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Correlation between Lethal Toxin-Neutralizing Antibody Titers and Protection from Intranasal Challenge with *Bacillus anthracis* Ames Strain Spores in Mice after Transcutaneous Immunization with Recombinant Anthrax Protective Antigen

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Transcutaneous immunization of mice with recombinant protective antigen (rPA) of *Bacillus anthracis* resulted in significantly higher lethal toxin-neutralizing antibody titers than did intramuscular injection of alum-adsorbed rPA. Immunized mice were partially protected against intranasal challenge with 235,000 (10 50% lethal doses) Ames strain *B. anthracis* spores. A highly significant correlation was observed between toxin-neutralizing antibody titer and survival after challenge. Future experiments with rabbits and nonhuman primates should confirm the significance of protection by this vaccine strategy.

Development of the mouse as a model animal for testing new types of vaccines to *Bacillus anthracis* has presented a problem in that spores from encapsulated strains, such as the Ames strain, exhibit much higher virulence than do unencapsulated strains, such as the STI and Sterne strains. Recent descriptions of the pulmonary infection model of *B. anthracis* in mice and the requirement of capsule formation by *B. anthracis* for dissemination in murine inhalation anthrax further support the need for a capsulated anthrax strain such as Ames in inhalation models (1, 8). The extreme virulence of the Ames strain isolate has made it a logical choice as a challenge strain for examining the efficacy of proposed improved vaccines (2, 8, 13). Delivery of Ames strain spores intranasally instead of by the previously reported subcutaneous or intraperitoneal routes more closely mimics natural transmission pathways. In previous studies we and others demonstrated that transcutaneous immunization (TCI) with recombinant protective antigen (rPA) induced long-lasting neutralizing antibody titers in mice that were superior to those obtained with intramuscular (i.m.) injection of alum-adsorbed rPA and completely protected the immunized mice against challenge with spores of the Sterne and STI strains, which are avirulent strains (6, 9). In the present study we demonstrate a clear improvement of TCI over injection with alum-adsorbed rPA for achieving protective efficacy against intranasally administered Ames strain spores. We also demonstrate a very strong correlation between protection and the quantitative level of toxin-neutralizing antibodies by TCI.

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Recombinant protective antigen was either purchased from List Biological Laboratories, Campbell, CA, or manufactured directly at NIH (12). Aluminum hydroxide gel (alum) (Rehydrogel) was provided as a gift by Reheis Inc., Berkeley Heights, N.J. Heat-labile *Escherichia coli* enterotoxin (HLT) was kindly supplied by John Clements at Tulane University. Female CBA/J mice (6 weeks old; 15/group) were purchased from the Jackson Laboratory (Bar Harbor, ME) and maintained with food and water ad libitum. The backs of the mice were shaved, hydrated with 10% glycerol in 0.9% saline for 5 min, blotted dry, mildly abraded with fine-grade emery paper strips (GE Medical Systems, Milwaukee, WI), rehydrated for 5 min, and blotted dry. The rPA was mixed with 4 μg of HLT in phosphate-buffered saline in a total volume of 50 μl and applied as a liquid to the backs of mice for 1 hour as indicated in each figure legend or Table 1. Positive-control mice were immunized by i.m. injection with 20 μg of rPA mixed with alum. Animals were immunized at week 0 and boosted at weeks 2 and 4. Animals were bled at 2-week intervals, and sera were analyzed for rPA-specific immunoglobulin G (IgG) by enzyme-linked immunosorbent assay (9) or for toxin-neutralizing antibodies as measured by the dilution of antiserum required for 50% reduction in cellular cytotoxicity (ED_{50}) as described before (slightly modified from the work of Herring et al. [3]). At week 9 postimmunization the mice were challenged by the intranasal route with 234,000 spores (10 50% lethal doses) of *B. anthracis* Ames spores administered in a 50-μl volume in the nasal cavity with a pipette. Mice were challenged at the Southern Research Institute in Frederick, MD.

rPA-specific IgG endpoint titers were low after the primary immunization (Fig. 1, week 2). There was a 1,000-fold increase in rPA-specific IgG after the first boost (Fig. 1, week 4). However, there was no further increase in antibody titers after the second boost in any of the groups (compare weeks 4 and 6). All

