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Evaluation of *Pseudomonas aeruginosa* Toxin A in Experimental Rat Burn Wound Sepsis

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The search for methods to achieve control of *Pseudomonas aeruginosa* infection continues with the introduction of aluminum-absorbed toxoid developed from *P. aeruginosa* exotoxin. This toxoid induces significant titers of neutralizing and precipitating antibodies for toxin A when given with appropriate adjuvants. These experiments show that immunization with aluminum phosphate-absorbed toxoid failed to protect burned rats infected with *P. aeruginosa*. These and previous experiments show that active immunization with live *P. aeruginosa* provides good strain-specific protection in the same model. No cross-protection was demonstrated between strains of *P. aeruginosa* in these experiments.

Pseudomonas aeruginosa infection is a recognized common cause of death in burned patients. Although its incidence has been diminished by topical chemotherapy, this infection is not completely prevented by such treatment and it has maintained its incidence during recent years.

Many virulent strains of *P. aeruginosa* produce a potent protein exotoxin (approximately 90% of all *P. aeruginosa* strains produce exotoxin A [6] and these exotoxins appear identical; no serologically definite subtypes have been detected), which is lethal in a number of animal models as well as cultured mammalian cells (4). The exotoxin has been purified to homogeneity (3) and apparently acts within cells by inactivating elongation factor 2 in the same fashion as diptheria toxin (2). Iglewski et al. (2) have suggested that exotoxin plays an important role in the pathogenesis of tissue destruction and death related to *Pseudomonas* infection in a mouse burn model. The evidence that supports this hypothesis includes demonstrations that: (i) levels of elongation factor 2 were decreased in burned infected mice before death (2, 7); (ii) passive immunization with antiserum specific for exotoxin increased the mean time to death of burned, infected mice and also prevented the decrease in elongation factor 2 levels (5, 8); and (iii) a non-exotoxin-producing strain (WR-5) of *P. aeruginosa* was less virulent for burned mice than strains producing exotoxin (5). It is suspected that exotoxin is not the sole cause of death in burned, infected mice, but that it damages host defense systems and permits the occurrence of overwhelming bacteremia, which is the immediate cause of death.

A toxoid was recently prepared by controlled glutaraldehyde treatment of exotoxin (S. H. Leppla, O. C. Martin, and O. R. Pavlovskis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B96, p. 29). This toxoid induces significant titers of neutralizing and precipitating antibodies for toxin A in several species of animals when given with appropriate adjuvants. The purpose of this study was to test the purified toxoid of *P. aeruginosa* in a well characterized model of burn wound infection in the rat (10). For comparison, groups of animals actively immunized with live *P. aeruginosa* were also studied.

MATERIALS AND METHODS

Animals. Adult Sprague-Dawley (Holtzman strain) rats were used throughout this investigation. The rats were conditioned on Wayne's Lab Blox and water ad libitum for at least 2 weeks before any experiment. The weight of individual rats was within 10 g of the average weight for the group. At the start of the immunization period the rats weighed 100 g. Weights at the time of burn and challenge ranged from 350 to 370 g.

Experimental and control animals were anesthetized with sodium pentobarbital (1 mg/25 g) before burning. Burns were inflicted on the dorsum after clipping the hair with a no. 40 blade in an Oster animal clipper. Each animal was placed in a fixed-area shield (10), and the dorsum was immersed in boiling water for 10 s. This procedure produces a uniform full-thickness burn covering 18 to 22% of the total body surface.

After burning, water and food were provided ad libitum for all test animals. An ambient temperature range of 21 to 27°C was maintained. Survivors were observed for 30 days after inoculation.

