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Effect of aluminum hydroxide adjuvant and formaldehyde in the formulation of rPA anthrax vaccine☆

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Abstract

The serological response and efficacy of *Bacillus anthracis* recombinant protective antigen (rPA) vaccines formulated with aluminum hydroxide adjuvant, either with or without formaldehyde, were evaluated in rabbits. Rabbits that had been injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum in aluminum hydroxide gel (Alhydrogel) had a significantly higher quantitative anti-rPA IgG ELISA titers ($p < 0.0001$) and toxin neutralizing antibody (TNA) assay titers ($p < 0.0001$) than rabbits tested at the next lowest concentration of aluminum (158 μg). Rabbits injected with two doses of 50 μg of rPA formulated with 500 μg of aluminum also had significantly higher serological responses, as measured by a quantitative anti-rPA IgG ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$), than sera from rabbits injected with a rPA vaccine formulated without adjuvant. Short-term protection against an aerosol spore challenge (448 LD_{50}), however, was not significantly different between the two groups (12/12 and 11/12, respectively). Rabbits injected with a single dose of 50 μg of rPA formulated with 500 μg of aluminum and 0.2% formaldehyde had significantly higher ELISA ($p < 0.0001$) and TNA assay ($p < 0.0001$) titers than rabbits that had been injected with a rPA vaccine formulated with adjuvant but without formaldehyde. Short-term protection against a 125 LD_{50} parenteral spore challenge, however, was not significantly different between the two groups (14/24 and 9/24, respectively; $p = 0.2476$). Under the conditions tested in the rabbit animal model, significantly higher serological responses were observed in rabbits that had been injected with rPA formulated with aluminum hydroxide gel adjuvant and formaldehyde. However, differences in short-term efficacy were not observed.

Keywords: Bacillus anthracis PA vaccine formulation; Rabbit animal model; Aluminum hydroxide gel adjuvant; Formaldehyde

1. Introduction

Anthrax is an infection that may result after exposure to spores of *Bacillus anthracis* by the cutaneous, gastrointestinal, or aerosol routes and may be characterized by an extensive bacteremia and toxemia. A small number of cutaneous infections, which are usually self-limiting, as well as gastrointestinal and aerosol infections, are life threatening. The bacteremia is facilitated by the expression of a poly-D-glutamic acid capsule which interferes with phagocytosis of the vegetative bacterium. Toxemia is the result of two separate binary toxins, lethal toxin (LeTx) and edema toxin. A central component of both toxins is protective antigen (PA). After PA binds to a cellular receptor,

☆ Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. Opinions, interpretations, conclusions, and recommendations are those of the author and are not necessarily endorsed by the U.S. Army. The research described herein was sponsored by the U.S. Army Medical Research and Materiel Command, Project 02-4-CC-008.

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14. ABSTRACT
The serological response and efficacy of Bacillus anthracis recombinant protective antigen (rPA) vaccines formulated with aluminum hydroxide adjuvant, either with or without formaldehyde, were evaluated in rabbits. Rabbits that had been injected with a single dose of 25mug of rPA adsorbed to 500mug of aluminum in aluminum hydroxide gel (Alhydrogel) had a significantly higher quantitative anti-rPA IgG ELISA titers ($p<0.0001$) and toxin neutralizing antibody (TNA) assay titers ($p<0.0001$) than rabbits tested at the next lowest concentration of aluminum (158mug). Rabbits injected with two doses of 50mug of rPA formulated with 500mug of aluminum also had significantly higher serological responses, as measured by a quantitative anti-rPA IgG ELISA ($p<0.0001$) and TNA assay ($p<0.0001$), than sera from rabbits injected with a rPA vaccine formulated without adjuvant. Short-term protection against an aerosol spore challenge (448 LD(50)), however, was not significantly different between the two groups (12/12 and 11/12, respectively). Rabbits injected with a single dose of 50mug of rPA formulated with 500mug of aluminum and 0.2% formaldehyde had significantly higher ELISA ($p<0.0001$) and TNA assay ($p<0.0001$) titers than rabbits that had been injected with a rPA vaccine formulated with adjuvant but without formaldehyde. Short-term protection against a 125 LD(50) parenteral spore challenge, however, was not significantly different between the two groups (14/24 and 9/24, respectively; $p=0.2476$). Under the conditions tested in the rabbit animal model, significantly higher serological responses were observed in rabbits that had been injected with rPA formulated with aluminum hydroxide gel adjuvant and formaldehyde. However, differences in short-term efficacy were not observed.