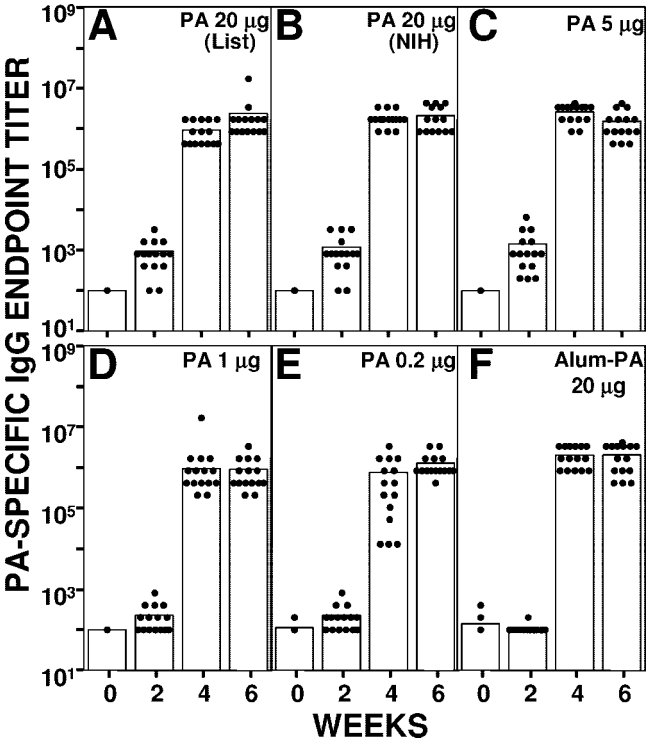

FIG. 1. Time course of PA-specific IgG antibodies. Individual serum samples (15 CBA/J mice/group/time point) were analyzed for PA-specific IgG antibodies by ELISA at 0, 2, 4, and 6 weeks postimmunization. rPA-specific IgG endpoint titers are similar at 4 and 6 weeks for both TCI and i.m. immunized mice. Bars represent arithmetic mean endpoint titers, and symbols represent endpoint titers of individual mice. The endpoint titer is defined as the highest dilution that yielded an optical density reading greater than or equal to twice the background value. The titers were calculated after subtracting the mean absorbance of triplicate wells lacking antigen from wells containing antigen.

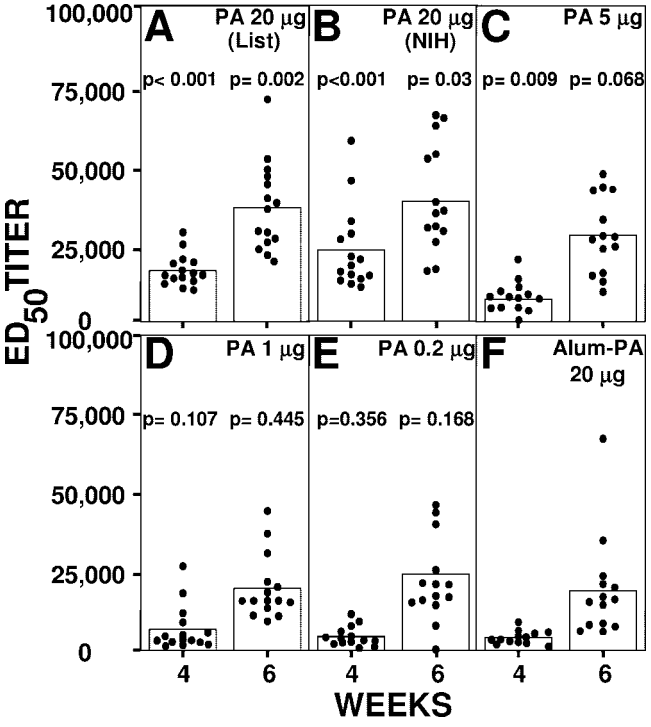

FIG. 2. Lethal toxin-neutralizing antibodies (ED_{50}). Sera from mice described in Fig. 1 were also measured for lethal toxin neutralization antibodies (3). Bars represent arithmetic mean ED_{50} titers, and symbols are four-parametric ED_{50} titers of individual mice. *P* values represent a two-tailed *t* test between week 4 rPA-HLT TCI groups and week 4 rPA-alum i.m. immunized mice with the values included on the graph or week 6 TCI versus week 6 i.m. immunized mice.

