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In vitro correlate of immunity in a rabbit model of inhalational anthrax☆

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Abstract

A serological correlate of vaccine-induced immunity was identified in the rabbit model of inhalational anthrax. Animals were inoculated intramuscularly at 0 and 4 weeks with varying doses of Anthrax Vaccine Adsorbed (AVA) ranging from a human dose to a 1:256 dilution in phosphate-buffered saline (PBS). At 6 and 10 weeks, both the quantitative anti-protective antigen (PA) IgG ELISA and the toxin-neutralizing antibody (TNA) assays were used to measure antibody levels to PA. Rabbits were aerosol-challenged at 10 weeks with a lethal dose (84–133 LD_{50}) of *Bacillus anthracis* spores. All the rabbits that received the undiluted and 1:4 dilution of vaccine survived, whereas those receiving the higher dilutions of vaccine (1:16, 1:64 and 1:256) had deaths in their groups. Results showed that antibody levels to PA at both 6 and 10 weeks were significant ($P < 0.0001$) predictors of survival. Published by Elsevier Science Ltd.

Keywords: Anthrax; *Bacillus anthracis*; Serological correlate

1. Introduction

Anthrax, caused by *Bacillus anthracis*, is an ancient disease of animals and humans [1]. It occurs under natural circumstances in three forms: cutaneous, accounting for 95% of human cases; and gastrointestinal [2] or inhalational [3,4], which occurs only rarely. In recent years, there has been a heightened concern of the use of *B. anthracis* as a bioterrorist or biowarfare agent, in part due to the revelation that Iraq produced and fielded *B. anthracis* spores for use in the Gulf War [5]. It is inhalational anthrax that would result from a biowarfare attack with *B. anthracis* spores.

B. anthracis possesses three plasmid-encoded virulence factors: a *poly*-D-glutamic acid capsule that inhibits phagocytosis (encoded by pXO2) [6] and two binary toxins (encoded by pXO1), lethal toxin and edema toxin [7]. These two toxins possess a common cell receptor-binding component, protective antigen (PA), which combined with lethal factor (LF) or edema factor (EF), form the active toxins. PA by itself is sufficient without other *B. anthracis* antigens to protect experimental animals against anthrax [8]. The human vaccine licensed in the United States, Anthrax Vaccine Adsorbed (AVA), is prepared from the sterile culture filtrate of a toxigenic, nonencapsulated strain of *B. anthracis*, V770-NP1-R. The PA-containing filtrate is adsorbed onto aluminum hydroxide [9]. This vaccine has been used for the past 30 years to protect people at risk of exposure to *B. anthracis* and was recently used to vaccinate the US armed forces against anthrax.

The efficacy of AVA varies in different animal models. AVA is poorly protective against inhalational an-

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thrax in guinea pigs [10] while it is very highly effective in rhesus monkeys [11,12]. Rabbits are similar to rhesus monkeys in that AVA is highly efficacious against inhalational anthrax [13].

In this study, we used the rabbit model to demonstrate that anti-PA antibodies levels are a serological in vitro correlate of AVA-induced immunity against inhalational anthrax.

2. Materials and methods

2.1. Experimental animals

Pasteurella-free New Zealand white (NZW) rabbits, 2–3.5 kg, were obtained from Charles River Laboratories, Wilmington, MA. Equal numbers of males and females were used in the study.

2.2. Vaccine

AVA was procured from BioPort (Lansing, MI). Lot FAV 008 was used in the first study and Lot FAV 032 in the second study.

2.3. Vaccination and challenge schedule

In two separate experiments, rabbits were vaccinated at 0 and 4 weeks intramuscularly (i.m.) with 0.5 ml of AVA (undiluted or diluted in phosphate-buffered saline (PBS)) or placebo (PBS with Alhydrogel containing 0.725 mg of metallic aluminum) (Superfos Biosector a/s Denmark). At both 6 weeks and between weeks 9 and 10 (prior to challenge) sera were examined by an enzyme-linked immunosorbent assay (ELISA) for antibodies to PA, and by a cell cytotoxicity assay for toxin-neutralizing antibodies. At 10 weeks all the rabbits were challenged with an aerosol of *B. anthracis* Ames spores.

