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Immunological Correlates for Protection against Intranasal Challenge of *Bacillus anthracis* Spores Conferred by a Protective Antigen-Based Vaccine in Rabbits

Shay Weiss,[1] David Kobiler,[1] Haim Levy,[1] Hadar Marcus,[2] Avi Pass,[2] Nili Rothschild,[1]
and Zeev Altboum[1]*

Departments of Infectious Diseases[1] and Biotechnology,[2] Israel Institute for Biological Research, Ness-Ziona, Israel

Correlates between immunological parameters and protection against *Bacillus anthracis* infection in animals vaccinated with protective antigen (PA)-based vaccines could provide surrogate markers to evaluate the putative protective efficiency of immunization in humans. In previous studies we demonstrated that neutralizing antibody levels serve as correlates for protection in guinea pigs (S. Reuveny et al., Infect. Immun. 69:2888–2893, 2001; H. Marcus et al., Infect. Immun. 72:3471–3477, 2004). In this study we evaluated similar correlates for protection by active and passive immunization of New Zealand White rabbits. Full immunization and partial immunization were achieved by single and multiple injections of standard and diluted doses of a PA-based vaccine. Passive immunization was carried out by injection of immune sera from rabbits vaccinated with PA-based vaccine prior to challenge with *B. anthracis* spores. Immunized rabbits were challenged by intranasal spore instillation with one of two virulent strains (strains Vollum and ATCC 6605). The immune competence was estimated by measuring the level of total anti-PA antibodies, the neutralizing antibody titers, and the conferred protective immunity. The results indicate that total anti-PA antibody titers greater than 1×10^5 conferred protection, whereas lower titers (between 10^4 and 10^5) provided partial protection but failed to predict protection. Neutralizing antibody titers between 500 and 800 provided partial protection, while titers higher than 1,000 conferred protection. In conclusion, this study emphasizes that regardless of the immunization regimen or the time of challenge, neutralizing antibody titers are better predictors of protection than total anti-PA titers.

The virulence of *Bacillus anthracis*, the causative agent of anthrax, is attributed to the anthrax tripartite toxin (composed of protective antigen [PA], lethal factor [LF], and edema factor) and its capsule. Toxic activity is expressed only when PA is combined with LF and with edema factor, forming the lethal toxin and edema toxin, respectively (2, 11). Consistent with the central role of PA in anthrax toxin action, preexposure vaccination with PA-based vaccines induces protective immunity to anthrax (4).

The protective efficacy of PA-based vaccines against the onset of fatal disease following challenge with *B. anthracis* spores varies in different animal models. For example, anthrax vaccine adsorbed (AVA), the human vaccine licensed in the United States, fails to protect mice (17), only partially protects guinea pigs from challenge with several *B. anthracis* isolates (3, 9), and is highly efficacious in rabbits and rhesus monkeys (3). Although the rhesus monkey model has been suggested as the best model for human disease, the rabbit model has lately gained acceptance as an appropriate model for evaluation of the anti-anthrax vaccines (8, 12, 13, 18).

Studies with rabbits vaccinated with either AVA or recombinant PA-based vaccines (8, 13) revealed that quantitative anti-PA immunoglobulin G (IgG) enzyme-linked immunosorbent assay (ELISA) and neutralizing antibody titers could be used as serological correlates for protection against challenge by the aerosol route with Ames spores.

In a previous study (16) we demonstrated that in PA-vaccinated guinea pigs, neutralizing antibody titers could predict protection against intradermal challenge with Vollum spores.

Here, we demonstrated the efficacy of a PA-based vaccine to prevent the onset of inhalation anthrax in rabbits challenged intranasally with either Vollum or ATCC 6605 spores and found that neutralizing antibody titers are better serological correlates than anti-PA titers for efficient protection.

