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Immunogenicity of Recombinant Protective Antigen and Efficacy against Aerosol Challenge with Anthrax

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Immunization with a recombinant form of the protective antigen (rPA) from *Bacillus anthracis* has been carried out with rhesus macaques. Rhesus macaques immunized with 25 μg or more of *B. subtilis*-expressed rPA bound to alhydrogel had a significantly increased immunoglobulin G (IgG) response to rPA compared with macaques receiving the existing licensed vaccine from the United Kingdom (anthrax vaccine precipitated [AVP]), although the isotype profile was unchanged, with bias towards the IgG1 and IgG2 subclasses. Immune macaque sera from all immunized groups contained toxin-neutralizing antibody and recognized all the domains of PA. While the recognition of the N terminus of PA (domains 1 to 3) was predominant in macaques immunized with the existing vaccines (AVP and the U.S. vaccine anthrax vaccine adsorbed), macaques immunized with rPA recognized the N- and C-terminal domains of PA. Antiserum derived from immunized macaques protected macrophages in vitro against the cytotoxic effects of lethal toxin. Passive transfer of IgG purified from immune macaque serum into naive A/J mice conferred protection against challenge with *B. anthracis* in a dose-related manner. The protection conferred by passive transfer of 500 μg macaque IgG correlated significantly ($P = 0.003$; $r = 0.4$) with the titers of neutralizing antibody in donor macaques. Subsequently, a separate group of rhesus macaques immunized with 50 μg of *Escherichia coli*-derived rPA adsorbed to alhydrogel was fully protected against a target dose of 200 50% lethal doses of aerosolized *B. anthracis*. These data provide some preliminary evidence for the existence of immune correlates of protection against anthrax infection in rhesus macaques immunized with rPA.

Bacillus anthracis is the causative agent of anthrax, a disease that in the inhalation form has a fatal outcome in humans when untreated. *Bacillus anthracis* presents a bioterrorist threat, partly due to its recent history of use in the United States and partly because the spore form of the organism is very stable in the environment and can be difficult to eradicate. Existing vaccines in the United Kingdom and United States are based on cell-free filtrates of nonencapsulated strains of *Bacillus anthracis* adsorbed to alum-based adjuvants. Attributed to these vaccines are a number of transient side effects (28), poor protection from challenge with aerosolized spores in certain animal models (4), and the need for repeated immunization in order to achieve protection (21). Therefore, there is a requirement for a defined anthrax vaccine that requires fewer doses to achieve protective immunity much more rapidly and which has an improved safety profile (14). Since protective antigen (PA) is known to be the key immunogen in the existing vaccines, the simplest initial approach to achieving a defined vaccine is to use PA in conjunction with a suitable adjuvant.

Recombinant PA (rPA) has previously been expressed and purified from *Bacillus subtilis* (16) and has been demonstrated to be immunogenic and protective in small animal models (15, 16). However *B. subtilis* is not an ideal expression system for scale-up manufacture, due to its sporulating nature and thus persistence in manufacturing plants. *Escherichia coli* provides an attractive alternative, although the export and authentic folding of a heterologous protein from this gram-negative expression system may not be straightforward. In order to circumvent such potential problems, the PA gene (*pagA*) has been resynthesized with altered codon bias to achieve satisfactory expression from *E. coli* (8).

As a part of the requirement to develop a defined and rapidly acting vaccine, there is a need to understand the mechanisms of protective immunity operating in an appropriate animal model. This will eventually allow the identification of immunological correlates of protection, so that the equivalent immune response data can be sought in humans as surrogate markers of efficacy. Small animal models used in the development of anthrax vaccines have included mice (7, 28, 29, 30), guinea pigs (6, 15), and rabbits (20), while rhesus macaques have been used as a nonhuman primate model (9–11). Rhesus macaques have analogous major histocompatibility complex class I and II and cross-reactive immunoglobulin G (IgG) compared with humans (5, 13, 25) and have been used as a surrogate model for human immune responses to anthrax vaccination (6, 10, 11).

Numerous studies have shown that PA is the most important antigen in natural and vaccine-induced immunity. PA is an 83-kDa protein which combines with lethal factor (LF) and edema factor (EF) to produce the *B. anthracis* binary toxins. PA has four domains that are functionally and structurally distinct (19). Domain 4 binds PA to a host cell receptor(s) via its carboxyl terminus (29), while the amino-terminal of domain

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1 of PA is cleaved by furin-like proteases to release a 20-kDa fragment and permit binding of either LF or EF to the cleavage site (19). The remaining portion of PA (PA63) heptamerizes on the cell surface to form a pore, thus allowing access of PA bound to LF or EF into the cytoplasm. Once intracellular, the lethal toxin complex of PA with LF is cytotoxic. Domains 2 and 3 of PA are thought to be involved in the interactions between PA63 monomers and the assembly of heptamers (17), while domain 4 alone has been demonstrated to be a potent immunogen that is protective against whole-organism challenge (7).

When presented to the immune system in an appropriate adjuvant, rPA derived from either *B. subtilis* (16) or *B. anthracis* has also been shown to protect rodents and nonhuman primates from an aerosol challenge with fully virulent *B. anthracis* spores (9, 11, 15). Protection by rPA in rodent and nonhuman primate models is likely to be T-cell dependent (15, 31, 32) and also is mediated by the presence of neutralizing antibody (2, 9).

Some studies have shown that there is not a positive correlation between the amount of total circulating IgG to PA and protection against *B. anthracis* in the guinea pig or rhesus macaque, but a direct correlation has been found between the titer of neutralizing antibody and protection against challenge in the rabbit model (20). Other studies have used cytotoxicity assays to correlate protective immunity in guinea pigs with the levels of neutralizing antibodies present in serum samples (23).

Here we have demonstrated that rPA from both *B. subtilis* and *E. coli* is immunogenic and protective in the A/J mouse. The immunogenicity of rPA derived from either source in nonhuman primates has also been compared with that of the PA in the existing licensed anthrax vaccines. Improved immunogenicity of rPA compared with the existing anthrax vaccines was observed in nonhuman primates. Using survival data from mice passively immunized with IgG purified from rhesus macaque serum and also from immunized rhesus macaques challenged by the aerosol route with *B. anthracis*, we have made some initial observations on the immune correlates of the protection observed.

MATERIALS AND METHODS

Sources of recombinant PA. (i) Expression and purification from *B. subtilis*. Recombinant PA was expressed and purified from *B. subtilis* as previously described (16). WB600(pPA101) was cultured in tryptone medium. Cultures were incubated with shaking at 37°C for 8 h prior to harvesting by centrifugation. The supernatant was subjected to ammonium sulfate precipitation prior to purification by anion-exchange chromatography and gel filtration chromatography.