2.4. B. anthracis challenge

Spores of the *B. anthracis* Ames strain were grown in Leighton and Doi medium, purified by centrifugation through 58% Renografin-76, resuspended in sterile water-for-injection (McGraw) containing 1% phenol and stored at 2–8 °C until used, as described earlier [10]. Immediately before challenge, the spores were diluted in sterile water-for-injection to $2.2–2.8 \times 10^9$ CFU/ml, heat-shocked at 60 °C for 45 min, and divided into 8 ml aliquots.

Each rabbit was placed in a nylon transport bag (Cat Sack; Four Flags over Aspen, Janesville, MN) for the challenge. Respiratory minute volumes were measured by whole body plethysmography using a Buxco Biosystem XA (Buxco Electronics, Sharon, CT), immediately before challenge. The rabbits were then exposed to the spore aerosol, muzzle-only (i.e. nose and mouth), in a dynamic aerosol chamber. The aerosol (mass median aerosol diameter, 1.2 μm) was generated by a three-jet Collison nebulizer [14,15]. The exposures were 10 min long, and the aerosol was sampled continuously by an all-glass impinger (AGI-30; Ace Glass, Inc., Vineland, NJ). For each animal, the aerosol concentration of spores was calculated by plating out dilutions of a sample from the AGI onto tryptic soy agar plates (Difco, Detroit, MI). The inhaled doses were then determined (expressed as LD_{50}). One aerosol LD_{50} in NZW rabbits is 1.1×10^5 spores (L. Pitt, unpublished observation). Animals challenged in study 1 received an average inhaled dose of 133 ± 51 (mean ± S.D.) LD_{50} while those in study 2 received 84 ± 42 LD_{50}.

2.5. Assay of rabbit anti-PA IgG by ELISA

Wells of polyvinyl chloride microtiter plates (Dynex Technologies, Chantilly, VA) were coated with 100 μl of purified recombinant PA (rPA) antigen [16] at 1 μg/ml in 10 mM PBS, pH 7.4 (PBS; Sigma Chemical Company, St. Louis, MO), and were incubated at 6–10 °C for 12–48 h. The plates were washed three times in PBS containing 0.1% Tween 20 (PBST). Standards, control sera, and test sera, at appropriate dilutions prepared in PBST containing 5% non-fat dry milk (PBSTM), were added to the plate (100 μl per well) in triplicate and the plates were incubated at 37 °C for 1 h. The plates were washed with PBST and 100 μl of horseradish peroxidase-labeled goat anti-rabbit IgG(H + L) (Kirkegaard and Perry Laboratories, Gaithersburg, MD) diluted 1:1000 in PBSTM was added per well. After the plates were incubated for 1 h at 37 °C, the plates were washed and 2,2′-azino-bis(3-ethylbenzthiozoline-6-sulfonic acid) (ABTS; Kirkegaard and Perry Laboratories) was added (100 μl per well) and the plates were incubated for 30 min at 37 °C. The absorbance values were obtained using a BioTek 312e microplate reader (BioTek Instruments, Winooski, VT) with a 405 nm filter. The concentration of IgG was calculated by interpolating the average absorbance value for triplicate wells with the absorbance values of a standard curve generated from seven dilutions of affinity purified rabbit anti-rPA IgG. Results, expressed as μg IgG per ml, are the mean of two separate assays.

2.6. Anthrax lethal toxin-neutralizing assay

Antibodies to lethal toxin were measured by the ability of sera to neutralize the cytotoxicity of lethal toxin for J774A.1 cells. Antisera were diluted in cell culture medium and added in triplicate to cell culture-treated 96-well microtiter plates (Corning Costar, Acton, MA), then preincubated with purified rPA (50

ng/ml final concentration) and lethal factor (40 ng/ml final concentration) for 1 h at 37 °C. A plate-to-plate transfer from the titration plate to another 96-well plate containing a monolayer of J774A.1 cells, plated the day before the assay at 5×10^5 cells per ml, was then performed. Plates were incubated for 4 h at 37 °C in 5% CO_2. Twenty-five microliters of 3-[4,5-dimethyl-thiazol-2-y-]-2,5-diphenyltetrazolium bromide (MTT; Sigma Chemical Company) at 5 mg/ml in PBS was then added per well. After incubating for 2 h, the cells were lysed and the reduced purple formazan was solubilized by adding 20% (w/v) SDS in 50% dimethylformamide, pH 4.7 [17]. Optical density readings were obtained at 570 nm with a reference wavelength at 690 nm with a BioTek 312e microplate reader (BioTek Instruments). The ratio between toxin plus antibody versus medium alone, expressed as a percentage of cell viability, was calculated for each dilution and plotted. The lethal toxin-neutralizing antibody titers of individual serum, calculated by linear regression analysis, were expressed as the reciprocal of the antibody dilution preventing 50% of cell death.