MATERIALS AND METHODS

***B. anthracis* strains.** The virulent strains used in this study were ATCC 14578 (Vollum) (Tox+ Cap+) and ATCC 6605 (Tox+ Cap+) from the IIBR collection (1, 6). The intranasal 50% lethal dose (LD_{50}) for each strain in rabbits was estimated by using three inoculum doses (three animals per group) and a refinement experiment with three intermediate doses (three animals per group). The ATCC 14578 and ATCC 6605 LD_{50}s were then calculated by the method of Reed and Muench (15), and the estimated doses were 3×10^5 and 2×10^4 spores, respectively, as determined by direct plate counting. The strain used for PA vaccine preparation was strain ATCC 14185 (Tox+ Cap−) from the IIBR collection (6, 16).

Animals. New Zealand White rabbits (2.5 to 3.5 kg) were obtained from Harlan (Israel). The animals received food and water ad libitum. All animals were cared for according to the 1997 NIH guidelines for the care and use of laboratory animals; the IIBR animal use committee approved all experimental protocols.

Vaccination and challenge. Purified PA isolated from strain ATCC 14185 was absorbed to Alhydrogel (Superfos Biosector) to generate a 50-μg/ml PA vaccine dose, as previously described (16). The animals were vaccinated by intramuscular injection of 0.5 ml of the PA-based vaccine. The immunization regimens are shown in Table 1. Different concentrations of PA-based vaccines were generated by dilution of the original vaccine in phosphate-buffered saline (PBS). Serum samples were collected biweekly, and antibody titers were determined by ELISA and cytotoxicity neutralization assays. Prior to challenge, the rabbits were anesthetized by subcutaneous injection of a mixture of ketamine HCl (40 mg/kg of body weight) and xylazine (5 mg/kg). The animals were challenged intranasally

* Corresponding author. Mailing address: P.O. Box 19, Ness-Ziona, Israel, 74100. Phone: 972-8-9381414. Fax: 972-8-9381639. E-mail: altboum @iibr.gov.il.

TABLE 1. Determination of total anti-PA and cytotoxicity-neutralizing antibodies in rabbits immunized with PA-based vaccine by different immunization regimens

Immunization regimen[a]			Total anti-PA antibodies			Neutralizing antibodies		
			Maximal titer		Induction ratio[c]	Maximal titer		Induction ratio[c]
Group	Week(s)	n	Week(s)	GMT (10^3)[b]		Week(s)	GMT (10^3)	
1	0	10	6	64	1	4–6	1.6	1
2	0 + 2	4	6	256	4	6	6.4	4
3	0 + 4	10	6	724	11	6	18	11
4	0 + 2 + 4	4	9	512	8	6	12.8	8
5	0 + 13	6	17	380	6	17	7	4.35
6	0 + 4 + 13	6	6, 17	724	11	6, 17	18	11

[a] Rabbits were immunized with PA-based vaccine (0.5 ml, 50 μg/ml).
[b] GMT, geometric mean titer.
[c] The induction ratio was calculated with respect to group 1 (single immunization at day 0). The total anti-PA and neutralizing antibody titers were determined by ELISA and J774.1 cytotoxicity assays, respectively.

by unilateral instillation of either Vollum or ATCC 6605 spores (0.5 ml per nostril). Survival was recorded for 30 days after challenge.

ELISA for total anti-PA titers. Antibody titers were determined by direct ELISA in 96-well microtiter plates (Nunc, Rosklide, Denmark), using purified PA (16) as the capture antigen and an alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO) as the detection reagent. The plates were coated with 10 μg/ml of purified PA (1 μg/well) in NaHCO₃ buffer (50 mM, pH 9.6) and subsequently blocked with 5% skim milk (Becton Dickinson, Sparks, MD). The sera tested and the internal positive and negative controls were serially diluted in twofold steps, and the plates were then incubated for 1 h at 37°C. The plates were washed with PBS containing 0.05% Tween 20 and were developed with detecting antibody conjugate, with *p*-nitrophenyl phosphate (Sigma, St. Louis, MO) as the substrate. Absorbance at 405 nm was determined using a Spectramax 190 microplate reader (Molecular Devices, Sunnyvale, CA). The end point was defined as the highest dilution at which the absorbance was >3 standard deviations above that of the negative control (normal rabbit serum). Antibody titers were expressed as reciprocal end point dilutions.