(ii) Expression and purification from *E. coli*. The PA gene in plasmid pAG163 was resynthesized with *E. coli* codon bias (8) and subcloned from pAG163 into expression plasmid pET29A via NdeI and XhoI restriction sites. Subsequently, pAG163 was retransformed into an *E. coli* K-12 host strain (UT5600) and stored at −70°C until required. UT5600/pAG163 was grown in shake flask culture on Luria broth at 37°C prior to addition of 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) to induce expression of rPA as insoluble inclusion bodies. Recombinant PA was extracted from inclusion bodies by standard methods, followed by refolding of the protein and purification by conventional chromatographic methods, followed by ultrafiltration and diafiltration.

The rPAs from the two sources were compared by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Recombinant PA (1 μl diluted to 0.5 mg/ml) from each source was loaded onto a 10 to 15% gradient gel with molecular mass markers in the range from 14 to 97 kDa (Amersham Biosciences UK Ltd., Bucks). The gel was subsequently stained with PhastGel Blue R (Amersham Biosciences).

Existing anthrax vaccines. Anthrax vaccine adsorbed (AVA) (Biothrax, batch FAV 038) was obtained from MDPH/Bioport, Lansing, Mich. Anthrax vaccine precipitated (AVP) (batch 350/E) was obtained from the United Kingdom Department of Health.

Active immunization. All animal procedures adhered strictly to the Animals (Scientific Procedures) Act of 1986. Nonhuman primates and rodents were offered food and water ad libitum, and environmental enrichment appropriate to the species was provided. Specific-pathogen-free BALB/c and A/J mice were obtained from Charles River and Harlan Laboratories, respectively. Rhesus macaques (*Macaca mulatta*) in the weight range of 2 to 4.5 kg were quarantined for a minimum of 6 weeks prior to the study start and were certified to be of good health, free of any malformations, and exhibiting no clinical signs of disease prior to the study start.

Comparison of the protective efficacies of rPA produced from either *B. subtilis* or *E. coli* in the A/J mouse. Groups of 10 female A/J mice were immunized with rPA, derived from either *B. subtilis* or *E. coli*, in the range of 0.1 to 5 μg rPA per mouse adsorbed to 20% (vol/vol) alhydrogel (Brenntag Biosector, Frederikssund, Denmark) in phosphate-buffered saline (PBS). Immunization was given on a single occasion by the intramuscular (i.m.) route in 0.1 ml PBS. Mice were challenged at day 21 by the i.p. route with 4.9×10^5 CFU *B. anthracis* STI strain in 0.1 ml sterile distilled water, which is equivalent to 363 median lethal doses (MLD) (2). Animals were closely monitored postchallenge, and humane end points were strictly observed. The number of survivors at 14 days postchallenge was recorded.

Comparison of immunogenicities of rPA and existing anthrax vaccines in rhesus macaques. Rhesus macaques were captive bred and health screened prior to acceptance into the 50-week study. The macaques were group housed by sex in a dedicated room with full environmental enrichment, forage materials, and graduated lighting to simulate dawn and dusk.

(i) Fifty-week study to monitor development of immunity. Six groups, each comprising four rhesus macaques (two male and two female), were immunized with one of six vaccine formulations by the intramuscular route in a constant 0.5-ml volume into the muscles of one of the hind legs. The vaccines administered were the United Kingdom AVP, the U.S. AVA, and four dose levels of rPA in the range of 0.25 μg to 50 μg. For this study, rPA from *B. subtilis* was adsorbed to 20% (vol/vol) alhydrogel 1.3% (Brenntag Biosector) such that doses of 0.25, 2.5, 25, and 50 μg were delivered in 0.5 ml PBS (containing 650 μg alum) per animal. The groups of macaques vaccinated with AVA or AVP received a full human dose (0.5 ml) i.m. Blood samples were taken from macaques by superficial venipuncture prior to immunization; then at days 3, 7, 9, 12, and 14; then every 7 days until day 70; and then every 28 days until day 154 in order to identify the earliest time point at which a specific IgG titer could be detected, as well as the magnitude and duration of the primary immune response. Subsequently, the four groups of macaques which had responded optimally to the primary immunization were boosted, and the secondary immune response was monitored over time. At day 154, the groups receiving 25 μg rPA, 50 μg rPA, United Kingdom AVP, and U.S. AVA were given a booster dose of vaccine (exactly as for the priming dose), and regular blood sampling was continued until day 350 of the study.

(ii) Short-term efficacy study. In a subsequent small proof-of-principle study, a group of five female rhesus macaques was immunized with rPA expressed and purified from *E. coli*. Each animal received 50 μg rPA in alhydrogel in 0.5 ml saline (Thraxine [rPA vaccine]; Arecia Biotechnology, Billingham, United Kingdom) i.m. at one site in one hind leg on day 1, with the booster dose being delivered into the contralateral leg on day 28. A single female control animal received vehicle (alhydrogel in saline) alone i.m. on each of the dosing days, in the same volume. Blood samples were taken from animals prior to start of immunization and then weekly. At day 70 (6 weeks after the second immunization) the rhesus macaques were transferred to biosafety level 3 laboratories, where each of the rhesus macaques was anesthetized prior to aerosol challenge with a mean dose of 305 50% lethal doses (LD_{50}) *B. anthracis* Ames strain (standard deviation, 143 LD_{50}). The animals were carefully observed and monitored for clinical signs and symptoms over the subsequent 30 days.

Immune analysis. (i) Determination of specific IgG. Sera from macaques were analyzed for the presence of anti-PA IgG and IgG subclasses by enzyme-linked immunosorbent assay (ELISA) as described below. For the short-term rhesus macaque efficacy study, the sera were analyzed at the Centers for Disease Control and Prevention (CDC), Atlanta, Ga., in a slightly modified protocol developed by the CDC in order to standardize the serological analysis of sera arising from studies of AVA and rPA vaccine candidates. This protocol was very similar to that outlined below but included quality control and reference sera produced in macaques by the CDC and used anti-monkey IgG detection reagents. The lower limit of quantification (LLQ) of the ELISA was 2.5 μg/ml.

TABLE 1. Coating concentrations for equimolar amounts of fusion proteins of GST with PA domains applied to plates

Coating antigen	Molecular mass (Da)	Coating concn (μg/ml)[a]
GST-domain 1	57,400	3.45
GST-domains 1 + 2	82,600	4.97
GST-domains 3 + 4	53,300	3.21
GST-domain 4	41,400	2.49
GST-domains 1–3	94,500	5.69
GST-domains 1–4	109,000	6.56
GST	26,000	1.56

[a] The coating concentration was calculated on the basis of the requirement for 6.02×10^{-11} mol ml^{-1} (equivalent to 5 μg ml^{-1} rPA).