2.7. Statistical analysis

The association of survival and logarithm of anti-PA antibody responses, both the concentration of anti-PA IgG and the toxin neutralizing antibody titers present at week 6 and the concentration of anti-PA IgG at 9–10 weeks, was tested by logistic regression analysis (SAS Institute Inc., SAS OnlineDoc version 8, Cary, NC, 2000).

3. Results

In the first study, groups of rabbits were vaccinated at 0 and 4 weeks with either undiluted or various dilutions of AVA and challenged by aerosol at 10 weeks. All rabbits that received undiluted AVA or 1:4 dilution of vaccine survived. Nine of the 10 rabbits in the 1:16 and 1:64 groups survived, whereas only one of the 10 in the 1:256 group survived (Table 1). All rabbits in the placebo group died.

The survival of the rabbits vaccinated with the varying doses of AVA was compared with the antibody responses measured at weeks 6 and 10 (Table 1). The response of individual animals is shown in Fig. 1. Both the concentration of anti-PA IgG and the TNA titer in sera at week 6 were significant predictors of survival ($P < 0.0001$ for both). In addition, the concentration of anti-PA IgG present at the time of challenge was also a significant predictor of survival ($P < 0.0001$).

In the second study, all the rabbits that received either the undiluted dose or the 1:4 dilution survived. Fifteen of 20 and 14 of 20 rabbits survived in the 1:16 and 1:64 groups, respectively (Table 1). All rabbits in the 1:256 dilution group and all the placebo controls died.

The antibody data were then compared with the survival data. Both the TNA titers and the concentration of anti-PA IgG in sera at week 6 were significant predictors of survival ($P < 0.0001$) as was the anti-PA IgG concentration in sera at the time of challenge (week 10) ($P < 0.0001$). The antibody response of individual animals is shown in Fig. 2.

Table 1
Summary of survival of rabbits vaccinated with varying doses of AVA after aerosol challenge with *B. anthracis* spores and the anti-PA IgG response

Dose of AVA	Survivors/Challenge (%)	Anti-PA IgG[a] (μg/ml)		TNA[b] (reciprocal titer)
		6 week	10 week	6 week
Study 1[c]				
Undiluted	8/8 (100)	1541	446	6490
1:4	10/10 (100)	574	230	2780
1:16	9/10 (90)	264	110	1248
1:64	9/10 (90)	124	48	602
1:256	1/10 (10)	13	5	55
Control	0/8 (0)	<1	<1	25
Study 2[d]				
Undiluted	10/10 (100)	550	384	4627
1:4	10/10 (100)	307	263	2928
1:16	15/20 (75)	147	122	1174
1:64	14/20 (70)	61	41	512
1:256	0/20 (0)	13	6	68
Control	0/10 (0)	<1	<1	25

[a] Group arithmetic mean.
[b] Group geometric mean reciprocal titer.
[c] Rabbits were vaccinated with varying doses of AVA Lot FAV 008.
[d] Rabbits were vaccinated with varying doses of AVA Lot FAV 032.

