Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques

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The authors examined the efficacy of Bacillus anthracis *protective antigen (PA) combined with adjuvants as vaccines against an aerosol challenge of virulent anthrax spores in rhesus macaques. Adjuvants tested included i) aluminum hydroxide (Alhydrogel), ii) saponin QS-21 and iii) monophosphoryl lipid A (MPL) in squalene/lecithin/Tween 80 emulsion (SLT). Animals were immunized once with either 50 mg of recombinant PA plus adjuvant, or with Anthrax Vaccine Adsorbed (AVA), the licensed human anthrax vaccine. The serological response to PA was measured by enzyme-linked immunosorbent assay. Lymphocyte proliferation and serum neutralization of* in vitro *lethal toxin cytotoxicity were also assayed. In all vaccine groups, anti-PA IgM and IgG titers peaked at 2 weeks and 4—5 weeks postimmunization, respectively. Five weeks postimmunization, animals in all vaccine groups demonstrated PA-specific lymphocyte proliferation and sera that neutralized* in vitro *cytotoxicity. Six weeks after immunization, the animals were challenged by aerosol with approximately 93 LD_{50} of virulent anthrax spores. Animals were bled daily for 1 week to monitor bacteremia, and deaths were recorded. Anti-PA ELISA titers in all groups of immunized animals were substantially increased 2 weeks after challenge. One dose of each vaccine provided significant protection (>90%) against inhalation anthrax in the rhesus macaques. Published by Elsevier Science Ltd.*

Keywords: anthrax; *Bacillus anthracis;* vaccine

The human anthrax vaccine currently licensed for use in the United States (Anthrax Vaccine Adsorbed, or AVA) is produced by the Michigan Biological Products Institute (formerly, the Michigan Department of Public Health) and consists primarily of protective antigen (PA) produced in fermentor cultures of a toxinogenic, nonencapsulated strain of *Bacillus anthracis*, V770-NP1-R[1,2], and adsorbed onto aluminum hydroxide. As licensed, the immunization schedule for the vaccine recommends six doses administered within 18 months, followed by yearly boosters. The culture supernatant from which the vaccine is made varies from lot to lot with respect to the concentration of PA, residual, small quantities of lethal factor and lesser amounts of edema factor and other undefined bacterial products (J. Ezzell and J. Novak, personal communications). Subcutaneous (s.c.) administration of the vaccine occasionally causes local reactions[3,4]. Although AVA has proven highly efficacious against inhalation anthrax in non-human primates[5] and safe in humans, there is an effort to develop an improved human vaccine. Indeed, considerable research over the past several years has focused upon the development and testing of anthrax vaccine candidates that could be characterized more precisely, would contain only bacterial components that were effective immunogens, and would require fewer doses[4,6-12].

Numerous immunological adjuvants have been tested in humans, but only aluminum salts such as aluminum hydroxide, aluminum phosphate and aluminum potassium sulfate are approved by the US Food and Drug Administration for human use[13,14]. In these studies the authors tested the following adjuvants combined with recombinant PA:

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aluminum hydroxide (Alhydrogel)[13,15], the saponin QS-21[16,17], and monophosphoryl lipid A (MPL) in a squalene/lecithin/Tween 80 emulsion (SLT)[12,18,19]. Although the authors have investigated several new human anthrax vaccine candidates in guinea pigs[11,12], they have found little efficacy or serological response data for these candidates in non-human primates. The rhesus macaque was used in recent studies as a model for human inhalation anthrax[20,21], and thus, the vaccine studies reported here were undertaken with rhesus macaques as the test animals.

MATERIALS AND METHODS

Experimental animals

Healthy male and female rhesus macaques *(Macaca mulatta)* that were 3-13.6 kg and had never been exposed to *B. anthracis* were selected for the study from the USAMRIID primate colony. Before immunizations, all animals were tested and found to be negative for simian retrovirus, simian immunodeficiency virus, and simian T-lymphotropic virus. Each vaccine group contained either 9 or 10 animals. Three animals given phosphate-buffered saline[22] (PBS) plus adjuvants were designated as controls.