(ii) ELISA of sera from 50-week rhesus macaque study. Doubly diluted serum was bound in duplicate to 96-well microtiter plates (Immulon 2; Dynex Technologies) precoated with rPA at 5 μg/ml in PBS. Each plate included nonimmune macaque serum as a negative control. Each plate was calibrated using 0.25 μg/ml of human IgG (The Binding Site Ltd., Birmingham, United Kingdom) or 0.3 μg/ml monkey IgG (Nordic Immunological Laboratories, Tilburg, The Netherlands) standard, doubly diluted in wells precoated with 15 μg/ml anti-human (Sigma) or 5 μg/ml anti-monkey (Nordic) Fab-specific IgG, respectively. Bound IgG was detected with anti-human IgG (Oxford Biotech Ltd., Oxford, United Kingdom) or anti-monkey IgG (Nordic Immunological Laboratories) conjugated to horseradish peroxidase. Assays were developed using 0.36 mM 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) (Sigma) in 61 mM citric acid and 70 mM disodium hydrogen phosphate with 0.88 mM hydrogen peroxide. The absorbance of each plate was measured at 414 nm after a standard (20-min) incubation at room temperature. Concentrations of IgG or IgG subclasses in samples from the 50-week study were determined from the standard curve incorporated in the assay, using Titertek software, and were expressed as micrograms of IgG $milliliter^{-1}$ serum, from which geometric mean titers (GMT) and 95% confidence limits (CL) were derived for each treatment group.

(iii) IgG subclass profiles. IgG subclass profiles were determined, at days 35 and 182, using the protocol described above for total IgG but modified for use with purified human IgG standards and cross-reactive anti-human IgG subclass reagents. Purified human IgG standards were purchased from Sigma (Poole, Dorset, United Kingdom) and used at the following starting concentrations: IgG1 and IgG2 at 2 μg ml^{-1}, IgG3 at 0.5 μg ml^{-1}, and IgG4 at 1.0 μg ml^{-1}. Horseradish peroxidase-conjugated anti-human IgG subclass reagents were purchased from The Binding Site (Birmingham, United Kingdom) and used at the following concentrations: anti-human IgG1 at 1:500, anti-human IgG2 at 1:250, anti-human IgG3 at 1:2,000, and anti-human IgG4 at 1:500.

Recognition of protective antigen domains. The standard ELISA protocol was modified so that plates were coated with rPA domains (expressed as fusion proteins with glutathione *S*-transferase [GST]) (7) at concentrations equimolar to the concentration of rPA (5 μg/ml) used in the ELISA for total IgG. Domain recognition was measured in sera collected at days 35, 49, and 182, which were pooled by treatment group for this analysis. The coating concentration of each domain protein is recorded in Table 1. The fusion proteins were applied to plates in PBS and left overnight at 4°C, and concentrations of total IgG against rPA domains were then determined using the standard ELISA protocol described above.

TNA assay. The titer of toxin-neutralizing antibody (TNA) in immune serum was determined as previously described (11, 12, 20) by the ability of the serum to inhibit the cytotoxicity of the combination of rPA with LF (List Biological Laboratories Inc., Ontario, Canada). This was assayed by exposing J774.1 murine macrophages (European Collection of Animal Cell Cultures, Porton Down, United Kingdom) to PA and LF in the presence or absence of immune serum. The end point of the assay was the measurement of released lactate dehydrogenase from dying cells. In brief, J774.1 cells were plated out on microtiter plates and incubated overnight at 37°C in 5% CO_2. They were allowed to form a confluent adherent monolayer prior to use in the TNA assay. Immune serum was added to the adherent cells in doubling dilutions prior to the addition of premixed rPA (12 μg) and LF (2 μg). The plates were incubated for 4 h (37°C, 5% CO_2) prior to decanting the tissue culture supernatant into a second plate for the measurement of lactate dehydrogenase release by addition of the tetrazolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide which is converted to formazan with red coloration in the presence of dehydrogenase. Thus, serum-mediated neutralization of anthrax toxin manifests as a suppression of cytotoxicity with no formazan production and low absorbance.

This assay was applied to determine the titer of toxin-neutralizing antibody in fractionated sera from macaques in the 50-week study. The results are presented as the reciprocal of the effective dose of purified IgG (micrograms) in fractionated serum which causes 50% inhibition (ED_{50}), from which the GMT and 95% CL were derived for each treatment group.

TNA assays on unfractionated sera arising from the short-term macaque study were performed at the CDC, using a modified protocol which incorporated quality control and reference sera produced at the CDC (22, 27). Assay end points were calculated using SAS version 8.0 (SAS Institute Inc., Cary, NC) running an end point calculation algorithm developed by the CDC (27). The primary end point used in this assay was the ED_{50}, derived from the reciprocal of the dilution of serum sample that results in 50% inhibition of lethal toxin cytotoxicity. The ED_{50} corresponded to the inflection point of a four-parameter logistic log fit of the neutralization curve. The TNA assay had a working range of 0.07 to 0.30 μg/ml of neutralizing anti-PA IgG, a lower limit of quantification of 0.07 μg/ml, and a positive-negative threshold of 2.5 μg/ml anti-PA IgG in undiluted serum. The assay has a diagnostic sensitivity of 97% and a diagnostic specificity of 100% (22).

Passive transfer of immunity. Individual blood samples collected at day 182 from macaques immunized with the highest dose of rPA (50 μg) or with AVA or AVP were allowed to clot with separation of the serum. The individual serum samples were precipitated with saturated ammonium sulfate and the pellet collected by centrifugation (1,000 rpm, 10 min). After washing, the pellets were individually resuspended in PBS and IgG was purified from each individual solution by affinity separation on a protein G column (Hi Trap; Pharmacia Biotech). IgG was eluted from each column in 0.1 M glycine (pH 2.7), and the yield of purified IgG was assessed by bicinchoninic acid assay (Pierce Chemical Co.), using purified simian IgG as the standard (Nordic Immunological Laboratories).