of the doses resulted in essentially identical high rPA-specific IgG endpoint titers that reached levels between 10^6 and 10^7 at weeks 4 and 6. As found previously (9), high levels of lethal toxin-neutralizing antibody levels were seen in the TCI mice compared to the i.m. injection of rPA adsorbed to alum. In this study, the four-parametric mean lethal toxin-neutralizing antibody levels were significantly higher at weeks 4 and 6 after TCI with 20 μg of rPA (Fig. 2A and 2B) than with i.m. injection of 20 μg of rPA adsorbed to alum (Fig. 2F). Additionally, we found significance at 4 weeks after boosting with 5 μg of rPA by TCI (Fig. 2C). Despite extraordinarily high neutralizing antibody titers observed in some of the mice, with ED_{50} levels reaching as high as 50,000 to 75,000, the variability of neutralizing antibody levels between individual mice within each of the TCI groups was quite large. The large differences between individual mice suggested that there were differences in the absorption and delivery efficiencies through the skin of different animals during the TCI procedure. An alternative explanation for the variability is suggested by the recent observation that certain murine monoclonal antibodies to PA actually cause enhancement of lethal toxin-mediated killing of murine macrophages (11). Different individual animals might produce different ratios of neutralizing and enhancing antibodies in response to the potent immunization stimulus provided by TCI with rPA, and the overall protective effect would then reflect the degree to which neutralizing antibodies were present in greater abundance than enhancing antibodies in each animal.

Mice (n = 10/group) were challenged intranasally at week 8 with spores (Table 1). Only one of the animals in the nonimmunized group and one of the animals immunized i.m. with 20 μg of alum-adsorbed rPA survived the challenge after 14 days. In contrast, in each of the five groups of TCI-immunized animals between three and six of the animals survived the chal-

TABLE 1. Survival of mice after intranasal challenge with *B. anthracis* spores

Group no.	Immunization method	rPA (μg)	Adjuvant (μg)	% Survival on day 14 (no. of survivors/ no. challenged) (P^b)	MTD[c]
1	TCI	20[a]	HLT (4)	50 (5/10) (0.007)	3.5
2	TCI	20	HLT (4)	60 (6/10) (0.010)	3.0
3	TCI	5	HLT (4)	20 (2/10) (0.281)	2.9
4	TCI	1	HLT (4)	30 (3/10) (0.055)	3.3
5	TCI	0.2	HLT (4)	60 (6/10) (0.010)	3.0
6	i.m.	20[a]	Alum	10 (1/10)	2.8
Nonimmunized	None	None	None	10 (1/10)	4.4

[a] rPA was purchased from List Biological Laboratories.
[b] Significance value represents a comparison of group 6 i.m. immunized mice versus TCI mice.
[c] MTD, mean time to death of animals that died, in days.