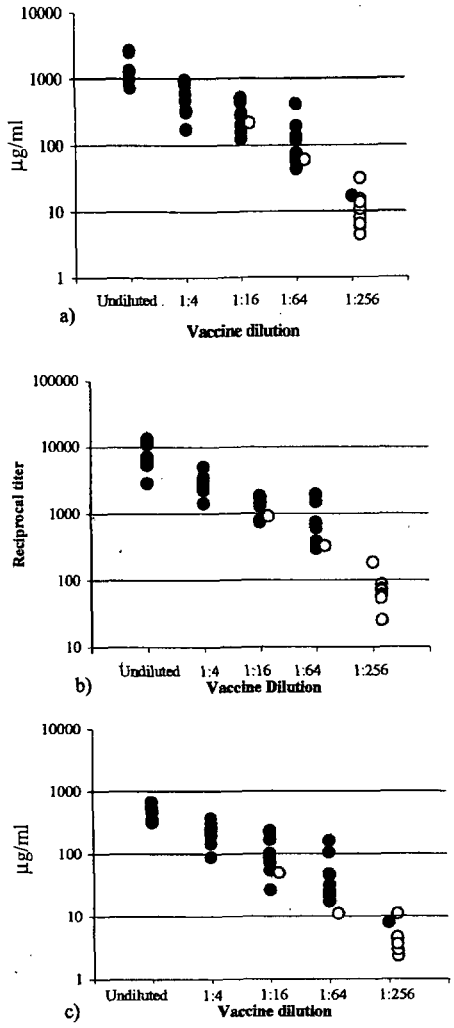

Fig. 1. Relationship between survival in AVA-vaccinated rabbits (Lot FAV 008) after aerosol challenge with *B. anthracis* spores and antibody response. The vaccine dilution is plotted against (a) the anti-PA IgG concentration (µg/ml) at 6 weeks; (b) the reciprocal TNA titer at 6 weeks; and (c) the anti-PA IgG concentration (µg/ml) at 9–10 weeks. Individual animals surviving (●) or dying (○) after challenge are indicated.

4. Discussion

This study was undertaken to develop a serological correlate of AVA-induced immunity against experimental inhalational anthrax.

The principal animal models used in laboratory investigations of experimental anthrax have been mice, rats, guinea pigs, rabbit and rhesus monkeys. Rhesus monkeys are considered the best model of inhalational anthrax in humans [3,18–21]. The disease induced by respiratory exposure to spores is a rapidly fatal illness, death occurring between the second and seventh days postexposure. In addition, rhesus monkeys inoculated with two doses of the licensed vaccine, AVA, were protected against a lethal aerosol challenge of anthrax spores for up to 2 years [11]. However, the necessity for large numbers of monkeys to produce statistically significant data for the evaluation of an in vitro correlate of immunity makes the use of rodents or lagomorphs desirable.

Mouse strains differ significantly in their innate susceptibility to lethal infection by both a fully virulent strain and the non-encapsulated Sterne vaccine strain [22,23]. The capsule appears to be important as a virulence factor in mice. Although capsule-positive, toxin-negative strains are avirulent in guinea pigs, they are virulent for mice [22]. Mice can be protected against attenuated, unencapsulated anthrax strains with various vaccines but not against virulent organisms [24]. Rats are resistant to *B. anthracis* infection but are sensitive to toxin [25]. Vaccinating naturally resistant rats only slightly increases their resistance to spore challenge [26].

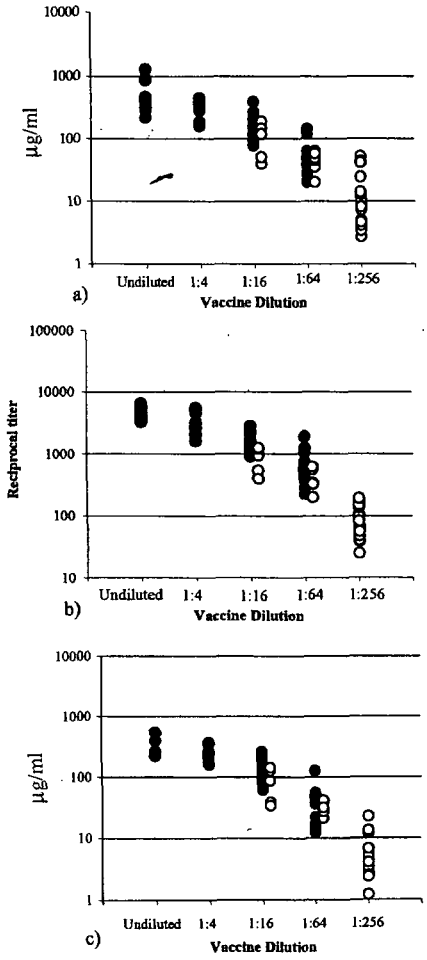

Fig. 2. Relationship between survival in AVA-vaccinated rabbits (Lot FAV 032) after aerosol challenge with *B. anthracis* spores and antibody response. The vaccine dilution is plotted against (a) the anti-PA IgG concentration (µg/ml) at 6 weeks; (b) the reciprocal TNA titer at 6 weeks; and (c) the anti-PA IgG concentration (µg/ml) at 9–10 weeks. Individual animals surviving (●) or dying (○) after challenge are indicated.