Subsequently, a dilution series was prepared from each sample such that 500-μg, 300-μg, and 100-μg doses of IgG were contained in 0.1 ml PBS. Aliquots (0.5 ml) of purified IgG comprising doses in the range from 100 to 500 μg were delivered by the i.p. route into groups of five A/J female mice. A further group of five mice received 0.5 ml of IgG purified from a nonimmunized macaque. The recipient mice were challenged by the i.p. route 2.5 h after the passive transfer with 4×10^4 CFU *B. anthracis* STI strain (equivalent to 30 MLD) (2) and their survival monitored over the subsequent 10 days.

Statistical analysis of results. In experiments determining the total IgG, individual samples were assayed and mean results were calculated for each group. However, in experiments determining IgG subclasses and recognition of rPA domains, equal aliquots from individual samples from each group were pooled and then analyzed. One-way analysis of variance (ANOVA) followed by Dunnett's *t* (two-sided) statistical tests was used to make statistical comparisons of the macaque responses to different doses of rPA or to AVA or AVP. Regression analysis was used to compare the significance of the difference between treatment groups. One-way ANOVA was carried out using Minitab13.1.

RESULTS

Comparison of the characteristics and protective efficacies of rPAs produced from either *B. subtilis* or *E. coli*. Recombinant PAs produced from *B. subtilis* and *E. coli* had similar electrophoretic mobilities and were purified to the same degree of homogeneity (Fig. 1). The protection afforded to A/J mice immunized with a single dose of either source of rPA in the same dose range (0.1 to 5 μg) in alhydrogel-PBS showed that both sources of rPA were protective (Table 2). All groups of mice were fully protected against injected challenge with 363 MLD *B. anthracis* STI spores when immunized with a least 0.5 μg rPA.

Immunogenicity of rPA compared with AVA and AVP in rhesus macaques. (i) Fifty-week immunization study. (a) Primary IgG response. All vaccines induced a primary anti-PA IgG response which was detected with an anti-human IgG reagent and which peaked at less than 10 μg per ml at day 14

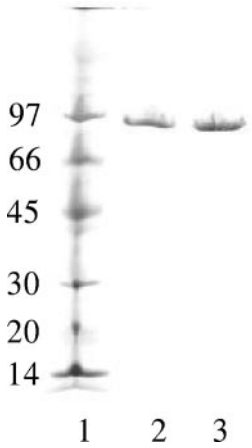

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of rPA expressed and purified from *B. subtilis* (lane 2) or *E. coli* (lane 3). One microliter of rPA, diluted to 0.5 mg/ml, from either source was loaded onto a 10 to 15% gradient gel. Molecular mass markers (lane 1) in the range from 14 to 97 kDa were run concurrently. The gel was stained subsequently with PhastGel Blue R.

and then decreased almost to baseline (<1.0 μg/ml IgG) by day 120 (Fig. 2a).

Anti-human IgG reagents were used for this analysis so that the values for total IgG could be compared with the concentration of PA-specific IgG subclasses, for which no monkey reagents could be obtained. When an anti-monkey IgG reagent was also used to detect antibody levels in macaque serum, the concentration of specific IgG detected in serum was approximately 10-fold higher but followed a similar profile of peak and decline to baseline (data not shown). One-way ANOVA indicated that the IgG responses to the top two doses of rPA were not significantly different overall from the response to either AVP or AVA vaccines when measured with either set of reagents. However, while the AVA vaccine induced a significantly ($P < 0.05$) greater IgG response than that induced to 0.25 μg rPA at days 21 and 42, the response to the AVP vaccine was not significantly different from the response to 0.25 μg rPA.

TABLE 2. Comparison of the immunogenicities and protective efficacies of rPA produced from *B. subtilis* and *E. coli* in the A/J mouse[a]

rPA immunizing dose (μg) administered per mouse in 0.26% alhydrogel in 0.1 ml PBS i.m.	Surviving A/J mice/no. challenged	
	B. subtilis rPA	*E. coli* rPA
5	10/10	10/10
2.5	10/10	10/10
1	10/10	10/10
0.5	10/10	10/10
0.25	9/10	10/10
0.1	10/10	6/10

[a] Groups of 10 female A/J mice were immunized with rPA derived from either *B. subtilis* or *E. coli*, in the range 0.1 to 5 μg rPA per mouse, in 20% (vol/vol) alhydrogel 1.3%. Immunization was by the i.m. route on a single occasion. Mice were challenged at day 21 by the i.p. route with 4.9×10^5 CFU *B. anthracis* STI strain (equivalent to 363 MLD) in 0.1 ml PBS. Survival at day 14 postchallenge is shown.

(b) Secondary IgG response. Groups of animals given the two highest doses of rPA or the AVP or AVA vaccines were boosted at day 154 with a second dose of vaccine. This induced a rapid and large secondary response in all groups (Fig. 2b). IgG levels in all vaccinees reached a peak at day 182 and then rapidly declined to levels equivalent to the primary response to these vaccines. These lower levels of serum antibody were maintained until the study was ended at day 350. IgG levels detected with anti-human IgG reagent in the secondary response to all vaccines were approximately 10 times greater than those in the primary response. In the secondary response, 10 to 20 times as much IgG was detected in assays using anti-monkey IgG reagents than in those with anti-human IgG reagents. When monkey reagents were used, there were no significant differences between treatments when they were compared in ascending pairs or when they were compared to the AVP vaccine response. In contrast, significantly greater ($P < 0.05$) IgG responses to rPA were observed using human reagents at days 266, 294, 322, and 350 when they were compared in ascending pairs with the IgG responses to animals given the AVP vaccine. In contrast to the trend in the primary response, end point titers did not decline to baseline in the secondary response, and significant differences were still measurable between groups at week 50 (day 350), so that the GMT for the 25-μg rPA group was significantly greater ($P < 0.05$) than that for the AVP group.

(c) IgG subclass profiles. The primary (day 35) anti-PA IgG response to any of the vaccines in the rhesus macaques was dominated by the IgG1 subclass, with smaller amounts of IgG2, as determined with anti-human IgG reagents (Fig. 3a). There were no measurable amounts of either IgG3 or IgG4. A similar pattern of subclasses was observed at day 182 at the peak of the secondary response, with a proportionately greater increase in IgG2 than in IgG1 for the 25-μg and 50-μg rPA groups which were boosted (Fig. 3b).