Determination of cytotoxicity neutralization titers. Neutralizing antibody titers were determined by assessment of the ability to prevent the death of murine macrophage J774.1 cells induced by the PA/LF toxin complex. The cytotoxicity assays were performed as described previously (10, 16). Antibody titers were expressed as reciprocals of the end point dilutions.

Passive immunization. Immune serum was collected (between weeks 6 and 10) from rabbits immunized with two injections (at zero time and 4 weeks) of the PA-based vaccine. Various doses of the immune serum were administered intramuscularly to naïve rabbits. Two days after serum administration, rabbits were bled for estimation of neutralizing antibodies prior to intranasal spore instillation.

Statistical analysis. The association of survival and the logarithm of neutralizing or total anti-PA titers was tested by an unpaired two-tailed *t* test. The correlation between neutralizing antibodies and total anti-PA was tested by linear regression. All tests were performed using GraphPad Prism, version 4.03 for Windows (GraphPad Software, San Diego, CA; www.graphpad.com). The hypothetical antibody titer that conferred protection to 50% of the immunized rabbits (hPT₅₀) was calculated by adapting the Reed-Muench method for deter-

mination of LD₅₀ (15). For the actual calculation, we used the dilutions of serum (ELISA or neutralizing titers) and the percentage of survivors (instead of the percentage of dead animals).

RESULTS

Evaluation of immunization regimen. In order to determine the optimal rabbit immunization regimen, using a PA-based vaccine, we examined the abilities of different regimens to induce anti-PA antibodies (defined by ELISA) and neutralizing antibodies (defined by inhibition of the lethal toxin effect in a cytotoxicity assay). As shown in Table 1, two injections that were 4 weeks apart generated the most efficient antibody response, which peaked between 6 and 10 weeks after the first injection (titers for total anti-PA and neutralizing antibodies, 724,000 and 18,000, respectively) and then gradually declined. Other vaccination regimens that included one injection (Table 1, group 1), two injections that were 2 weeks apart (Table 1, group 2), and two injections that were 13 weeks apart (Table 1, group 5) resulted in lower antibody responses ($P < 0.01$ compared to group 3). Three injections that were 2 weeks apart (Table 1, group 4) induced titers that did not differ significantly from group 3 titers ($P > 0.05$). The regimen consisting of two injections that were 4 weeks apart seemed to be optimal since addition of a third boost at week 13 (Table 1, group 6) resulted in titers of specific anti-PA antibodies that were similar to but not greater than those at week 6.

Protection conferred by PA-based vaccine. The protection conferred by the optimal immunization regimen described above was tested by intranasal challenges with spores of two virulent strains, strains Vollum and ATCC 6605. The prechallenge immune status of the animals at week 10 after initiation of vaccination and the challenge parameters are shown in Table 2. The PA-based vaccine conferred full protection against both strains (eight of eight animals in each group) when the animals were tested with various doses ranging from 26 to 2,300 LD₅₀s. In addition, no changes in the anti-PA antibody titers (both total and neutralizing) were detected within 30 days after challenge (data not shown), indicating that the animals were highly immunized against PA, which probably prevented bacterial growth.