15. SUBJECT TERMS
Bacillus anthracis, anthrax, adjuvant, aluminum hydroxide, recombinant vaccine, rPA, formaldehyde, effects, laboratory animals, rabbits

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it undergoes proteolysis by the cell-surface protease resulting in a receptor-bound fragment, PA63. The PA63 molecules form a heptameric pre-pore which complexes with the enzymatic components, either lethal factor (LF), forming LeTx, or edema factor (EF), to form edema toxin. The toxin complex is then endocytosed by the cell and within the endocytic compartment the pre-pore undergoes an acidic pH-dependent conformational rearrangement that allows translocation of LF and EF into the cytosol [1].

Anthrax vaccine adsorbed, Biothrax (AVA Biothrax; BioPort Corporation, Lansing, MI) is the current vaccine licensed for human use against exposure to *B. anthracis* spores in the U.S. The vaccine is prepared by adsorbing filtered culture supernatant fluids of the V770-NPR-1 strain of *B. anthracis* to an aluminum hydroxide gel. The major protective antigen in AVA BioThrax is PA [2–5], the central component of the *B. anthracis* exotoxins. Also present in the vaccine are LF and undefined bacterial proteins, which are present in the filtered culture supernatant and which are also adsorbed onto the adjuvant [6]. In addition to the adjuvant, AVA Biothrax is formulated to contain 25 μg/ml of benzethonium chloride as a preservative and 100 μg/ml of formaldehyde as a stabilizer. Several concerns have been raised in regards to the vaccine, including the lot-to-lot variation in the amounts of PA in the vaccine [6] and occasional reactogenicity after vaccination, which may be related to the presence of uncharacterized components and possibly formaldehyde [7–9] that have served as a stimulus to develop a more fully characterized vaccine. Several studies have demonstrated the efficacy of PA in vaccines to protect against anthrax intoxication or infection [10–13]. The importance of anti-PA serum also has been shown in the identification of in vitro correlates of immunity [14–17] and in passive antibody studies [18–20]. This report evaluates the role of the aluminum hydroxide gel adjuvant and the excepient formaldehyde in the formulation of rPA vaccines in the rabbit model using in vitro surrogate markers (the quantitative anti-rPA IgG ELISA and toxin neutralizing antibody (TNA) assay) and efficacy studies.

2. Materials and methods

2.1. Animals

An equal number of male and female New Zealand white (NZW) rabbits (3.0–3.5 kg) (Covance Research Products, Denver, Penn.) were used for each experiment. The animals received food and water *ad libitum*. Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations relating to animals and experiments involving animals and adheres to principles stated in the Guide for the Care and Use of Laboratory Animals, National Research Council, 1996. The facility where this research was conducted is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

2.2. Preparation of rPA vaccine, vaccination, and challenge

Recombinant PA (rPA), expressed in a *B. anthracis* background [21,22], was manufactured as a cGMP lot by the Biopharmaceutical Production Facility at NCI-FCRC (Frederick, MD) using a modification of a reported procedure [23]. The same lot of rPA was used throughout these experiments for vaccinations and serological analysis of antibody response. LF was prepared by chromatographic separation from V770-NP1-R culture supernatants as previously described [24].

The effect of aluminum hydroxide gel adjuvant on the serological response to rPA was evaluated by adsorbing 25 μg of rPA diluted in Dulbecco's phosphate buffered saline without Ca^{2+} or Mg^{2+} (PBS) to various concentrations of aluminum (500–15.8 μg per 0.5 ml dose in half-log dilutions) in Alhydrogel (2% Al_2O_3; HCl Biosector, Frederikssund, Denmark) for 20–24 h at 4 °C before use. NZW rabbits were inoculated intramuscularly (i.m.) with a single dose in 0.5 ml volumes and were bled weekly. The immunological status of the rabbits was measured by using a quantitative anti-rPA IgG ELISA and TNA assay [15].