(d) Recognition of PA domains by rhesus macaque serum. Sera collected from macaques immunized with AVA, AVP, or rPA were assayed for recognition of full-length rPA as well as GST fusions of individual domains of rPA and combinations of domains. The reactivities of sera collected from each of the treatment groups at days 35, 49, and 182 (through the primary immune response to the peak of the secondary response) are shown in Table 3. While the major reactivity of IgG in sera from all treatment groups was for full-length rPA, recognition of truncates comprising individual or combinations of domains did not occur until day 182. At day 182 there was substantial recognition of truncates comprising domains 1 to 3 by sera from both existing vaccine- and rPA vaccine-treated groups. At day 182 sera from the rPA treatment groups were also more reactive with a truncate incorporating domains 3 and 4 than were sera from the groups receiving the existing vaccines. Generally there was lesser recognition of the combination of domains 1 and 2 and the individual domains 1 and 4 by sera derived from all vaccinated groups. Thus, the general indications from this domain-screening analysis were that macaques immunized with rPA recognized both the N- and C-terminal domains of the PA molecule, while those immunized with the existing vaccines recognized the N-terminal region more extensively than the C-terminal region.

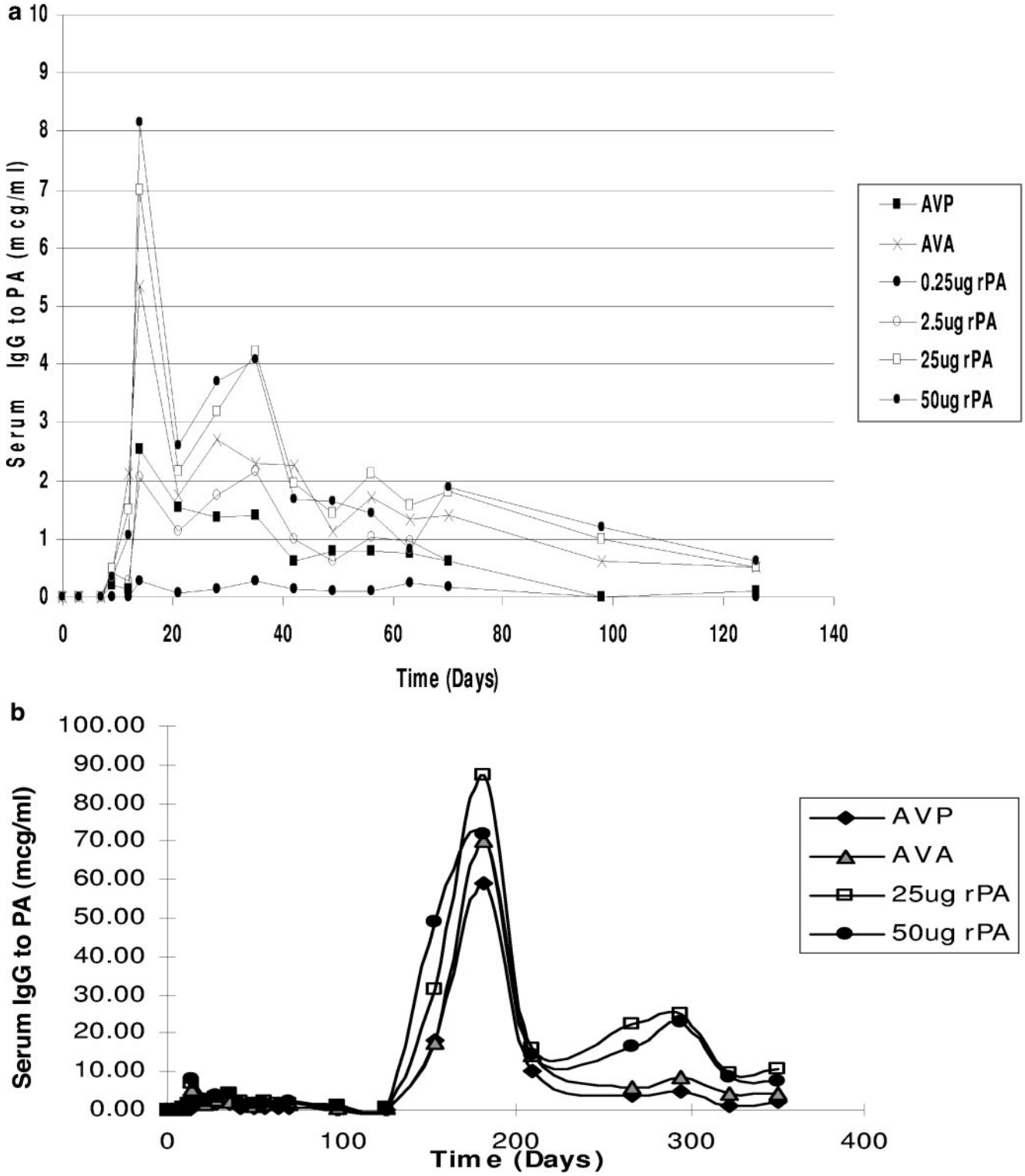

FIG. 2. (a) Primary anti-PA IgG response in sera from rhesus macaques immunized with rPA or existing vaccines. Individual samples from each treatment group were analyzed using an anti-human IgG secondary antibody, and geometric mean titers were derived. (b) Secondary anti-PA IgG response in sera from rhesus macaques immunized with rPA or existing vaccines. Individual samples from each treatment group were analyzed using an anti-human IgG secondary antibody, and geometric mean titers were derived.