Protective antigen

A single lot of recombinant PA was used in these studies. It was produced at the US Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD, from a fermentor culture of the *B. anthracis* ΔSterne-1(pPA1O2)CR4 strain, purified by anion exchange high pressure liquid chromatography, and stored at −70°C (J. Farchaus, submitted for publication). It was administered intramuscularly (i.m.) with the various adjuvants in PBS at a total volume of 0.5 ml. The dose of PA in the various recombinant vaccine preparations was 50 μg.

AVA

The licensed human anthrax vaccine was purchased from the Michigan Department of Public Health. It was injected i.m. in 0.5 ml doses.

Adjuvants

Alhydrogel, a 3% aluminum hydroxide gel suspension, was manufactured by Superfos Biosector a/s, Denmark, and supplied by E. M. Sargeant Pulp and Chemical Co., Inc., Clifton, NJ. PA in PBS was adsorbed to a suspension of the aluminum hydroxide[12], then administered i.m. to rhesus macaques in 0.5 ml doses. Each vaccine dose contained 0.725 mg of metallic aluminum.

Monophosphoryl lipid A (MPL, Ribi Immunochem Research, Inc., Hamilton, MT) was administered with PA in an emulsion (SLT) containing 2% (v/v) squalene, 0.24% (w/v) lecithin and 0.08% (v/v) Tween 80[12]. MPL is a derivative of diphosphoryl lipid A from *Salmonella minnesota* R595, detoxified by removal of the phosphate group from the reducing end of the molecule. The final concentration of MPL in the vaccine was 100 μg per 0.5 ml dose.

QS-21 saponin (gift of Oscar Kenshala, Cambridge Biotech, Worcester, MA) was dissolved in 10 mM sodium phosphate, pH 6.0, then added to PBS+PA to give a final concentration of 50 μg of QS-21 per 0.5 ml dose[12].

Immunization

Six weeks before challenge, the animals were given a single, 0.5 ml, i.m. dose of either AVA, PA+Alhydrogel, PA+QS-21, or PA+MPL in SLT. Each of three control animals received PBS+either Alhydrogel, QS-21, or MPL in SLT.

B.anthracis challenge

Spores of the virulent *B. anthracis* Ames strain[23] were harvested from shaking broth cultures of Leighton and Doi medium, purified by centrifugation through 58% Renografin-76, and resuspended in sterile water for injection containing 1% phenol[5,24,25]. Six weeks after immunization and immediately before aerosol challenge, the spores were diluted in sterile water for injection to 1.5 x 10^9 CFU ml^{-1}, heat-shocked at 60 C for 45 min, and divided into 8 ml aliquots. After minute respiratory volumes were measured, animals were exposed in a head-only chamber to a spore aerosol generated by a three-jet Collison nebulizer[5,20,26,27]. For each animal, the concentration of spores in the aerosol inhaled dose (expressed as LD_{50}) was determined by plating a sample from an all glass impinger onto tryptic soy agar plates (Difco, Detroit, MI). One aerosol LD_{50}, in rhesus macaques is 5.5 x 10^4 spores[5]. Animals challenged by aerosol received approximately 93±63 (mean±S.D.) LD_{50} of spores and were observed for 90 days.

Lymphocyte proliferation studies

Immediately prior to immunization, as well as 5 weeks afterward, animals were anaesthetized with Telazol (3mg kg^{-1}, administered i.m.), and 10 ml of heparinized blood were obtained from each monkey by venipuncture and diluted to 30 ml with sterile Hanks Balanced Salt Solution (HBSS). This cell

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suspension was gently layered onto 12 ml of endotoxin-free Histopaque 1077 (Sigma Chemical Company, St. Louis, MO) in a sterile 50 ml centrifuge tube and centrifuged at 250 x g for 30 min at room temperature. The band containing leukocytes was transferred to a second 50 ml conical centrifuge tube and HBSS added to a volume of 20 ml. The cells were centrifuged at 180 x g for 10 min at room temperature and the supernatant discarded. After an additional wash, the cells were suspended in 5 ml of growth medium (RPMI 1640 containing 5% pooled human AB plasma (BioWhittaker, Walkersville, MD), 50 mM 2-mercaptoethanol, 2mM L-glutamine, 25mM HEPES, and 0.5 mg ml^{-1} gentamycin sulfate).