Guinea pigs have been used extensively to characterize the pathogenesis of the disease [27], elucidate the role of toxin [28], and test the immunogenicity of anthrax vaccines [10,29,30]. The licensed vaccine, AVA, only partially protects guinea pigs against a parental challenge and is poorly protective against an aerosol spore challenge [10,30]. However, when PA is combined with certain new adjuvants other than aluminum, full protection can be obtained [29–31].

Guinea pigs inoculated with rPA/Ribi adjuvant were completely protected against a lethal aerosol challenge but were only poorly protected when vaccinated with either rPA/Alhydrogel or the licensed UK human vaccine [31]. Analysis of the immunological parameters showed an overall higher lymphocyte proliferation response, higher PA neutralizing titers and IgG2 levels in the rPA/Ribi group compared with other vaccine groups, but no significant differences in PA-specific IgG1 levels, and no correlation of antibody levels with survival after challenge.

Different adjuvants stimulate diverse immune responses when combined with protein antigens. They may either protect via various mechanisms or not be capable of inducing protective immunity, and these responses do vary between species [32,33]. The guinea pig immune system may be incapable of recognizing the protective epitopes of PA when it is combined with aluminum. They are difficult to protect by vaccination with aluminum-containing human vaccines.

Rabbits are also used for anthrax research [34,35]. These animals are extremely sensitive to lethal infection by *B. anthracis*. The pathology of anthrax in the rabbit model appears remarkably similar to that of inhalational anthrax in humans, although the disease progresses more rapidly [36]. In addition, rabbits are a good predictor for vaccine efficacy in rhesus monkeys [13]. AVA is efficacious in rabbits given two doses of the vaccine 4 weeks apart and aerosol challenged 3 months later with a lethal dose of anthrax spores. We found a similar high degree of efficacy of AVA in rabbits in this study and another study of challenge with other *B. anthracis* [37]. Thus, we chose this animal model to develop a surrogate marker of efficacy for inhalational anthrax with the licensed anthrax vaccine, AVA.

While it has become clear that PA is the principal protective immunogen in vaccine-induced immunity, prediction of immunity based on anti-PA antibody levels has not been shown conclusively. Several studies have, however, provided some evidence for this. Survival of guinea pigs after intramuscular (i.m.) challenge correlates with anti-PA antibodies after vaccination with an attenuated live *B. anthracis* strain producing PA, LF, and EF [38]. More recently, survival of guinea pigs challenged i.m. also correlates with anti-PA antibodies after vaccination with attenuated recombinant *B. anthracis* strains producing varying amounts of PA [39]. In addition, serum anti-toxin levels in rabbits hyperimmunized with PA, measured with a guinea pig intradermal toxin neutralization assay, correlated with survival after intradermal challenge [35].

The importance of antibodies to PA in protection was shown by the demonstration that antiserum to recombinant PA provided passive protection, comparable to that achieved after active vaccination with AVA, in guinea pigs [40]. In addition, in a pilot study, we found that anti-AVA sera elicited in rabbits could passively protect naïve rabbits from a lethal anthrax infection (data not shown).

In this study, we inoculated dilutions of AVA to several groups of rabbits to get a gradation in both the serological response to PA and survival after challenge (Table 1). Statistical analysis demonstrated a significant ($P < 0.0001$) correlation between survival and the anti-PA antibody levels achieved at week 6 (2 weeks after the second dose), and at the time of challenge. Both the total anti-PA IgG and the toxin-neutralizing antibody at 6 weeks and the anti-PA IgG just prior to challenge were predictive of survival.

We also investigated the lymphocyte memory responses to PA at 6 weeks in the first study (data not shown). We found no correlation between the lymphocyte proliferative response to PA and survival.

The present study demonstrates for the first time a serological correlate of vaccine-induced immunity against inhalational anthrax. These results will be of great value in choosing an in vitro test or surrogate marker to determine the immune status of humans vaccinated with AVA.

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