Correlates for protection. To define a serological marker that could predict protection, two experimental approaches were used: active immunizations and pretreatment with specific anti-PA antibodies. We determined the protection conferred by immunization with decreasing amounts of PA-based vaccine in the regimen selected, using undiluted or PBS-diluted PA-based vaccine. Ten weeks after the first injection, the animals were challenged by intranasal instillation of 300 LD₅₀s of ei-

TABLE 2. Antibody titers and efficacy of protection conferred by PA-based vaccine against intranasal instillation of *B. anthracis* spores

Group	Prechallenge immune status (week 10)[a]		Challenge parameters[b]			% Survival (no. of survivors/ no. challenged)
	Total anti-PA GMT, 10^3 (range)	Neutralizing antibody GMT, 10^3 (range)	Strain	Challenge dose		
				CFU	LD₅₀	
1	395 (282–552)	11 (7–16)	Vollum	8×10^6	26	100 (8/8)
2	215 (136–340)	13 (13)	ATCC 6605	5×10^7	2,300	100 (8/8)

[a] Animals were vaccinated with PA-based vaccine (0.5 ml, 50 μg/ml) by two immunizations that were 4 weeks apart. GMT, geometric mean titer.
[b] The animals were challenged at week 10 after immunization by intranasal spore instillation. Nonimmunized animals (*n* = 4 for each strain) died within 2 to 3 days after infection.
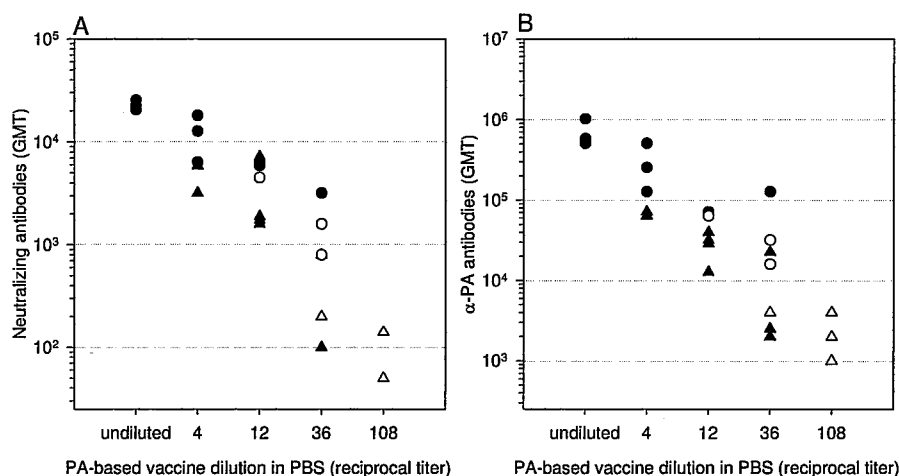
FIG. 1. Relationship between antibody titer of rabbits immunized with PA-based vaccine and survival upon challenge via intranasal spore instillation. Rabbits were immunized with either undiluted or PBS-diluted vaccines by two immunizations that were 4 weeks apart, and at week 10 their neutralizing (A) and total anti-PA (α-PA) (B) antibody titers were determined. The rabbits were then challenged with 300 LD_{50}s of either Vollum (circles) ($n = 12$) or ATCC 6605 (triangles) ($n = 13$). Solid and open symbols represent surviving and dead animals, respectively. GMT, geometric mean titer.

ther Vollum or ATCC 6605 spores. The prechallenge immune response (neutralizing antibody and total anti-PA antibody titers) and protection efficiency were determined (Fig. 1). All the rabbits that were immunized with undiluted vaccine (25 μg PA) or with PA-based vaccine diluted 1:4 ($\sim$6.25 μg PA) survived. Further dilution of the PA-based vaccine resulted in decreasing titers of anti-PA and neutralizing antibodies along with partial protection. Neutralizing antibody titers higher than 1,000 conferred protection to 14/16 rabbits (Fig. 1A). Total anti-PA titers higher than 100,000 conferred protection to 7/7 rabbits, and titers between 10^4 and 10^5 protected 8/11 rabbits (Fig. 1B).

We assessed the level of protection conferred with greater intervals between the last vaccination and challenge. Rabbits that were immunized using six immunization regimens (Table 1) were monitored up to weeks 19 and 23 after initiation of immunization and then challenged with 10 and 160 LD_{50}s of Vollum spores, respectively (Table 3).