Leukocytes were adjusted to a concentration of 2 x 10^6 ml in growth medium and added to individual wells of 96-well cell culture plates in a volume of 180 ml. 20ml containing 0.55 mg of PA were added to appropriate wells. Cell culture plates were incubated at 37^0C with 90% relative humidity and 5% CO_2 for 5days. On the fourth day, 1 mCi ^{3}H-thymidine (specific activity 5 Ci $mmole^{-1}$) was added to each well, and 18—20 h later the cells were harvested onto glass fiber filters. Radiolabel uptake was determined by standard scintillation techniques. Stimulation indices (SI) were calculated by dividing the mean cpm of 3 PA-stimulated wells by the mean cpm of three corresponding control wells.

Cytotoxicity neuti'alization (TN) studies

An *in vitro* colorimetric assay for cell growth and viability[28,29] was used as described previously[30] with the following modifications. J774A.l cells were suspended to 5 x 10^5 ml^{-1}, and 0.2 ml aliquots were added to each well of a multiwell cell culture plate. The cells were cultured for 2 days. Antiserum from the samples at week 5 was diluted in medium and preincubated for 1 h with PA added to a concentration of 100 ng ml^{-1}. Lethal factor was added to the wells at a concentration of 40 ng ml^{-1}. Antiserum samples in PA were then transferred to the J774A.l cells. Control wells received medium without PA or LF. Cell lysis was achieved by adding 20% (w/v) sodium dodecyl sulfate in 50% dimethylformamide, pH 4.7, 2 h after adding 3-[4.5-dimethyl-thiazol-2-y-]2,5-diphenyltitrazolium bromide (MTT)[31]. Cell viability was calculated after determining on a multiwell plate autoreader the mean A_{570} of pairs of wells receiving either toxin+antiserum or antiserum alone. The authors selected the dilution of antiserum that inhibited 50% of cell death as a quantitative measure of the *in vitro* neutralizing ability of the antiserum.

Serological studies

Animals were anaesthetized as above, bled by venous puncture, and sera were analyzed for antibody to PA by enzyme-linked immunosorbent assay (ELISA)[5,32]. with IgG measured by direct ELISA [5,32], and IgM measured by an indirect capture ELISA[32]. Reciprocal geometric mean anti-PA ELISA titers were determined.

Bacteremia studies

After challenge, approximately 1 ml of blood was drawn daily by venous puncture from each anaesthetized animal for 1 week. 1/10 ml aliquots of serial dilutions of the blood were plated in triplicate onto tryptic soy agar. After incubating for 18 h at 37^0C, the plates were examined and *B. anthracis* colonies counted.

Statistical analysis

Mortality differences among immunization groups were compared by using Fisher's exact test. Differences in ELISA, cytotoxicity neutralization (TN), and SI titers among the 4 vaccine groups were examined by analysis of variance, followed by multiple tests of pair-wise treatments adjusted for multiple comparisons by the Tukey—Kramer method. The association of ELISA IgM titers 2 weeks postimmunization and IgG ELISA. CN and SI titers 5 weeks postimmunization with bacteremia was examined by calculating the odds ratio of an animal being non-bacteremic versus bacteremic after stepwise logistic regression for the four assays.

Table 1 Vaccine efficacy against *B. anthracis* Ames spore aerosol challenge[a].

Vaccine	Survived/ Challenged	(%)	Mean of weight animals	Mean dose (LD_{50})
Control[b]	0/3	(0)	8.20 kg	68.3
AVA[C]	10/10	(100)	5.88 kg	74.4
PA.+Alhydrogel	10/10	(100)	5.41 kg	116.9
PA+QS-21[c]	9/9	(100)	5.97 kg	78.3
PA+MPL in SLT[c,d]	9/10	(90)	621 kg	96.0

[a]Rhesus macaques were given a single intramuscular dose of vaccine, then challenged by aerosol 6 weeks later with approximately 93 LD_{50} of spores. Survival or death was noted.
[b]Two deaths on day 3; one death on day 5. Terminal bacteremia range = 5.4 x 10^7—1.0 x 10^8 CFU $m1^{-1}$.
[c]Survival value significantly greater (P<O.O5) than that of the control group.
[d]One death on day 5, with a terminal bacteremia of 7.0 x 10^7 CFU ml^{-1}.