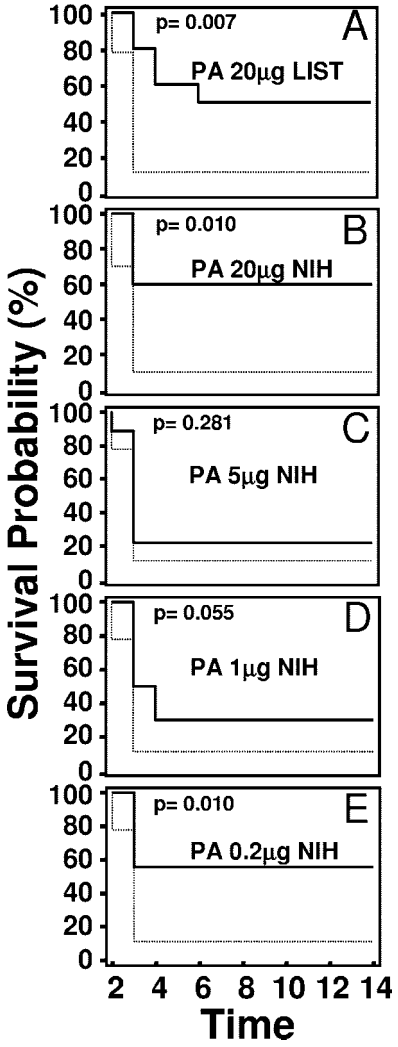

FIG. 3. Kaplan-Meier survival curves: TCI versus i.m. Four weeks after the final boost, mice were challenged intranasally with 234,000 *Bacillus anthracis* Ames strain spores. Survival of mice immunized i.m. with 20 μg alum-adsorbed rPA is represented as dotted lines, and survival of mice immunized with (0.2 to 20 μg) rPA by TCI is represented as solid lines. Time is shown in days.

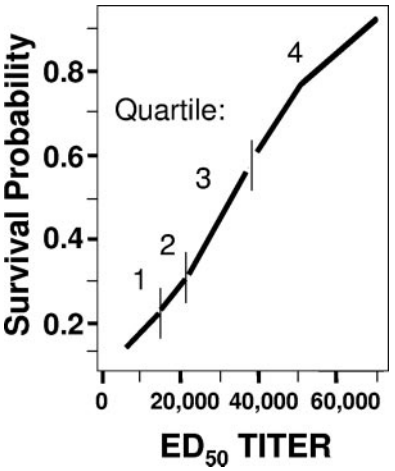

FIG. 4. Probability analysis of survival correlated with ED_{50} titers. Lethal toxin neutralization titers (ED_{50}) from all six groups in Fig. 3 were divided into quartiles, each containing 14 individual mice, and combined with survival rates from Table 1 to derive the mathematical probability of survival compared to ED_{50} titers using a binary logistic regression model ($P = 0.003$).

lenge. In the two groups that received 20 μg of either List rPA or NIH rPA, 5 of 10 and 6 of 10 animals survived, respectively, a survival rate that was significantly higher than that in the group immunized i.m. with 20 μg of rPA adsorbed to alum ($P = 0.02$ and 0.03, respectively [Table 1]) and also significantly higher than that in the nonimmunized control group. The survival after Ames spore challenge was similar to that observed previously after Ames strain spore challenge of animals immunized by injection of PA combined with potent adjuvants (4, 5).

Kaplan-Meier survival curves over the 14-day observation interval confirmed enhanced survival of animals immunized by TCI with 20 μg and 0.2 μg of rPA compared to the animals immunized i.m. with 20 μg of alum-adsorbed rPA (Fig. 3A, 3B, and 3E). The data therefore demonstrate that immunization by TCI significantly enhanced the survival rate compared to the alum-adsorbed i.m. injection procedure. To test the relationship between neutralizing antibodies and survival, we grouped the data from all of the toxin-neutralizing antibodies together for statistical analysis. Binary logistic regression is used when the dependent, survival in our case, is a dichotomy and the independents, neutralization titer in this case, are of any type. Logistic regression estimates the probability of a certain event occurring. The toxin-neutralizing antibody data were grouped in ascending order into equal quartiles of 14 animals each, based on neutralizing antibody titers. ED_{50} titers were then compared with the 14-day survival data in each quartile. The values were used in a binary logistic regression analysis to determine the estimated survival probability. The mathematical model derived from this analysis is shown in Fig. 4. A highly significant probability relationship existed between the ED_{50} titers and survival ($P = 0.003$). Based on the probability relationship, it is predicted that an ED_{50} of >75,000 is required for >90% probability of survival after challenge with a dose of 10 50% lethal doses of Ames strain spores. In view of the striking correlation between neutralizing antibodies and survival after challenge, we believe that the neutralizing antibodies were the predominant immunological mechanism related to survival in this study. We conclude therefore that the neutralizing antibody titer is a highly significant predictor of survival from challenge with Ames strain spores in mice immunized transcutaneously with PA-based vaccines. This observation is compatible with similar findings in a rabbit model (7). We further conclude that TCI induces significant elevation of neutralizing antibodies to rPA and enhanced survival to challenge with virulent Ames strain spores, thus highlighting the potential benefits of skin immunization (6, 9, 10).

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