The effect of adjuvant (Alhydrogel) on the serological response and protection was evaluated in NZW rabbits, which were inoculated i.m. at 0 and 4 weeks with 50 μg of rPA formulated with either 500 μg of aluminum per injection, final concentration, or without adjuvant in 0.5-ml volumes. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week to determine serological titers to PA (quantitative anti-rPA IgG ELISA and TNA assay). At 10 weeks, rabbits were exposed to a small-particle aerosol in a modified Henderson exposure system contained within a Class III biological safety cabinet to the head only (nose and mouth) with a lethal dose of spores from the Ames isolate of *B. anthracis* [25]. Inhaled doses were calculated using the aerosol exposure concentration obtained from plate counts from the all-glass impinger which continuously sampled the test atmosphere during the 10 min exposure time and the respiratory minute volume for each animal measured by plethysmography. Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The aerosol LD_{50} of Ames spores in NZW rabbits is 1.1×10^5 spores [25]. The average inhaled dose (average $LD_{50} \pm$ S.D.) of spores was 448 ± 214.6 LD_{50}.

The effect of formaldehyde on the immune response in NZW rabbits after injection of a rPA vaccine preparation was also evaluated. Vaccine preparations were formulated with 50 μg of rPA adsorbed to 500 μg of aluminum in Alhydrogel with or without 0.02% formaldehyde. Rabbits were inoculated i.m. with a single dose of vaccine. Control rabbits were injected with PBS and Alhydrogel. Rabbits were bled every other week after inoculation, and the sera were tested by a quantitative anti-rPA IgG ELISA and TNA assay. Rabbits were challenged subcutaneously (s.c.) at week 6 with 125

LD_{50} spores from the Ames isolate of *B. anthracis* (actual count was 1.9×10^5 spores). Spores were prepared as previously described [25]. Survival was noted for 21 days after challenge. The s.c. LD_{50} of Ames spores in NZW rabbits is 1.5×10^3 spores.

2.3. Serological analysis of antibodies

Blood was collected periodically for analysis of serum antibodies by a quantitative anti-rPA IgG ELISA and TNA assay [15]. ELISA titers were determined by interpolating the average absorbance value for triplicate wells of each sample with the absorbance values of a standard curve generated from seven dilutions of affinity purified rabbit anti-rPA IgG by linear regression analysis and reported as micrograms of anti-rPA IgG per ml (KC4 software, BioTek Instruments, Winooski, VT) [15]. Titers were presented as the geometric mean and ×/÷ standard error of the geometric mean (S.E.M.). For the TNA assay, the average absorbance value of triplicate wells for each test sample dilution, less the average absorbance value of triplicate wells incubated with LeTx, was divided by the average absorbance value of control wells that contained only medium, less the average absorbance value of triplicate wells incubated with LeTx, and the ratio multiplied by 100 to obtain the percent viability of the test wells compared to the control wells:

$$\%\text{Control} = \left(\frac{\text{sample avg} - \text{LeTx avg}}{\text{medium control avg} - \text{LeTx avg}} \right) \times 100.$$

The percent control values were plotted against each respective test dilution using a four-parameter logistic equation algorithm. TNA assay titers were expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) using XLfit software (IDBS Inc., Emeryville, CA). Titers were presented as the geometric mean and ×/÷ S.E.M.

2.4. Statistical analysis

Log_{10} transformations were applied to all ELISA and TNA assay ED_{50} titers except for TNA assay ED_{50} titers from results presented below in Section 3.1. After transformation, the dependent variable met assumptions of normality and homogeneity of variance. Mixed model analysis of variance (RM-ANOVA) or ANOVA were used to compare titers over time and between challenge groups. Fisher exact tests and Chi-square tests for proportions were used to compare survival rates, which are the ratio between survivors and the total number of test animals at the end of the study. Kaplan-Meier survival analysis, which is a plot of the percent survival as a function of time, was used to compare survival curves between groups. Analyses were conducted using SAS Version 8.2 (SAS Institute Inc., SAS OnlineDoc, Version 8, Cary, NC).