Statistical tests were conducted at the 95% confidence level.

(3)

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RESULTS

PA+Alhydrogel, PA+QS-2l and the licensed human anthrax vaccine, AVA, completely protected the monkeys from an aerosol challenge by spores of the *B. anthracis* Ames strain *(Table 1)*. PA+MPL in SLT protected 9 out of 10 animals. The one animal in this group that died 5 days after challenge had a terminal bacteremia of 7.0 x 10^7 CFU ml^{-1}. The 3 control animals died 3—5 days after challenge, with terminal bacteremias ranging from 5.4 x 10^7—1.0 x 10^8 CFU ml^{-1}. All 4 vaccines were significantly better than the controls (PBS+adjuvant) in protecting against mortality. None of the experimental vaccines appeared to elicit any remarkable local or systemic reactions.

B. anthracis bacteremia was seen in all groups of animals except those immunized with PA+QS-21 *(Table 2)*. All 3 controls were bacteremic beginning 2 days after challenge until death at either day 3 or day *5*. Two animals in the AVA-immunized group and two in the group given PA+Alhydrogel demonstrated transient bacteremias for 1 day only. Four of the surviving nine animals immunized with PA+MPL in SLT had transient bacteremias lasting 1—3 days. The single animal that died in this group was bacteremic from day 2 until death on day 5.

Table 2 Bacteremia in vaccinated rhesus macaques after *B. anthracis* aerosol spore challenge[a]

	No. Bacteremic	
Vaccine	Total	(%)
Controls[b]	3/3	(100)
AVA[c]	2/10	(20)
PA+Alhydrogel[d]	2/10	(20)
PA+QS-21	0/9	(0)
PA+MPL in SLT[e]	5/10	(50)

[a]After challenge, vaccinated animals were bled daily for 1 week after challenge. Levels of bacteremia in living monkeys were noted after plating dilutions of blood onto tryptic soy agar and counting colonies after incubation at 37°C.
[b]Bacteremia range = 3—20,000 CFU ml^{-1}.
[c]Bacteremia range = 30—230 CFU ml^{-1}.
[d]Bacteremia range = 3—27 CFU ml^{-1}.
[e]Bacteremia range = 13—2270 CFU ml^{-1}.

Animals exhibited no anti-PA IgM titer until 2 weeks after immunization, when titers had reached their maximum *(Figure 1)*. Thereafter, the IgM titers steadily declined until they reached preimmunization baseline levels 6 weeks after immunization. Prechallenge IgG titers to PA were also first demonstrable 2 weeks after immunization *(Figure* 2). Reciprocal geometric mean IgG titers from all groups of immunized animals peaked at either 4 or 5 weeks, then decreased at 6 weeks. (No sera was obtained at 3 weeks.) With sera from the animals at the 5-week time point, the only significant difference between groups was between PA+MPL in SLT and PA+Alhydrogel *(P* = 0.0213). After challenge at 6 weeks, mean anti-PA IgG titers increased dramatically in all immunization groups *(Figure 3)*. Comparison of mean titers 2 weeks after challenge with titers immediately before challenge gave increases of 19-, 23-, *57*- and 62-fold for animals vaccinated with AVA, PA+Alhydrogel, PA+QS-21, and PA+MPL in SLT, respectively. The standard error of the geometric mean titers plotted in *Figures 1—3* varied from 1.0 to 1.9.

In the lymphocyte proliferation assay *(Table 3)*, there was a statistically significant elevation in SI at *5* weeks postimmunization compared with the baseline pre-immunization SI in animals immunized with either AVA, PA+Alhydrogel or PA+QS-21 *(P<0.05)*. The SI increase seen in the group immunized with PA+MPL in SLT was not significant *(P* = 0.69). At 5 weeks post-immunization, the mean SI of animals in all of the immunized groups were higher than the mean of the control group, although the differences in SI from the control group were not statistically significant. Indeed, some of the animals from the immunized groups had a lower SI than one of the controls. The single animal that died in the group immunized with PA+MPL in SLT had a SI of 4.67, which was higher than that of 6 of the surviving animals in the same group.