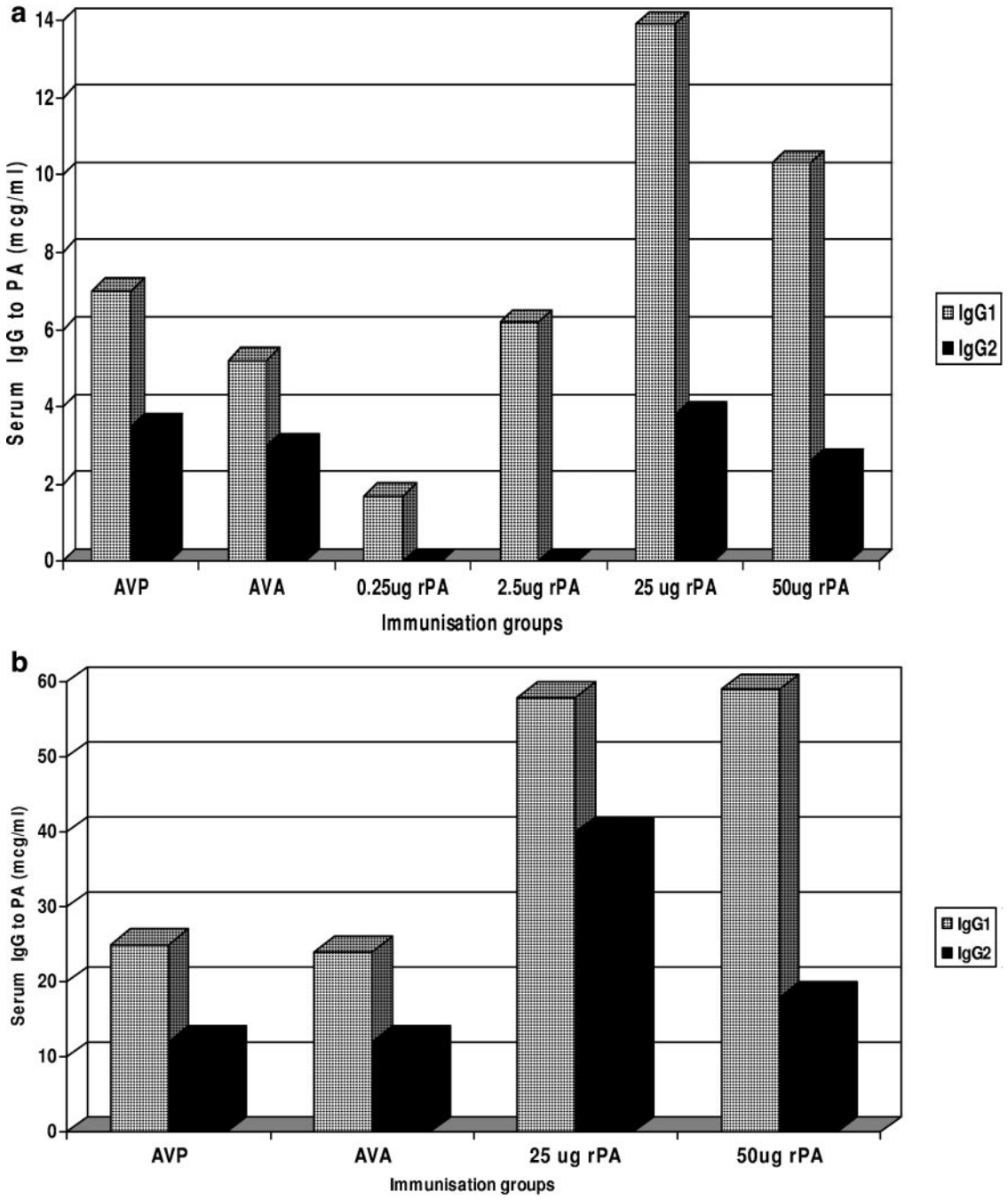

FIG. 3. (a) Anti-PA IgG subclass profile in sera from immunized rhesus macaques during the primary response. Equal aliquots of sera from individuals in each treatment group were pooled for analysis by ELISA using anti-human IgG secondary antibody. Geometric mean titers are presented. (b) Anti-PA IgG subclass profile in sera from immunized rhesus macaques during the secondary response. Equal aliquots of sera from individuals in each treatment group were pooled for analysis by ELISA using anti-human IgG secondary antibody. Geometric mean titers are presented.

(e) Toxin-neutralizing antibody. Serum samples collected at day 182 were assayed for their titer of specific IgG prior to fractionation of the individual samples and measurement of neutralizing antibody by TNA. At this time point, there were no significant differences in PA-specific IgG titer between treatment groups (Table 4). The ED_{50} was measured for individual samples of purified IgG in the TNA assay and is presented as the reciprocal of the concentration of purified IgG in the sample which inhibits the cytotoxic effect of lethal toxin by 50%. The higher the numerical value for the reciprocal, the more inhibitory is the sample. All the purified IgG samples tested were inhibitory in this assay, with the exception of IgG purified from animal 219 in the 25-μg rPA dose group; however, no significant differences in ED_{50} were found between treatment groups.

TABLE 3. Recognition of domains of PA by serum IgG from rhesus macaques immunized with existing and subunit vaccines[a]

Day	Group[b]	Total IgG (μg ml^{-1})[c]					
		GST-PA 1–4	GST-PA 1–3	GST-PA 3–4	GST-PA 1–2	GST-PA4	GST-PA1
35	1	15.0	3.1	3.7	1.4	<LLQ	0.9
	2	23.7	22.0	3.1	2.2	<LLQ	3.7
	3	<LLQ	0.9	1.0	0.2	<LLQ	<LLQ
	4	16.9	8.1	1.5	0.5	0.6	1.1
	5	34.6	5.1	4.5	2.7	1.4	4.9
	6	42.1	5.2	6.8	1.6	0.9	2.4
49	1	15.2	2.9	3.2	0.4	<LLQ	0.4
	2	28.1	5.1	3.4	2.2	0.3	3.2
	3	<LLQ	0.9	1.2	0.2	<LLQ	<LLQ
	4	1.1	1.9	3.1	1.6	0.4	0.2
	5	27.9	3.9	4.6	3.0	0.7	2.6
	6	23.2	3.3	4.6	1.6	0.8	0.8
182	1	216.6	106.2	53.2	58.8	5.7	16.0
	2	198.0	132.5	13.1	108.5	2.4	43.5
	5	338.4	180.2	156.8	110.1	8.7	27.0
	6	246.4	140.2	149.6	56.2	9.0	12.0

[a] Sera collected at days 35, 49, and 182 were pooled by treatment group and screened for reactivity with fusion proteins of GST with domains of PA by ELISA. Reactivity was measured using purified monkey IgG standards and anti-monkey IgG detection reagent.
[b] Groups were immunized as follows: 1, AVP; 2, AVA; 3, 0.25 μg rPA; 4, 2.5 μg rPA; 5, 25 μg rPA; 6, 50 μg rPA.
[c] Concentration of macaque IgG per milliliter of serum, where LLQ is <0.2 μg/ml IgG.

(f) Passive transfer assay. Groups of 20 naive A/J mice were passively immunized with IgG purified from sera collected on day 182 from the individual macaques in each of the groups immunized with 50 μg rPA, AVA, or AVP. Individual mice received purified IgG in the dose range from 100 to 500 μg at 2.5 h prior to challenge i.p. with approximately 30 MLD *B. anthracis* STI strain. The survival of passively immunized animals at 10 days postchallenge is shown in Table 4. In this assay,

TABLE 4. Inhibition of in vitro cytotoxicity by immune macaque IgG purified from serum taken at day 182 (week 26) of the 50-week study