3. Results and discussion

3.1. Dose effect of Alhydrogel adjuvant on serological responses

During the development of an anthrax vaccine based upon rPA, numerous adjuvants were evaluated for their efficacy in guinea pigs [5,26] and non-human primates [12]. Aluminum-based adjuvants however, are the only class of adjuvants that have been approved for use in humans. AVA Biothrax is prepared from V770-NP1-R filtered culture supernatants adsorbed to 650 μg of aluminum hydroxide gel per 0.5 ml dose [6]. The British vaccine approved for human use consists of filtered culture supernatants of the Sterne strain of *B. anthracis* precipitated with aluminum phosphate gel (alum) [27]. Although the maximal amount of aluminum that is allowed for the U.S. vaccine is 850 μg per dose, the recommended maximum concentration for anthrax vaccines based upon rPA is 500 μg per dose. Our first experiment examined the serological response of NZW rabbits injected with a rPA vaccine formulated with various concentrations of aluminum present in aluminum hydroxide gel (Alhydrogel). Animals that were injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum per 0.5 ml dose had a geometric mean peak ELISA titer at week 2 of 31.0 μg of anti-rPA IgG per ml (Table 1). At a half-log lower dose of adjuvant (158 μg of aluminum), the geometric mean week 2 anti-rPA ELISA titer dropped significantly ($p < 0.0001$) by eight-fold to 4.0 μg of anti-rPA IgG per ml. Each half-log lower concentration of aluminum adjuvant in the rPA vaccine preparations resulted in two-fold decreases in week 2 anti-rPA ELISA titers (Table 1). The geometric mean TNA assay ED_{50} titer at week 2 for rabbits that had been injected with a single dose of 25 μg of rPA adsorbed to 500 μg of aluminum was 360 (Table 1). The TNA assay ED_{50} titer dropped significantly ($p < 0.0001$) by five-fold to 69.4 for rabbits injected with 158 μg of aluminum per dose (Table 1). At lower concentrations of adjuvant, a decrease in TNA assay ED_{50} titers were also observed (Table 1). It would appear that for a maximal serological response for rabbits, the maximum recommended concentration of 500 μg of aluminum per dose was the most effective among the doses tested.

3.2. Effect of Alhydrogel on serological response and protection

The effect of the adjuvant on the serological response and protective efficacy was examined in NZW rabbits that were injected with rPA vaccines formulated either with or without Alhydrogel. Rabbits were inoculated with two doses of 50 μg of rPA at 0 and 4 weeks. For one group, rPA was adsorbed to aluminum hydroxide adjuvant at 500 μg of aluminum per dose while the second group did not receive adjuvant. Control rabbits were injected with PBS and Alhydrogel. Titers peaked 2 weeks after the second dose of vaccine on week 6 for both groups (Tables 2a and 2b). The anti-rPA IgG

Table 1
Quantitative anti-rPA IgG ELISA and TNA assay ED_{50} titers of rabbits after a single inoculation with 25 μg of rPA adsorbed to various concentrations of aluminum

Aluminum concentration (μg)	Quantitative anti-rPA IgG ELISA titer[a]				TNA assay ED_{50} titer[b]
	Week 1	Week 2	Week 3	Week 4	Week 2
500	2.5 (1.409)	31.0 (1.209)	18.2 (1.158)	14.2 (1.195)	360 (1.133)
158	0.56 (1.344)	4.0 (1.372)	3.5 (1.302)	3.2 (1.294)	69.4 (1.649)
50	0.49 (1.075)	2.3 (1.251)	2.1 (1.321)	2.1 (1.217)	17.0 (1.782)
15.8	0.51 (1.272)	1.1 (1.262)	0.87 (1.233)	1.1 (1.251)	7.0 (1.641)
0	0.15 (1.213)	BLQ[c]	0.10 (1.254)	0.09 (1.085)	1.0 (1.00)

[a] Titer expressed as μg of anti-rPA IgG per ml and standard error of the mean (S.E.M.) in parenthesis.
[b] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.
[c] BLQ, below the limit of quantitation which was 0.072 μg/ml of IgG, the concentration of the lowest standard (1.44 ng/ml of IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

Table 2a
Geometric mean quantitative anti-rPA IgG ELISA titers and survival ratio of rabbits inoculated with 50 μg of rPA formulated with or without aluminum hydroxide gel adjuvant[a]