Organisms. *P. aeruginosa* strains ISR 12-4-4 (59)

and ISR 8-28-3 (63) were the challenge microorganisms employed throughout the study. ISR 12-4-4 (59) was tested and found to be an exotoxin A producer. Partially purified culture supernatants were highly toxic for mouse L929 fibroblasts, and this toxicity was completely blocked by a higher titered monospecific pony antitoxin. ISR strain 8-28-3 (63) was not tested for exotoxin A production. These microorganisms were originally isolated from burn patients who expired with septicemia and are known to be virulent in this model. A sufficient quantity was grown on Trypticase soy agar (BBL Microbiology Systems) to be used for the entire series of experiments. After 18 h of incubation, the microorganisms were harvested and added to sterile evaporated milk. Two-milliliter volumes of this mixture were placed in sterile 5-ml bottles. The bottles were sealed with sterile rubber gaskets and aluminum caps. This sealed-bottle procedure prevented contamination and simplified the removal of the bacterial suspension. The sealed bottles were quick-frozen and placed in storage at −80°C for preservation.

Cultures for use in the experiments were prepared from the frozen, preserved samples; 0.1 ml of the microorganism-milk mixture was placed in 9.9 ml of Trypticase soy broth and incubated for 18 h before use.

Challenge technique. The usual seeding culture contained 10^8 organisms per ml, and 1 ml of this 18-h culture was used. The standard area topically seeded was 105 cm^2. The dose was evenly distributed over the entire burned area.

Immunization. Groups of animals were immunized with either live *P. aeruginosa* or with glutaraldehyde toxoid. For immunization with live organisms, the animals received a single intraperitoneal injection of *P. aeruginosa* each week for 4 weeks. Immunization (11) was initiated with 0.02 ml of an 18-h culture of *P. aeruginosa*; the second dose was 0.05 ml, the third was 0.1 ml, and the fourth was 0.2 ml. For the induction of antitoxin immunity, 0.5 ml of toxoid was injected intramuscularly each week for 4 weeks. Toxoid was prepared by glutaraldehyde treatment of exotoxin A that had been purified as previously described (3). The toxoid suspension contained 100 μg of toxoid per ml and 500 μg of protamine sulfate per ml adsorbed on an aluminum phosphate precipitate (3 mg of aluminum per ml). Control groups received an aluminum phosphate precipitate in which the toxoid was replaced by an equal weight of bovine serum albumin.

RESULTS

Active immunization with *P. aeruginosa* ISR strains 12-4-4 (59) and 8-28-3 (63). Table 1 summarizes the results of immunization and challenges. Twenty-four animals were immunized with ISR strain 12-4-4 (59) and challenged with the standard burn procedure. In the first group of 17 animals immunized with ISR strain 12-4-4 (59) and challenged with ISR strain 12-4-4 (59), no deaths occurred during the 30-day period of observation. The second group of seven animals was immunized with ISR strain 12-4-4 (59) and challenged with ISR strain 8-28-3 (63). All died between days 9 and 17. Twenty-two animals were immunized with ISR strain 8-28-3 (63) and 12 were challenged by the standard procedure using ISR strain 8-28-3 (63). In this group of 12 animals, no deaths occurred during the 30-day period of observation. The second group of 10 animals immunized with ISR strain 8-28-3 (63) was burned and challenged with ISR strain 12-4-4 (59), and all died between days 9 and 17.

TABLE 1. *Protection of burned rats by immunization*

Immunization	Challenge strain	No. of animals	No. died	Time to death (days)[a]
12-4-4 (59)	12-4-4 (59)	17	0	
12-4-4 (59)	8-28-3 (63)	7	7	9-17 (12.1)
8-28-3 (63)	8-28-3 (63)	12	0	
8-28-3 (63)	12-4-4 (59)	10	10	9-17 (12.8)
Toxoid	12-4-4 (59)	20	20	6-18 (10.6)
Toxoid	8-28-3 (63)	19	19	5-12 (7.6)
BSA[b]	12-4-4 (59)	22	22	7-15 (10.2)
BSA	8-28-3 (63)	19	19	5-17 (8.3)
None	12-4-4 (59)	21	21	6-17 (10.3)
None	8-28-3 (63)	20	20	5-11 (7.5)

[a] Numbers in parentheses indicates the mean.
[b] BSA, Bovine serum albumin.