Immunization group	Macaque sample	IgG in serum samples (μg/ml)[a]	Reciprocal ED50[b]	Survivors/total in groups of naive mice passively immunized with IgG purified from immune macaque serum[c]		
				500 μg	300 μg	100 μg
AVP	201	31.2	0.16	1/5	1/5	2/5
	202	94.6	0.64	5/5	3/5	0/5
	203	14.2	0.32	3/5	0/5	1/5
	204	95.4	0.32	2/5	3/5	4/5
	Geometric mean for group (95% CL)	44.7 (22.4, 107.2)	0.32 (0.18, 0.57)	11/20	7/20	7/20
AVA	205	25.2	0.16	1/5	2/5	2/5
	206	98.5	0.16	1/5	2/5	0/5
	207	48.9	0.32	5/5	3/5	0/5
	208	108.3	0.64	3/5	2/5	0/5
	Geometric mean for group (95% CL)	60.2 (26.9, 151.4)	0.27 (0.13, 0.48)	10/20	9/20	2/20
25 μg rPA	217	101.5	0.64	ND[d]	ND	ND
	218	48.6	0.64	ND	ND	ND
	219	106.4	negative	ND	ND	ND
	220	92.9	0.32	ND	ND	ND
	Geometric mean for group (95% CL)	77.1 (29.5, 213.8) (n = 3)	0.51 (0.24, 1.0) (n = 3)			
50 μg rPA	221	176.9	0.32	4/5	4/5	3/5
	222	25.8	0.16	3/5	0/5	0/5
	223	26.2	0.32	3/5	3/5	1/5
	224	58.3	0.64	4/5	2/5	2/5
	Geometric mean for group (95% CL)	51.4 (22.4, 125.9)	0.32 (0.18, 0.57)	14/20	9/20	6/20

[a] The rPA-specific IgG titer of the serum was tested prior to fractionation.
[b] Macaque IgG preparations were tested in the TNA assay for the concentration of IgG which inhibits by 50% the cytotoxic effect of lethal toxin for J774.1 cells in tissue culture, and this ED_{50} value is presented as a reciprocal.
[c] The protective capacity of IgG purified from each of the sera was determined by passive immunization of A/J mice, which were subsequently challenged with 30 MLD (4×10^4 CFU) of *B. anthracis* (STI strain) by the i.p. route. The survival of mice passively immunized with IgG preparations from different sources at 10 days postchallenge is shown.
[d] ND, not done.

the protection achieved was dose related, so that 500 μg IgG derived from the group immunized with 50 μg rPA protected 14/20 mice, 300 μg IgG protected 9/20 mice, and 100 μg IgG protected 6/20 mice. A similar dose-response effect was seen for the AVA and AVP groups.

(g) Correlation of protective efficacies of purified macaque IgG measured in vitro and in vivo. The protective efficacies of IgG purified from individual macaques in the 50-μg rPA, AVA, and AVP groups in the in vitro TNA assay and the in vivo passive transfer assay are recorded in Table 4. When all the ED_{50} data across treatment groups were combined, a significant correlation was found with the protection conferred by passive transfer of 500 μg IgG purified from donor macaque serum into the mouse ($P = 0.003$), although the regression coefficient was low ($r = 0.4$). No significant correlation was found between the combined ED_{50} data and the protection conferred by passive transfer of 300 μg or 100 μg macaque IgG.

(ii) Short–term efficacy study. (a) IgG response. In a subsequent study, the IgG responses of five individual macaques immunized with 50 μg rPA in alhydrogel on days 0 and 28 were followed. Titers, detected with anti-monkey IgG reagents, increased between 5- and 25-fold following the booster dose, peaking at day 42, and were still elevated at the time of challenge at day 72 (Fig. 4a). The titer of toxin-neutralizing antibody (presented as the reciprocal of the dilution of serum which caused 50% inhibition in the TNA assay) in these sera increased in proportion to the increase in total IgG titer (Fig. 4b). All the immunized macaques were positive by ELISA and TNA by day 14, with the lower bound of the 95% confidence limits around the GMT at this time point being 20-fold and 4-fold over the LLQ, respectively, for these assays. The naive macaque had no detectable IgG titer and no positive ED_{50} value at any time point.

(b) Protection against challenge. The immunized macaques were challenged by the aerosol route with a mean dose of 305 LD_{50} *B. anthracis* Ames strain (range, 179 to 539 LD_{50}). All the immunized animals survived challenge without any symptoms of infection. A single control animal which had been treated with alhydrogel in saline died. Postchallenge, the immunized macaques were monitored for bacteremia and expected clinical signs for 1 month postchallenge, none of which were observed.

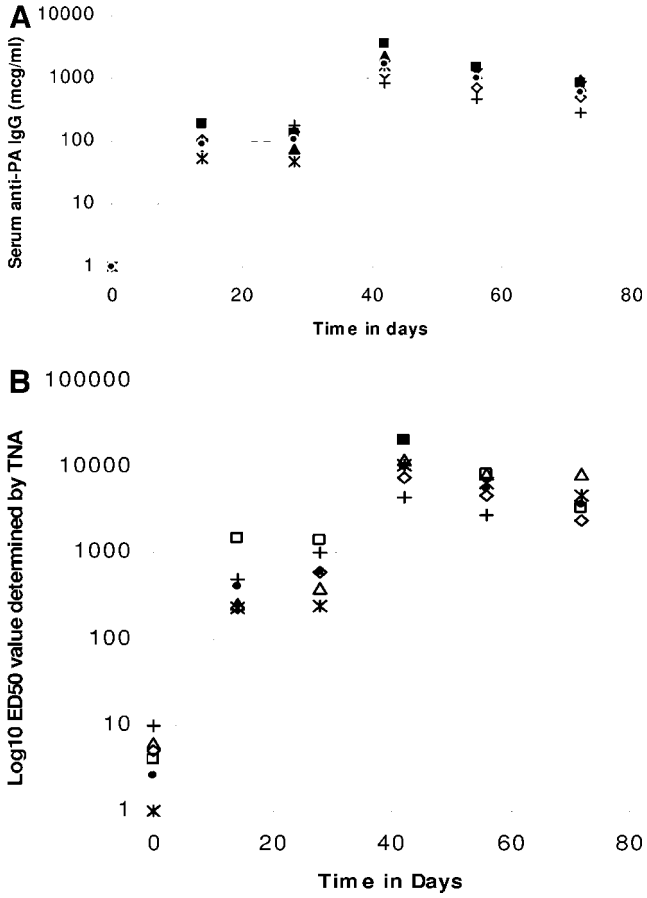

FIG. 4. (a) Development of serum IgG to rPA with time in five individual rhesus macaques. Each animal received 50 μg rPA in 0.26% alhydrogel in saline (Thraxine) on days 0 and 28. The geometric mean titer is shown as a solid line. (b) Increase in $\log_{10}$ ED_{50} value (reciprocal of serum dilution giving 50% inhibition) with time in five rhesus macaques immunized with 50 μg rPA (days 0 and 28). The geometric mean of the ED_{50} values is shown by the solid line.

