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

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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 protective efficacy 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 × 10^9 conferred protection, whereas lower titers (between 10^8 and 10^9) 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 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 tripartite toxin (composed of protective antigen [PA], lethal factor [LF], and edema factor). Consistent with the central role of PA in anthrax toxin action, preexposure vaccination with PA-based vaccines induces protective immunity to anthrax.

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). The rhesus monkey model has been suggested as the best model for human disease, the rabbit model has largely 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 (LD50) 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 LD50s were then calculated by the method of Reed and Muench (15), and the estimated doses were 3 × 10^8 and 2 × 10^8 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 by the aerosol route with Ames spores.
TABLE 1. Determination of total anti-PA and cytotoxicity-neutralizing antibodies in rabbits immunized with PA-based vaccine by different immunization regimens

<table>
<thead>
<tr>
<th>Immunization regimen</th>
<th>Total anti-PA antibodies</th>
<th>Neutralizing antibodies</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Maximal titer</td>
<td>Induction ratio</td>
</tr>
<tr>
<td></td>
<td>Maximal titer</td>
<td>Induction ratio</td>
</tr>
<tr>
<td>Group</td>
<td>Week(s)</td>
<td>n</td>
</tr>
<tr>
<td>1</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>0 + 2</td>
<td>4</td>
</tr>
<tr>
<td>3</td>
<td>0 + 4</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>0 + 2 + 4</td>
<td>4</td>
</tr>
<tr>
<td>5</td>
<td>0 + 13</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>0 + 4 + 13</td>
<td>6</td>
</tr>
</tbody>
</table>

* RABBITS WERE IMMUNIZED WITH PA-BASED VACCINE (0.5 ml, 50 µg/ml).

** GMT: GEOMETRIC MEAN TITER. **

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**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 immunization 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 LD₅₀. 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 LD₅₀ of either strain of B. anthracis spores.

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

<table>
<thead>
<tr>
<th>Group</th>
<th>Prechallenge immune status (week 10)*</th>
<th>Challenge parameters b</th>
<th>% Survival (no. of survivors/no. challenged)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total anti-PA GMT, 10³ (range)</td>
<td>Neutralizing antibody GMT, 10³ (range)</td>
<td>Strain</td>
</tr>
<tr>
<td>1</td>
<td>395 (282–552)</td>
<td>11 (7–16)</td>
<td>Vollum</td>
</tr>
<tr>
<td>2</td>
<td>215 (136–340)</td>
<td>13 (13)</td>
<td>ATCC 6605</td>
</tr>
</tbody>
</table>

* 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.
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 (~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 10⁴ and 10⁵ 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 LD₅₀s 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 10⁴ and 10⁵) 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-

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

<table>
<thead>
<tr>
<th>Immunization regimen</th>
<th>Group</th>
<th>Week(s)</th>
<th>Time of challenge (week)</th>
<th>Antibody GMT at challenge, 10^3 (range)</th>
<th>% Survival (no. of survivors/no. challenged)</th>
<th>MTTD (days) (±SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>0</td>
<td>19</td>
<td>12.7 (8–32)</td>
<td>0.9 (0.4–0.8)</td>
<td>33.3 (2/6)</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0 + 2</td>
<td>23</td>
<td>33.6 (20–40)</td>
<td>0.6 (0.4–0.8)</td>
<td>25 (1/4)</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0 + 4</td>
<td>19</td>
<td>161 (64–512)</td>
<td>5 (3.2–6.4)</td>
<td>100 (6/6)</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0 + 2 + 4</td>
<td>23</td>
<td>67.3 (40–80)</td>
<td>0.9 (0.8–1.6)</td>
<td>50 (2/4)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>0 + 13</td>
<td>19</td>
<td>23.6 (20–40)</td>
<td>0.87 (0.4–1.6)</td>
<td>50 (2/4)</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>0 + 4 + 13</td>
<td>19</td>
<td>256 (128–512)</td>
<td>3.7 (3.2–6.4)</td>
<td>100 (6/6)</td>
</tr>
<tr>
<td>Control</td>
<td>Naive</td>
<td></td>
<td>19</td>
<td>BDL</td>
<td>0 (0/2)</td>
<td>2.0 ± 0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>23</td>
<td>BDL</td>
<td>0 (0/2)</td>
<td>2.0 ± 0</td>
</tr>
</tbody>
</table>

a The challenge parameters were as follows: at week 19 the animals were challenged with 10 LD₅₀, and at week 23 they were challenged with 160 LD₅₀s 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.
theoretical antibody titers (15) that conferred protection to 50% of the immunized rabbits. The calculated hPT50s obtained were 940 for neutralizing antibodies and 25,000 for total anti-PA antibodies. These hPT50s 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 hPT50 were protected against challenge (37/39 survivors [94.5%]) (Fig. 3, upper right quadrant). Animals with antibody titers that were lower than the calculated hPT50 were not protected (5/17 survivors [29.4%]) (Fig. 3, lower left quadrant). According to the neutralizing antibody titer criteria, titers higher than the hPT50 indicated survival of 37 of 39 animals (~94.5%). All the animals that qualified for the neutralizing hPT50 criteria also qualified for the total anti-PA hPT50 criteria. On the other hand, total anti-PA titers higher than the calculated hPT50 indicated that 39 of 48 animals (~81.2%) survived. Of the nine animals that qualified for the total anti-PA hPT50 criteria but failed to qualify for the neutralizing hPT50 criteria, only two survived (~22.2%) (Fig. 3, lower right quadrant). 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 hPT50, 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 LD50s). 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 (~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-
neutralizing 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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**REFERENCES**