The prechallenge PA-specific antibody levels, survival rates, and mean times to death are shown in Table 3. Full protection was conferred when there was a 6-week interval between the last vaccination and challenge (groups 5 and 6) and when there was a 15-week interval (group 3); all the other immunization regimens (15-, 19-, and 23-week intervals) conferred partial protection with a marked delay in the mean time to death (Table 3). The individual total anti-PA and neutralizing antibody titers were plotted against protective ability (Fig. 2). The data demonstrate that neutralizing antibody titers higher than 1,000 conferred full protection ($P < 0.001$) (Fig. 2A). Total anti-PA antibody titers higher than 100,000 conferred protection ($P < 0.001$), whereas lower titers (between 10^4 and 10^5) provided partial protection but failed to predict protection ($P = 0.2929$) (Fig. 2B).

The data from the active immunization experiments (Tables 1 and 2 and Fig. 1 and 2) were analyzed to determine the hypo-

TABLE 3. Antibody titers and efficiency of protection for PA-based vaccine-immunized rabbits with different intervals between the last vaccination and challenge

Immunization regimen		Time of challenge (week)[a]	Antibody GMT at challenge, 10^3 (range)[b]		% Survival (no. of survivors/ no. challenged)	MTTD (days) ($\pm$SD)[c]
Group	Week(s)		Total anti-PA	Neutralizing antibody		
1	0	19	12.7 (8–32)	0.9 (0.4–0.8)	33.3 (2/6)	8.25 ± 4.35
		23	33.6 (20–40)	0.6 (0.4–0.8)	25 (1/4)	5.0 ± 1.0
2	0 + 2	23	33.6 (20–80)	0.6 (0.4–0.8)	25 (1/4)	7.3 ± 4.2
3	0 + 4	19	161 (64–512)	5 (3.2–6.4)	100 (6/6)	
		23	67.3 (40–80)	0.9 (0.8–1.6)	50 (2/4)	5.0 ± 1.4
4	0 + 2 + 4	23	33.6 (20–40)	0.87 (0.4–1.6)	50 (2/4)	4.5 ± 0.7
5	0 + 13	19	228 (128–256)	7 (6.4–12.8)	100 (6/6)	
6	0 + 4 + 13	19	256 (128–512)	5.7 (3.2–6.4)	100 (6/6)	
Control	Naive	19	BDL[d]	BDL	0 (0/2)	2.0 ± 0
		23	BDL	BDL	0 (0/2)	2.0 ± 0

[a] The challenge parameters were as follows: at week 19 the animals were challenged with 10 LD_{50}s, and at week 23 they were challenged with 160 LD_{50}s of Vollum spores.
[b] GMT, geometric mean titer.
[c] The mean time to death (MTTD) was determined by determining the average time to death for each group, excluding the survivors.
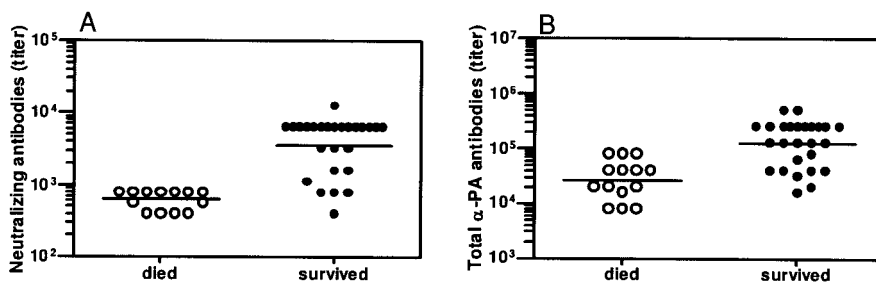[d] BDL, below the detection limit.