Vaccination group	Anti-rPA IgG ELISA titer[b]						Survival ratio[a]
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge	
With adjuvant	14.1 (1.340)	8.1 (1.330)	342 (1.154)	207 (1.164)	158 (1.134)	322 (1.130)	12/12
Without adjuvant	1.5 (1.311)	1.2 (1.220)	72.0 (1.259)	50.1 (1.248)	31.8 (1.184)	511 (1.176)	11/12
Control	0.44 (1.392)	0.16 (1.616)	0.18 (1.260)	0.09 (1.138)	0.14 (1.741)	na[c]	0/4

[a] NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 μg of aluminum adjuvant (Alhydogel). Rabbits were challenged on week 10 by the aerosol route with spores from the Ames isolate of *B. anthracis*.
[b] Titer expressed as μg of anti-rPA IgG per ml and S.E.M. in parenthesis.
[c] na, sample not available.

ELISA antibody responses (Table 2a) and TNA assay ED_{50} titers (Table 2b) were significantly higher in rabbits that were injected with rPA adsorbed to aluminum hydroxide adjuvant than in rabbits that were injected with rPA without aluminum hydroxide gel adjuvant. There was a significant difference in ELISA titers between the two groups ($F(1,108) = 161.75$, $p < 0.0001$) over time ($F(4,108) = 143.45$, $p < 0.0001$) and at each week tested (p values $p < 0.0001$ for each week). There also was a significant difference in TNA assay ED_{50} titers between the two groups ($F(4,110) = 145.43$, $p < 0.0001$) over time ($F(4,110) = 118.60$, $p < 0.0001$) and at each week tested (p values $p < 0.004$ for each week). Protection against an aerosol exposure to *B. anthracis* spores was measured 6 weeks after the booster inoculation (week 10). Rabbits were challenged by aerosols consisting of spores of the Ames isolate of *B. anthracis*. The average inhaled dose (average $LD_{50} \pm$ S.D.) of spores that was measured for the rabbits was 448 ± 214.6 LD_{50}. Rabbits that were inoculated with two doses of rPA adsorbed to Alhydrogel were fully protected against the aerosol challenge (100%; 12/12), whereas 92% of rabbits (11/12) inoculated with rPA without adjuvant were protected (Table 2a). None of the control rabbits survived the challenge (0%; 0/4). The serological responses of the rabbits from each group were also compared 21 days after challenge. The post-challenge quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers from the vaccination group receiving rPA adsorbed to Alhydrogel (322 μg anti-rPA IgG per ml and 6151, respectively) were similar to quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers measured 2 weeks after the booster injection (342 μg anti-rPA IgG per ml and 4367, respectively). The post-challenge quantitative anti-rPA IgG ELISA titers and TNA

Table 2b
Geometric mean TNA assay ED_{50} titers of rabbits inoculated with 50 μg of rPA formulated with or without aluminum hydroxide gel adjuvant[a]

Vaccination group	TNA assay ED_{50} titer[b]					
	Week 2	Week 4	Week 6	Week 8	Week 10	Post-challenge
With adjuvant	203 (1.360)	74.5 (1.435)	4367 (1.125)	2029 (1.100)	1065 (1.100)	6151 (1.146)
Without adjuvant	4.5 (1.766)	2.3 (1.489)	1090 (1.218)	542 (1.226)	312 (1.235)	10422 (1.183)
Control	1.3 (1.172)	1.0 (1.00)	2.0 (1.478)	4.3 (1.626)	1.3 (1.147)	na[c]

[a] NZW rabbits were inoculated i.m. at 0 and 4 weeks with rPA vaccine formulated either with or without 500 μg of aluminum adjuvant (Alhydogel).
[b] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the 4-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.
[c] na, sample not available.