Mean cytotoxic neutralization (TN) titers *(Table* 4) were highest for the group of animals immunized with PA+QS-2l and lowest for the animals that had received PA+MPL in SLT. The latter group of animals also had the highest rate of bacteremia and the lowest mean IgM and IgG ELISA titers.

Correlations among the ELISA IgM, ELISA IgG, CN and SI titers were low *(P> 0.05)*. Analysis of the four assays with respect to bacteremia (stepwise selection of predictors of non-bacteremic status) determined that IgM titers followed by IgG titers were predictors of being non-bacteremic *(P<0.05)*. The odds of being non-bacteremic increased 5.66-fold for every one $\log_{10}$ increase in IgM titer and 6.46-fold for every one $\log_{10}$ increase in IgG titer when each was adjusted for the other. (The odds ratios are estimates of the independent effect of the two variables.) Neither CN nor SI titers had a statistically significant effect on predicting bacteremia *(P* > 0.05) when all four variables were considered in the model, that is, when IgM and IgG titers were known, or when they were considered by themselves.

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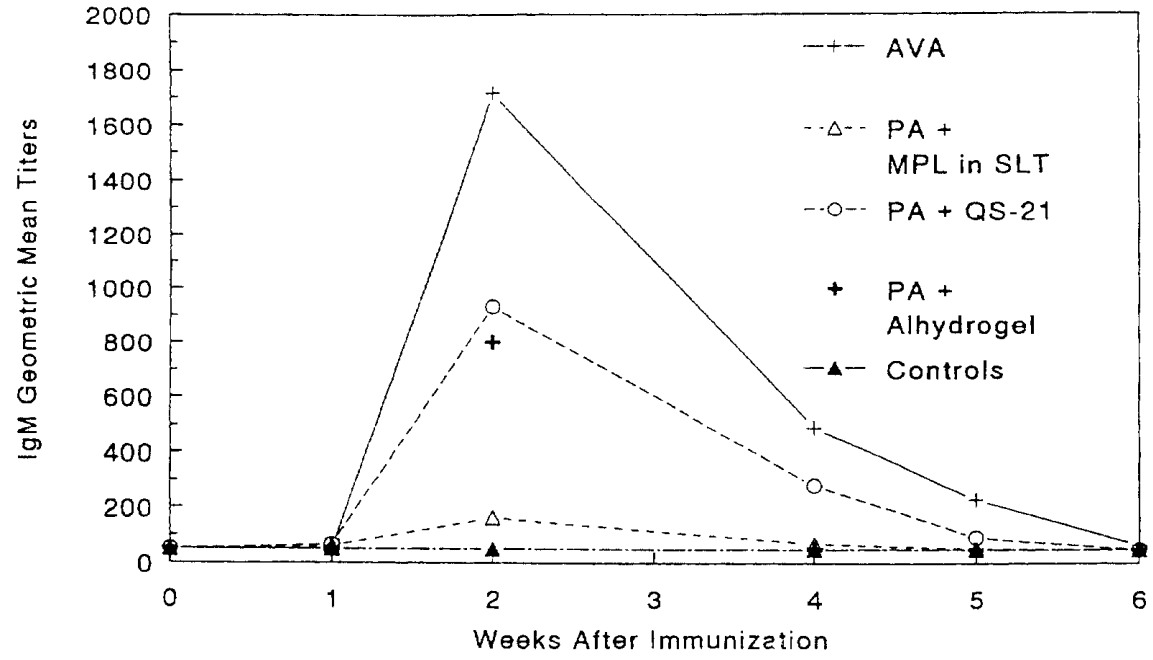

Figure 1 Prechallenge reciprocal geometric mean anti-PA IgM ELISA titers.