FIG. 2. Relationship between antibody titers of rabbits immunized with PA-based vaccine and survival upon challenge via intranasal spore instillation. Rabbits were immunized by using various regimens (Table 1) ($n = 40$). At weeks 19 and 23, the neutralizing (A) and total anti-PA (α-PA) (B) antibody titers were determined, and the animals were challenged with 10 to 160 LD_{50}s of Vollum spores. Solid and open circles represent surviving and dead animals, respectively. For each group the solid line indicates the geometric mean titer.

thetical antibody titers (15) that conferred protection to 50% of the immunized rabbits. The calculated hPT_{50}s obtained were 940 for neutralizing antibodies and 25,000 for total anti-PA antibodies. These hPT_{50}s were then used as central axes to analyze the relationship between neutralizing or total anti-PA titers and survival (Fig. 3). Most of the animals that developed antibody titers higher than the calculated hPT_{50} were protected against challenge (37/39 survivors [94.5%]) (Fig. 3, upper right quadrant). Animals with antibody titers that were lower than the calculated hPT_{50} were not protected (5/17 survivors [29.4%]) (Fig. 3, lower left quadrant). According to the neutralizing antibody titer criteria, titers higher than the hPT_{50} indicated survival of 37 of 39 animals ($\sim$94.5%). All the animals that qualified for the neutralizing hPT_{50} criteria also qualified for the total anti-PA hPT_{50} criteria. On the other hand, total anti-PA titers higher than the calculated hPT_{50} indicated that 39 of 48 animals ($\sim$81.2%) survived. Of the nine animals that qualified for the total anti-PA hPT_{50} criteria but failed to qualify for the neutralizing hPT_{50} criteria, only two survived ($\sim$22.2%) (Fig. 3, lower right quad-

rant). Although there is a good correlation between neutralizing and total anti-PA titers ($r^2 = 0.7961$), our data indicate that neutralizing antibody titers are better predictors for protection than total anti-PA titers and that neutralizing antibody conferred protection regardless of the immunization protocol or the time of challenge.

In order to corroborate the neutralizing antibody hPT_{50}, we next studied the protection conferred by passive immunization of rabbits. Various amounts (0.375 to 12 ml/kg) of rabbit anti-PA-based vaccine hyperimmune sera (with a neutralizing antibody titer of 12,800) were administered subcutaneously 48 h before intranasal Vollum spore instillation (10 to 40 LD_{50}s). The relationship between neutralizing antibody titers and protection was analyzed (Fig. 4). Rabbits with neutralizing antibody titers higher than 1,000 resisted challenge, and antibody titers between 500 and 800 conferred protection to 8 of 11 animals ($\sim$72.7%), while lower antibody titers did not provide any protection (Fig. 4). These results suggest that in rabbits neutralizing antibody titers of at least 500 to 800 are necessary for protection against intranasal spore instillation.
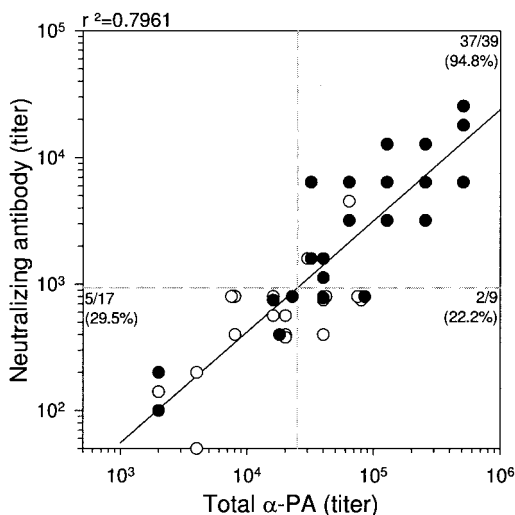
DISCUSSION

The correlates between antibody titers and protection against *B. anthracis* infection in different animal species vaccinated with PA-based vaccines could provide surrogate markers to evaluate the protective efficiency of immunization in humans. In previous studies we determined the immunological correlates for protection of guinea pigs vaccinated with a PA-based vaccine against challenge with Vollum spores injected intradermally (10, 16). In this study we evaluated the correlates for protection by active immunization of New Zealand White rabbits with a PA-based vaccine or passive immunization of rabbits with anti-PA-based vaccine hyperimmune sera against challenge via intranasal instillation of lethal doses of *B. anthracis* spores.