assay ED_{50} titers however, from the rPA vaccination group formulated without Alhydrogel (511 μg anti-rPA IgG per ml and 10,422, respectively) were much higher than the quantitative anti-rPA IgG ELISA titers and TNA assay ED_{50} titers measured 2 weeks after the booster injection (72 μg anti-rPA IgG per ml and 1090, respectively). A significant increase in ELISA or TNA assay titers suggests an absence of sterile immunity. Rabbits inoculated with rPA adsorbed to Alhydrogel had post-challenge ELISA titers and TNA assay ED_{50} titers that were two-fold higher ($p = 0.0014$) and six-fold higher ($p < 0.0001$), respectively, than those measured at week 10. Significantly higher titers were measured also in rabbits that had been inoculated with rPA without Alhydrogel, which had a seven-fold increase in week 10 ELISA titers ($p < 0.0001$) and a 33-fold increase in week 10 TNA assay ED_{50} titers ($p < 0.0001$) post-challenge. The difference in post-challenge ELISA titers and TNA assay ED_{50} titers between the two groups was significantly different ($F(1,21) = 5.28$, $p = 0.0319$ and ($F(1,21) = 6.05$, $p = 0.0227$, respectively). In addition to resulting in higher quantitative anti-rPA IgG ELISA titer and TNA assay ED_{50} titers, the formulation of rPA with aluminum adjuvant in the vaccine resulted in a lower increase in the post-challenge serological responses than what was observed in rabbits that had been injected with the rPA vaccine formulated without aluminum hydroxide gel adjuvant (Tables 2a and 2b).

3.3. Effect of formaldehyde on serological response and protection

AVA Biothrax is formulated to contain 0.01% formaldehyde as a stabilizer and 0.0025% benzethonium chloride as a preservative [6]. Studies that had been conducted in evaluating rPA vaccine preparations, including serological correlates of immunity in rabbits [15], potency assay [28], and duration of immunity in rabbits [29], were not formulated with components other than aluminum hydroxide gel adjuvant. The serological response and efficacy of vaccines containing 50 μg of rPA adsorbed to Alhydrogel (500 μg of aluminum) and formulated either with 0.02% formaldehyde or without formaldehyde were compared in rabbits inoculated i.m. with a single injection of vaccine (Table 3). Geometric mean anti-rPA IgG ELISA titers between the two groups were significantly different at week 2, ($p = 0.0001$) and week 4 ($p = 0.0002$) but not at week 6 ($p = 0.0652$). Similarly, geometric mean TNA assay ED_{50} titers between the two groups were significantly different at week 2 ($p < 0.0001$) and week 4 ($p = 0.0003$) but not at week 6 ($p = 0.1119$). Rabbits were challenged s.c. at 6 weeks with spores from the Ames isolate of *B. anthracis*. The rPA vaccine formulated with 500 μg of aluminum and 0.02% formaldehyde protected 58% of rabbits (14/24) against the parenteral challenge, while 37.5% of rabbits (9/24) inoculated with rPA formulated with 500 μg of aluminum but without formaldehyde were protected. None of the control rabbits survived the challenge (0%; 0/4). The s.c. route of challenge was evaluated because it provided greater control of the number of spores that were in the challenge. Neither the difference in survival rates ($p = 0.1486$), survival curves ($\chi^2(1) = 1.62$, $p = 0.2037$), nor mean time-to-death (4.2 days for rPA with formaldehyde and 4.5 days for rPA without formaldehyde; $p = 0.7959$) between the two groups was significant. Our data do not suggest that the inclusion of formaldehyde is a necessary additive to the rPA vaccine. From these results, it appears that formaldehyde's action, in addition to acting as a stabilizer, may also be one of an adjuvant. Studies from a booster injection were not investigated.

Aluminum compounds, aluminum hydroxide ($Al(OH)_3$), aluminum phosphate ($AlPO_4$), and alum ($KAl(SO_4)_2$), are the only adjuvants currently approved for use in human vaccines and are used in the formulation of many veterinary vaccines. The currently licensed U.S. anthrax vaccine, AVA Biothrax, is prepared by adsorbing filtered culture supernatants of the *B. anthracis* V770-NP1-R strain to aluminum hydroxide gel [6]. The current British anthrax vaccine, AVP, is prepared by precipitating filtered culture supernatants of the *B. anthracis* Sterne strain with aluminum phosphate (alum) [27]. AVP contains more LF and EF than AVA Biothrax as measured by antibody response to these components [30]. Studies have shown that AVA Biothrax provides

Table 3
Survival ratio, quantitative anti-rPA IgG ELISA titer, and TNA assay ED_{50} titer of rabbits injected with 50 μg of rPA formulated with or without formaldehyde[a]