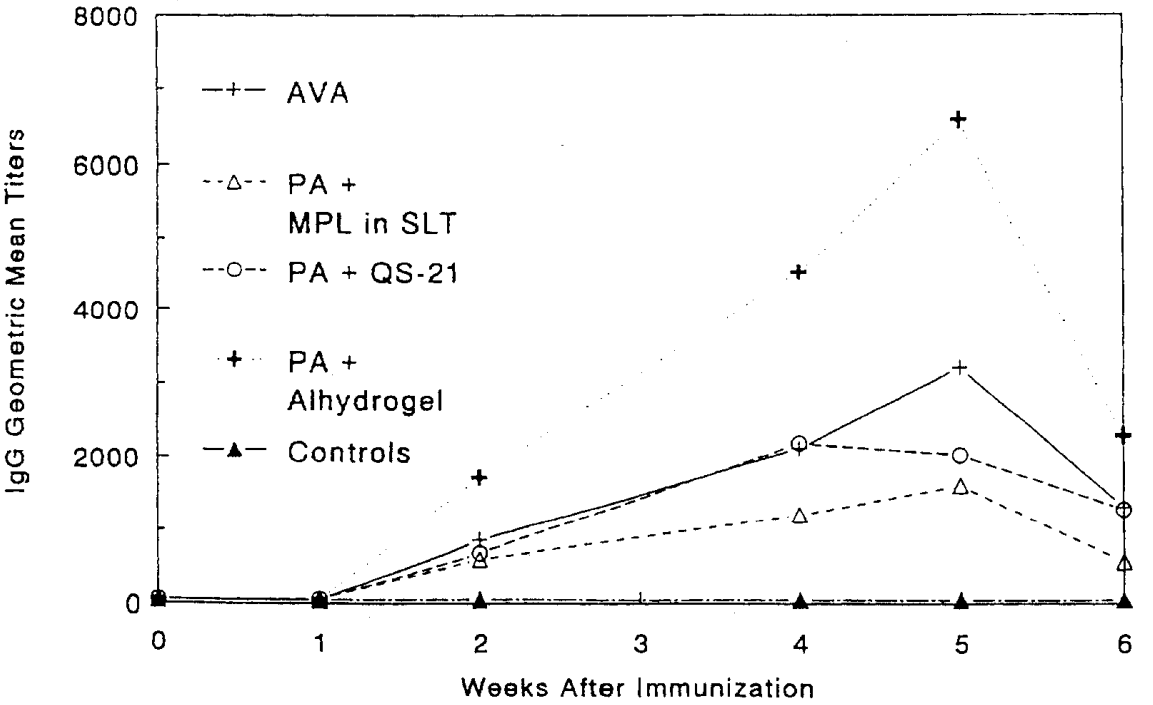

Figure 2 Prechallenge reciprocal geometric mean anti-PA IgG ELISA titers. **(5)**

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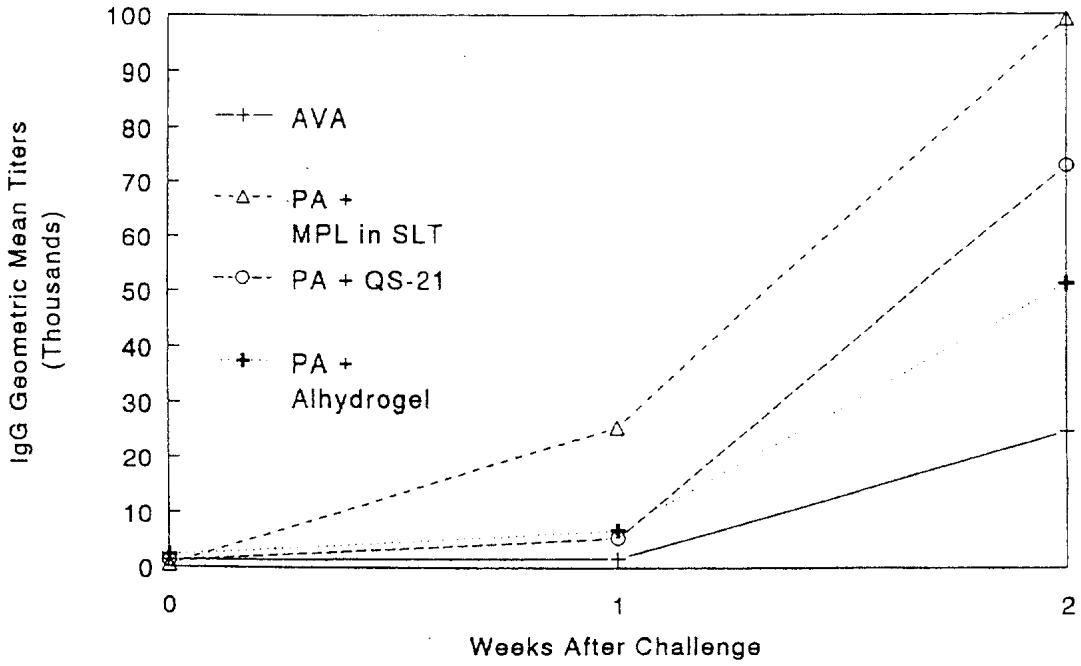