Antitoxic immunization with toxoid. Thirty-nine animals were immunized with toxoid and challenged with the standard burn procedure and ISR strain 12-4-4 (59) or ISR strain 8-28-3 (63). In the first group of 20 animals burned and challenged with ISR 12-4-4 (59), all died between days 6 and 18. The second toxoid-immunized group of 19 animals was challenged with the standard procedure and ISR strain 8-28-3 (63). All the animals in this group died between days 5 and 12. Forty-one immunization control animals were injected with bovine serum albumin adsorbed on aluminum phosphate and challenged with the standard procedure and ISR strains 12-4-4 (59) or 8-28-3 (63). In the first group of 22 animals challenged with ISR strain 12-4-4 (59), all died between days 7 and 15. A second group of 19 animals was challenged with the standard burn procedure and strain ISR 8-28-3 (63), and all died between days 5 and 17.

Burn-challenged controls. Twenty-one control animals were challenged with the standard burn procedure and ISR strain 12-4-4 (59). All died between day 6 and 17. A second group of 20 animals was challenged with the standard burn procedure and ISR strain 8-28-3 (63). All died between days 5 and 11.

Pathology. Selected rats from each test group were examined for gross and microscopic lesions. Pathological changes were similar in all groups that died.

The dorsal burn wounds were edematous and hemorrhagic at the junction of normal and burned skin. Suppuration and necrosis were seen at the eschar base after 1 week. Invasive bacterial infection was found in all skin sections examined histologically. Focal hemorrhagic and necrotic lesions were seen in various organs including the lung, kidney, spleen, and liver, and occasionally the intestine. Microscopically, all the hemorrhagic and necrotic lesions were found to contain gram-negative rod-shaped bacteria. A pattern of necrotic bacterial vasculitis typical of *P. aeruginosa* was seen in many of the skin and hematogenous lesions. *P. aeruginosa* was cultured from lung, liver, and spleen in these animals.

Serology. Sera were collected before challenge from animals in each of the immunized groups of Table 1. Assays were made for neutralizing antibodies in a cytotoxicity test employing mouse L929 fibroblasts (3). Rats immunized with toxoid ($n = 4$) had an average titer (the number of serial threefold dilutions of serum needed to abolish neutralization of cytotoxicity) of 5. Animals not immunized or immunized with bovine serum albumin or liver organisms had no antitoxic antibodies. A high-titered pony antitoxin previously described (3) had a titer of 9.

DISCUSSION

These experiments demonstrate that immunization with aluminum phosphate-absorbed toxoid prepared from *Pseudomonas* exotoxin failed to protect burned rats infected with *P. aeruginosa*. Previous experiments (11) have shown that active immunization utilizing live *P. aeruginosa* provides good strain-specific protection in the same infection model. Those experiments showed no cross-protection between strains of *P. aeruginosa*. This study confirms that active immunization utilizing *P. aeruginosa* provides strain-specific protection in this model and no cross-protection. Deaths were consistently caused by invasive infection and hematogenous spread of the infection to various organs including the lung, kidney, spleen, and heart. These inflammatory lesions do not occur in the burned mouse model (9) suggesting that the latter may be a toxic rather than an infectious model.

Immunization with toxoid as described here induced moderate but not high levels of antitoxin. Higher titers of antitoxic immunity have been obtained in rats given toxoid in Freund complete adjuvant (unpublished studies), but the use of this adjuvant was precluded here because it is known to induce a nonspecific bacterial resistance (1). The apparent inability of this moderate antitoxic immunity to protect rats which are readily protected by immunization with live organisms suggests that exotoxin A may not be a primary virulence factor in *Pseudomonas* infection. Passive immunization has been shown to have a protective effect in burned, infected mice (5, 8), but these animals are about eightfold more sensitive than rats to exotoxin A on a body weight basis.

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