DISCUSSION

In this study, rPA expressed from either *B. subtilis* or *E. coli* has been demonstrated to be efficacious in protecting A/J mice against injected challenge with *B. anthracis*. The dose range tested in the A/J mouse (0.1 to 5 μg) also bridged to the lower doses tested in the rhesus macaque. Transfer to the rhesus macaque model has allowed an evaluation and comparison of rPAs from both expression systems with PAs present in the existing licensed anthrax vaccines. The source of recombinant PA for the first rhesus macaque study was from *B. subtilis* expression, whereas in the short-term efficacy study, rPA was derived from *E. coli* expression after resynthesis of the PA gene with optimized codon bias for this gram-negative expression system. Both sources of rPA have been found to induce similar immune responses in immunized macaques, suggesting that each source of the rPA protein has adopted a conformation with conserved epitopes.

Rhesus macaques immunized with the existing anthrax vaccines developed immune responses qualitatively similar to those induced to either source of rPA. The existing anthrax vaccines and the rPA vaccine formulations used here are all adjuvanted with alum salts, so there is a common baseline against which to compare these responses. The time scales of development of an IgG titer specific for PA were similar in all immunized macaques, during both the primary and secondary immune responses, despite quantitative differences in the peak titers achieved. The IgG subclass response comprised IgG1 and IgG2 in all groups, with no evidence of IgG3 or IgG4. All the vaccines induced toxin-neutralizing antibody in the macaques, as demonstrated by the ability of immune macaque serum to protect macrophages in tissue culture from the cytotoxicity of lethal toxin. Additionally, IgG purified from immune macaque serum collected during the secondary immune response protected mice against anthrax challenge by passive transfer.

Existing vaccines (from the United Kingdom and the United

States) are produced as microbial culture filtrates with downstream purification followed by alum adsoprtion (AVA) or precipitation (AVP). Due to the method of production of AVP and AVA, there will be batch-to-batch variation within, as well as between, these vaccines in the contents of residual bacterial products and of PA, which makes comparison with each other and with rPA formulations difficult. One of the advantages of recombinant subunit vaccines is the ability to vary the dose of the recombinant immunogen until an optimum is achieved. The data from this study indicate that enrichment of the vaccine formulation for rPA can induce a statistically significant increase ($P < 0.05$) in anti-PA IgG levels in the secondary response to the vaccine compared with the existing AVP vaccine. During the primary immune response, the titer induced to AVP did not significantly differ from that induced to 0.25 μg rPA. However, immunization with 25 μg rPA resulted in a secondary IgG response which was significantly greater than that induced to AVP at time points after the peak response at day 182.

Dosing macaques with 50 μg rPA in a shortened immunization schedule so that the booster dose was given 28 days after the primary immunization resulted in a peak secondary response of magnitude and duration comparable to that achieved in the more protracted schedule. The small cohort of macaques immunized on this shortened schedule were fully protected against challenge by the inhalational route with *B. anthracis* Ames strain in the range of 179 to 539 LD_{50} at 10 weeks after the first vaccination. At the time of challenge, serum IgG titers had peaked but were still elevated in these animals, and affinity maturation of the residual IgG would be expected to be ongoing. All of the immunized macaques had developed a titer of toxin-neutralizing antibody. The neutralizing antibody titer measured by the TNA assay varied with the individual and was proportional to the total IgG titer developed. The challenge level used here was very high, and the data gained regarding the level of protection achievable with rPA extend previous findings with lower challenge levels following single-dose immunization with AVA or rPA in macaques (11).

Comparative immunological studies of rhesus macaques with humans or other animals are few. However, it has been suggested that rhesus macaques possess three IgG subclasses (26) and that these are homologous with human IgG1, IgG2, and IgG4 (5). Investigations into IgG responses in United Kingdom vaccinees and individuals convalescent following a cutaneous anthrax infection have shown a predominantly IgG1 response in both groups, smaller amounts of IgG3 in convalescent-phase sera, and IgG4 > IgG3 > IgG2 (1). In this study, a predominance of IgG1 was also observed in all vaccine groups, with IgG2 also present in significant amounts, particularly during the secondary immune response, while IgG3 and IgG4 subclasses were completely absent. The absence of IgG3 may be explained by the lack of cross-reactivity between macaque and human IgG3, as described in other studies. However, the differences in IgG2 and IgG4 levels are not easily explained. In the human, IgG4 is thought to indicate a Th2-type response (24), while alum-precipitated proteins are known to stimulate a strong Th2-type response in mice (3, 32), so it is perhaps surprising that the IgG4 subclass is not represented in the results described here. Preliminary data from this study indicated minor differences in the reactivity of macaque antiserum which had been raised to PA in existing vaccines, or to rPA, with proteins representing the domains of the PA molecule. Whereas antiserum raised in macaques immunized with rPA recognized the N-and C-terminal regions of the PA molecule equally, antiserum raised in macaques to PA contained in the existing vaccines recognized the N-terminal region of the molecule better than the C-terminal region. Although the current data are preliminary, any differences in recognition of domains of PA by vaccinees could be of practical significance if they lead to better neutralization of the toxigenic effects of anthrax infection. After furin cleavage of the whole PA molecule and loss of the 20-kDa N-terminal fragment, the N-terminal region of the PA molecule provides a binding site for the toxin component LF or EF, so that the development of immunity to domains 1 and 2 or 1 to 3 is protective (7). The C-terminal region of the molecule contains domain 4, which has been shown to be a protective immunogen (7) and which binds host cell receptors (29). Accessibility to the domains of PA may also have significance for the development of cell-mediated immunity, since all the domains of PA (with the exception of domain 2, which is thought to be involved in oligomerization of PA63 with membrane insertion) have been found to contain murine T-cell epitopes (18). However, the interaction of T-cell epitope-containing regions of PA with the host cell during development of the immune response can take place in the absence of domain 4 binding to the anthrax toxin receptor and thus appears to differ from the interaction of PA with the host cell during the toxigenic process of anthrax infection.

In conclusion, the immune response data gained from the rhesus macaques indicate that rPA, derived from either *B. subtilis* or *E. coli* expression, induces immune responses qualitatively similar to those induced to PA in the existing anthrax vaccines. Recombinant PA derived from *E. coli* expression has been demonstrated to induce protective immunity against inhalation exposure to multiple lethal doses of anthrax in a small proof-of-principle study in rhesus macaques. This study provides the basis for further work to be undertaken with *E. coli*-produced rPA to identify the immunological correlates of the protective immunity demonstrated here.

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