Figure 3 Postchallenge reciprocal geometric mean anti-PA IgG ELISA titers.

Table 3 Mean prechallenge stimulation indices in vaccinated rhesus macaques[a]

	Stimulation Index			
Vaccine	Before immunization	(range)	After immunization	(range)
Controls	1.37	(0.57—2.29)	1.11	(0.55—1.68)
AVA[b]	1.04	(0.44—1.71)	6.11	(1.06—13.47)
PA+Alhydrogel[b]	1.16	(0.64—2.35)	4.02	(1.48—7.47)
PA+QS-21[b]	1.34	(1.03—2.19)	7.10	(1.55—11.58)
PA+MPL in SLT	1.40	(0.71—2.29)	6.46	(1.17-25.62)

[a]Immediately before immunization and 5 weeks afterward, animals were bled and the leukocytes separated and purified. They were incubated with PA and ^{3}H-thymidine, and stimulation indices determined after measurement of radiolabel uptake.
[b]Mean SI values after immunization were significantly greater than before immunization ($P < 0.05$).

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Table 4 Mean cytotoxic neutralization (TN) titers in vaccinated rhesus macaques[a]

Vaccine	Geometric Mean TN Titer
Controls	50
AVA	424
PA+Alhydrogel	823
PA+QS-21	947
PA+MPL in SLT[b]	57

[a]Five weeks after immunization, animals were bled and serum was collected. Lethal toxin-neutralizing activity was assayed in J774A.1 cells.
[b]Significantly different from other vaccine groups *(P* <0.001). No differences were found among other test groups.

DISCUSSION

Results of recent studies show that anthrax vaccines vary in their efficacy among different species[11,12,23,21]. Mice[4,11,33-35], rabbits[27,36-40], guinea pigs[4,6-9,11,12,23,24,37,41-48], monkeys [5,20,38,49,50] and other animal species have all been used as test animals in anthrax vaccine investigations. Mice are difficult to immunize against anthrax[10,33]. Indeed, studies by Welkos et al[51,52] demonstrated that toxin-negative, capsule-positive variants of the Ames strain of *B. anthracis* retained more virulence for the strains of inbred mice tested than did toxin-positive, capsule-negative strains. Thus, in mice, the capsule rather than the toxin appears to be the primary virulence factor, and vaccines based on the PA moiety of lethal or edema toxin possess reduced efficacy in mice. This is supported by the finding that immunization with AVA is unable to protect mice from challenge with virulent *B. anthracis* spores[30,33]. Substantial protection of mice was obtained only when PA was combined with a potent adjuvant[10].

Guinea pigs have been used for most recent anthrax vaccine efficacy studies[4,6-9,11,12,21,23,24,41-45]. They are partially protected by AVA from either a parenteral or aerosol challenge of virulent *B. anthracis* spores[4,6-9,11,12,21,23,24,42,43],but immunization with PA combined with certain new adjuvants has yielded strong protection in several studies[11,12,45]. In recent studies, PA+MPL in SLT has induced strong protection against either a parenteral challenge (B. Ivins, unpublished observations) or an aerosol challenge of virulent anthrax spores[12]. Immunizing guinea pigs with PA+QS-21 has provided substantial protection against an aerosol spore challenge[12].