Two immunizations that were 4 weeks apart were used to demonstrate the efficacy of AVA (13) or recombinant anthrax vaccine (8) in rabbits against inhalation of *B. anthracis* Ames spores. In humans, postponement of the second AVA dose (from 2 to 4 weeks after immunization) resulted in an enhanced antibody response (14). The rabbit model, as presented in this study, supports and corroborates these findings since the same immunization protocol was found (i) to provoke the highest antibody response for either total anti-PA or neutral-

FIG. 3. Relationship between hPT_{50}s and survival in active immunization experiments. The individual prechallenge neutralizing titers were plotted against the matching prechallenge total anti-PA (α-PA) titers. Solid and open circles represent surviving and dead rabbits, respectively. The number of survivors/number challenged and the percentage of protection are indicated in the quadrants. The hPT_{50}s are indicated by dashed lines.
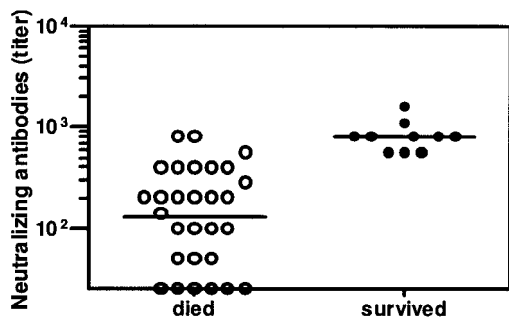
FIG. 4. Relationship between neutralizing antibody titers and survival of passively immunized rabbits. Decreasing amounts (12 to 0.375 ml/kg) of rabbit anti-PA-based vaccine hyperimmune sera were administered subcutaneously 48 h before intranasal spore instillation (10 to 40 $LD_{50}s$). Neutralizing antibody titers were determined by a cytotoxicity assay. The solid circles represent survivors, and the open circles represent dead rabbits. For each group the solid line indicates the geometric mean titer.

izing antibody titers, (ii) to maintain high levels of antibodies between weeks 6 and 10 after initiation of immunization, and (iii) to confer full protection against challenge with two virulent *B. anthracis* strains (strains Vollum and ATCC 6605) within 15 weeks after the last immunization.

The central role of anti-PA antibodies in protection against *B. anthracis* infection was demonstrated in passive protection experiments with guinea pigs challenged either intranasally with Vollum spores (5) or intramuscularly with Ames spores (7). In addition, PA-neutralizing antibodies were found to represent correlates for protection in guinea pigs (10, 16). Recently, both neutralizing and IgG anti-PA titers were reported to be correlates for protection in rabbits (8, 13). In these experiments the rabbits were challenged at week 10 after initiation of immunization. Our data support these findings since at this time the total anti-PA and neutralizing antibody titers were more than 100,000 and 1,000, respectively, in all the rabbits vaccinated. However, as demonstrated by late-challenge experiments, at weeks 19 or 23, our findings indicate that neutralizing antibody titers at the time of challenge should be employed as correlates for immunity in rabbits.

In conclusion, this study completed previous experiments to define correlates for protection induced by an experimental PA-based vaccine. First we defined the antibody titers that provide protection in a guinea pig model challenged by intradermal inoculation (10, 16), and in the present study we introduced an additional animal model, immunized rabbits challenged via intranasal spore instillation. Both animal models proved that neutralizing

antibody titers serve as a reliable surrogate marker for protection against *B. anthracis* spore challenge.

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