Vaccination Group	Survival ratio[b]	Anti-rPA IgG titer[c]			TNA assay titer[d]		
		Week 2	Week 4	Week 6	Week 2	Week 4	Week 6
With formaldehyde	14/24	53.9 (1.188)	36.3 (1.180)	12.7 (1.189)	611 (1.150)	289 (1.148)	157 (1.171)
Without formaldehyde	9/24	18.6 (1.235)	12.9 (1.217)	7.7 (1.239)	145 (1.404	86.6 (1.367)	93.4 (1.210)
Control	0/4	BLQ[e]	BLQ	BLQ	1.3 (1.316)	2.3 (2.300)	1.0 (1.000)

[a] Rabbits were inoculated with a single dose of 50 μg of rPA vaccine formulated with 500 μg aluminum adjuvant (Alhydrogel) and either with 0.02% formaldehyde or without formaldehyde.

[b] Survival ratio of rabbits challenged s.c. on week 6 with spores from the Ames isolate of *B. anthracis*.

[c] Titer expressed as μg of anti-rPA IgG per ml and S.E.M. in parenthesis.

[d] Titer expressed as the reciprocal of the dilution of antiserum at which neutralization of the cytotoxic activity of LeTx on J774A.1 cells was half-maximal (50%; ED_{50}) and S.E.M. in parenthesis. If the TNA assay ED_{50} titer could not be extrapolated from the four-parameter logistic regression curve, the value was arbitrarily assigned a value of '1.0'. The starting dilution for the TNA assay was 1:50.

[e] BLQ, below the limit of quantitation which was 0.072 μg/ml IgG, the concentration of the lowest standard (1.44 ng/ml IgG) multiplied by the lowest starting concentration of the sample (1:50) of the ELISA.

high-level, long-lasting protection in non-human primates [31]. Few studies have been performed on the formulation of rPA vaccines and the effect on the resulting immunological responses in animal models. Aluminum adjuvants are thought to enhance the immune response by localizing the deposition of the antigen, that desorption of antigen can occur in the interstitial fluid, and that both desorbed and adsorbed antigens are processed by antigen-presenting cells [32] and preferentially stimulate the Th2 immune (humoral) response. Anthrax vaccines formulated with either aluminum hydroxide gel or aluminum phosphate adjuvants result in comparable anti-PA titers in humans and guinea pigs [33,30]. Berthold et al. [34] reported a significant increase in ELISA titers to rPA in mice when their vaccine was formulated with either aluminum hydroxide gel or aluminum phosphate adjuvant compared to controls without adjuvant and that ELISA titers to rPA were comparable when either aluminum hydroxide gel or aluminum phosphate adjuvants were used to formulate the rPA vaccine. However, they also found that there was an optimal adjuvant concentration because at higher concentrations of aluminum hydroxide gel adjuvant, the neutralizing antibody titers decreased [34]. We did not observe a decrease in the TNA assay ED_{50} titers in the rabbit animal model at the highest concentration of aluminum tested (500 μg). Both the anti-PA ELISA titer and toxin neutralizing antibody titers have been identified as serological correlates of immunity in rabbits and guinea pigs [13–17,25] and are thus important measurements in developing effective vaccine strategies. Various formulations have been tested in preparing anthrax vaccines based upon rPA for its ability to elicit optimal immunological responses. Recent examples include Toll-like receptor ligands CpG ODN and Resiquimod R-848 [35,36], pluronic F127, a non-ionic, hydrophilic polyoxyethylene-polyoxypropylene block copolymer [37], additional vaccine antigens such as capsule [38] or EF [39,40], DNA vaccines [41], and mucosal vaccine strategies [42,43]. That antibodies have been recognized as important in protection is demonstrated by the number of immunotherapeutic reagents that have been recently suggested [44–49]. However, protection has not always been attributed to toxin-neutralizing antibodies. Brossier et al. [50] proposed that neutralizing anti-PA antibodies may be more important in animal models that are highly susceptible to toxemia than in animal models that are more susceptible to infection. The development of a new rPA vaccine will require the identification of an acceptable aluminum compound, optimal concentration of aluminum, and approved excipients that will enhance the immunological responses necessary for protection against infection in the proper surrogate animal models.

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