% of AVA and 83% of PBT) measured <50 mm, and only three (all AVA) >120 mm were recorded. Persistence of injection site reactions beyond 7 days was infrequent, although one volunteer experienced persistent induration to postvaccination day 47. These findings are generally consistent with those reported previously [4].

Two years after receiving one, two or three doses of AVA, the overall prevalence of detectable antibodies against anthrax PA was 30%. After boosting, 99.3% had measurable anti-PA antibodies in sera; only two volunteers did not elicit a detectable titer. The GMT among responders increased an impressive 33-fold (from 1:277 to 1:9183). Similarly, the prevalence of anti-type A botulinum toxin increased from 28 to 99% (97% for titer >0.25 IU/ml) after boosting, with an equally impressive rise in GMT. It should be noted, however, that responses to other toxin types present in the vaccine (i.e. types B, C, D, and E) were not measured and could well have been less prevalent. It will thus be important to extend these findings to other botulinum toxin types, as well as to observe the kinetics of antibody responses to both AVA and PBT to determine their persistence over

time and to assess the temporal requirement for additional boosts.

Using stored specimens, we previously showed that by increasing the interval between the first and second doses of AVA from 2 to 4 weeks, the seroconversion rate and the mean anti-PA antibody levels were statistically elevated [20]. This finding was confirmed recently in a randomized prospective clinical trial of AVA [21].

In this study, we showed that receipt of two or more doses of AVA and PBT can serve as an effective "primer" for safe and effective boosting at least 2 years afterward. Further studies are needed to assess the persistence of these boosted antibody levels, as well as to define the maximum length of time between priming and boosting where such effects can be observed. These findings should be considered in the design of vaccination strategies against anthrax, botulism, and possibly other biological warfare threat agents.

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References

[1] Kadlec RP, Zelicoff AP, Vrtis AM. Biological weapons control, prospects and implications for the future. JAMA 1997;278:351–6.

[2] Zilinskas RA. Iraq's biological weapons. The past as future? JAMA 1997;278:418–24.

[3] Smith RJ. Iraq had program for germ warfare: big stockpiles destroyed, UN team told. The Washington Post, 6 July 1995, Section A:01.

[4] Brachman PS, Gold H, Plotkin SA, Fekety FR, Werrin M, Ingraham NR. Field evaluation of a human anthrax vaccine. Am J Pub Health 1962;52:632–45.

[5] Friedlander AM, Pittman PR, Parker GW. Anthrax vaccine: evidence for safety and efficacy against inhalational anthrax. JAMA 1999;282:2104–6.

[6] Ivins BE, Fellows PF, Pitt MLM, Estep JE, Welkos SL, Worsham PL, et al. Efficacy of a standard human anthrax vaccine against *Bacillus anthracis* aerosol spore challenge in rhesus monkeys. Salisbury Med J Suppl 1995;87:125–6.

[7] Anderson JH, Lewis Jr. GE, Clinical evaluation of botulinum toxoids. In: Lewis Jr. GE, editor. Biomedical aspects of botulism. New York: Academic Press, 1981. p. 233–46.

[8] Brown JE, Parker GW, Pitt LM, Swauger JE, Elliott JJ, Ruble DL, et al. Protective efficacy in monkeys of pentavalent botulinum toxoid vaccine on an abbreviated immunization schedule. In: Rood J, McClane BA, Songer JG, Titball RW, editors. Abstracts of the ASM First International Conference on the Molecular Genetics and Pathogenesis of the Clostridia 11–14 Jan Rio Rico, AZ, 1995. p. 23.

[9] Brown JE, Williamson ED. Molecular approaches to novel vaccines for the control of clostridial toxemias and infections. In: Rood JI, McClane BA, Songer JG, Titball RW, editors. The clostridia: molecular biology and pathogenesis. New York: Academic Press, 1997. p. 505–24.

[10] Franz, DR, Pitt LM, Clayton MA, Hanes MA, Rose KJ. Efficacy of prophylactic and therapeutic administration of antitoxin for inhalation botulism. In: DasGupta BR, editor. Botulinum and Tetanus Neurotoxins. New York: Plenum Press, 1993. p. 473–6.

[11] Fiock MA, Cardella MA, Gearinger NF. Studies on immunity to toxins of *Clostridium botulinum*: IX. Immunologic response of man to purified pentavalent ABCDE botulinum toxoid. J Immunol 1963;90:697–702.

[12] Anthrax vaccine adsorbed. Lansing, Michigan Department of Public Health, 1978 [package insert].

[13] Iacono-Connors LC, Novak J, Rossi C, Mangiafico J, Ksiazek T. Enzyme-linked immunosorbent assay using a recombinant baculovirus-expressed *Bacillus anthracis* protective antigen (PA): measurement of human anti-PA antibodies. Clin Diag Lab Immunol 1994;1:78–82.

[14] Iacono-Connors LC, Welkos SL, Ivins BE, Dalrymple JM. Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals. Infect Immun 1991;59:1961–5.

[15] Hatheway CL, Dang C. Immunogenicity of the Neutrotoxins of *Clostridium botulinum*. In: Jankovic J, Hallett M, editors. Therapy with botulinum toxin. New York: Marcel Dekker, 1994. p. 93–107.

[16] Bowmer EJ. Preparation and assay of the international standards for *Clostridium botulinum* types A, B, C, D and E antitoxins. Bull World Health Organ 1963;29:701–9.

[17] SAS Institute. SAS/STAT User's Guide, 6th ed. vol. 2. Cary, NC: The Institute, 1990.

[18] Pitt ML, Little S, Ivins BE, Fellows P, Boles J, Barth J, et al. In vitro correlate of immunity in an animal model of inhalational anthrax. J Appl Microbiol 1999;87:304.

[19] Barnard IP, Friedlander AM. Vaccination against anthrax with attenuated recombinant strains of *Bacillus anthracis* that produce protective antigen. Infect Immun 1999;67:562–7.

[20] Pittman PR, Mangiaficio JA, Rossi CA, et al. Anthrax vaccine: increasing intervals between the first 2 doses enhances antibody response in humans. Vaccine 2001;19:213–6.

[21] Pittman PR, Gibbs PH, Cannon TL, Friedlander AM. Anthrax vaccine: short-term safety experience in humans. Vaccine 2001; 20:972–8.