Although rabbits were used for many early studies of anthrax vaccine efficacy[36-40], they have been generally replaced by guinea pigs as test animals. However, a recent study involving both species[21] suggested that rabbits were more easily immunized than guinea pigs against inhalation anthrax with either AVA or PA+Alhydrogel. PA+MPL in SLT was highly efficacious in both species.

A study in rhesus macaques demonstrated that immunization with AVA at 0 and 2 weeks protected them against an aerosol anthrax spore challenge for at least 2 years[5]. However, there was no data from non-human primates that compared AVA with other anthrax vaccine candidates. The data from this study show that rhesus macaques are protected from an aerosol challenge of anthrax spores by a single immunization with either AVA, PA+Alhvdrogel, PA+QS-21 or PA+MPL in SLT. The animals had a strong immune response to PA, as demonstrated by their ELISA titers to PA, by their TN titers, and by the PA-induced increase in their lymphocyte proliferation SI.

The high level of protection granted by immunization with the various vaccines made it difficult in this study to correlate anti-PA IgM and IgG ELISA titers, TN titer, or lymphocyte proliferation SI with levels of protection. Indeed, in these studies, there was no anti-PA ELISA titer, TN titer, or SI level that would serve as a surrogate marker of immunity to an aerosol anthrax spore challenge. However, IgM titers (2 weeks postimmunization) and IgG titers did combine in a stepwise logistic regression statistical model to form a strong predictor of bacteremia. Further work on development of an *in vitro* correlate of immunity to inhalation anthrax is obviously required.

The adjuvant in AVA is aluminum hydroxide, which is a potent stimulator of humoral, but not cell-mediated immunity[13,15]. The substantial protection granted rhesus macaques against an aerosol challenge of anthrax spores by AVA in this and previous research[5] not only demonstrates the high level of efficacy of the currently licensed vaccine against inhalation anthrax, it also suggests that humoral immunity is important in the specific resistance of rhesus macaques to anthrax. In mice and guinea pigs, however, immunization with AVA results in substantially lower protection than in rhesus macaques[4,6-12,21,23,24,33,42,43]. This data may reflect differences in host susceptibility or in the relative importance of the various mechanisms of immunity to anthrax among animal species.

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Alternatively, they may indicate that AVA stimulates certain critical parameters of cell-mediated immunity to a greater extent in rhesus macaques than in mice or guinea pigs. It is unknown what immune mechanisms in humans are important in specific resistance to anthrax. Perhaps both cellular and humoral immunity play a role. The efficacy in humans of a protective antigen vaccine containing an aluminum salt adjuvant points to a role for humoral immunity[3]. On the other hand, individuals who have been immunized against anthrax demonstrate delayed-type hypersensitivity to *B. anthracis* antigens, suggesting the importance of cell-mediated immune mechanisms[41]. The authors' efforts to find a single surrogate marker or correlate of immunity indicate that specific immunity to anthrax in primates may be complex and involve multiple mechanisms of immunity.

The various lots of recombinant PA used in these and other studies[12,21] have contained approximately 88—94% of the 83 kDA PA polypeptide, as determined with SDS-PAGE, SDS-CE, and reversed phase chromatography (J. Farchaus, submitted for publication). These lots of PA were fully immunogenic in guinea pigs, rabbits, and rhesus monkeys[12,21]. Other investigations on the crude fermentation supernatants used in making AVA have indicated considerable variability in the protein content, although 83 kDa PA was the major component in each case (J. Farchaus, unpublished data). The exact composition of the AVA used in these studies is difficult to assess due to non-quantitative desorption of protein from the Alhydrogel adjuvant. The formaldehyde excipient in AVA also complicates analysis, in that it causes considerable modification of the proteins in the licensed vaccine.

The results of these studies have been a critical prerequisite to initial tests in humans of an anthrax vaccine candidate. Indeed, the efficacy data specifically suggest that any of several potential new anthrax vaccines may be useful in humans as prophvlaxis against anthrax.

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