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Protection Against Experimental *Pseudomonas aeruginosa* Infection in Mice by Active Immunization with Exotoxin A Toxoids

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The immunoprophylactic effect of chemically inactivated *Pseudomonas aeruginosa* extoxin A in experimental pseudomonas infections was studied. Exotoxin A toxoids were prepared by Formalin (f-TXD) or glutaraldehyde (g-TXD) treatment. Immunization of mice with three or four doses (10 μg each) of f-TXD and the synthetic adjuvant *N*-acetylmuramyl-L-alanyl-D-isoglutamine (50 μg) induced high levels of antiexotoxin A antibodies as measured by passive hemagglutination assay and enzyme-linked immunosorbent assay. Immunization with toxoid alone did not elicit antitoxin. A significant increase in survival time and survival rate ($P < 0.01$) was seen in immunized (f-TXD) and in burned and infected mice (50 to 85%) as compared with control mice immunized with formalinized bovine serum albumin (6 to 20%). Virtually 100% survival was obtained when preinfection immunization was combined with single-dose gentamicin treatment within 24 h of infection. Immunization with g-TXD increased survival time ($P < 0.01$) but did not consistently increase survival rate, and the results were not as satisfactory as those with formalinized exotoxin. The data presented indicate that active immunization with formalinized exotoxin A toxoid and adjuvant induced protective immunity to various degrees against infections in mice and could be potentially useful in prophylaxis of *P. aeruginosa* infections.

Previous studies have shown that *Pseudomonas aeruginosa* exotoxin A (3, 4, 18, 22, 26) is an important virulence factor in experimental pseudomonas infections (27, 29, 30, 38, 42) and that passive immunization against exotoxin A confers a significant degree of protection (25, 29, 30, 38, 40). In human infection, high serum antitoxin levels have been correlated with a greater chance of survival (8, 37). These results suggest that active or passive immunization against exotoxin A may be of value in the prophylaxis and treatment of *P. aeruginosa* infections. However, protection against experimental infection by exotoxin A toxoid of no or very limited toxicity has not yet been demonstrated.

Our studies were prompted by the findings of Abe et al. (1) that a preparation of Formalin-treated exotoxin A protected mice against purified exotoxin A. We used a somewhat similar preparation to evaluate its immunoprophylactic potential in a burned-mouse *P. aeruginosa* infection model (16, 32, 41) that mimics human burn sepsis. The data presented indicate that the toxoid, in the amounts used, had no demonstrable toxicity for mice and that it may be useful in prophylaxis of pseudomonas infections.

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MATERIALS AND METHODS

Organisms. *P. aeruginosa* strains were obtained from the following sources. Strain PA103 (23) came from P. V. Liu, University of Louisville, School of Medicine, Louisville, Ky; PA220 came from M. Pollack (30), Uniformed Services University of the Health Services, Bethesda, Md.; and PA103-29, a non-toxin-producing mutant of PA103, came from B. H. Iglewski (28), University of Oregon Health Sciences Center, Portland. The median lethal dose (LD_{50}) for burned mice of strain PA103-29 was about 50-fold higher than that of PA103. The properties of the strains were summarized in previous publications (28, 30).

Animals. Swiss white mice of either sex, NIH/Nmri CV inbred strain weighing between 16 and 20 g (at the start of the experiment), and Hartley outbred male guinea pigs, weighing between 500 and 750 g, were used.

Toxoid preparation. Two different toxoid preparations were used: Formalin- and glutaraldehyde-inactivated exotoxin A (f-TXD and g-TXD, respectively).

The f-TXD preparation was made by a modification of the procedure of Abe et al. (1). Purified exotoxin A (2.5 ml, 200 μg/ml) was dialyzed against 125 ml of 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride

buffer, pH 7.9 to 8.0, containing 1% Formalin and 0.2 M L-lysine (Calbiochem, San Diego, Calif.) at 37°C for 72 h. The toxoid preparation was then dialyzed against a 0.2 M L-lysine–saline solution, pH 6.3, at 6°C for 48 h. Residual Formalin was removed by chromatography on prepacked, disposable 5-cm (volume, 9.1 ml) Sephadex G-25 (Pharmacia Fine Chemicals, Inc., Piscataway, N.J.) columns. Protein-containing fractions were pooled, adjusted to the desired concentration, divided, and stored at −20°C. Before use, each preparation of f-TXD was tested for residual toxicity by injecting 10 μg intravenously (i.v.) into mice.

The g-TXD preparation was made by controlled glutaraldehyde treatment of exotoxin A (22) and absorption on aluminum-phosphate (44; S. H. Leppla, O. C. Martin, and O. R. Pavlovskis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B96, p. 29). The final toxoid preparation, containing 100 μg of g-TXD plus 500 μg of protamine sulfate per ml, was absorbed on an aluminum-phosphate precipitate (3 mg/ml).

Control protein preparations used to immunize controls were prepared from bovine serum albumin (BSA) (Miles Laboratories, Inc., Kankakee, Ill.) by treating comparable concentrations of BSA in an identical manner by the Formalin (f-BSA) or the glutaraldehyde (g-BSA) procedure.

Immunization. The initial i.v. injection (0.1 ml given) on day zero with or without adjuvant, as indicated, was followed by comparable injections into the muscle of the right hind leg. Control mice received either f-BSA or g-BSA instead of toxoid (with or without adjuvant according to the experiment) or *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP) alone. At various times, the mice were anesthetized with methoxyflurane (Pittman-Moore, Inc., Washington Crossing, N.J.), bled by the retroorbital procedure (<0.5 ml), and immediately injected subcutaneously with 0.5 ml of Ringer-lactate (Travenol Laboratories, Inc., Deerfield, Ill.) to replace the lost fluids. Serum was separated by centrifugation and stored at −20°C.

Mouse infection model. The Holder and Jogan (16) and Stieritz and Holder (41) burned-mouse infection model modified as previously described (32) was used. Mice were burned for 11 s and immediately injected with a dose of *P. aeruginosa* resulting in 80 to 100% mortality of normal, untreated mice. Unless otherwise indicated, all experiments were done at least in triplicate.

Assays. The antitoxin titers of mouse sera were measured by an indirect enzyme-linked immunosorbent assay (ELISA) (D. C. Edman, and O. R. Pavlovskis, Abstr. Annu. Meet. Am. Soc. Microbiol. 1980, B94, p. 32). The procedure can be briefly described as follows. Microtitration plates (U-bottom; Microbiological Associates, Bethesda, Md.) were coated with 100 μl of purified exotoxin A in 0.1 M sodium carbonate buffer, pH 9.6, with 0.02% sodium azide. After incubation (6 h, 37°C) in a humidified chamber, the wells were aspirated with a multiple automated sample harvester (MASH II; Microbiological Associates). All subsequent incubations were done at 37°C. The wells were washed five times with working buffer (0.036 M sodium borate-saline, pH 7.85, containing 0.01% sodium azide, 0.5% BSA fraction V [Calbiochem-Behring Corp., La Jolla, Calif.], and 0.5% Tween 20 [Sigma Chemical Co., St. Louis, Mo.]) in the MASH II, as were all subsequent washings. Known mouse antitoxin serum dilutions or experimental immune serum dilutions in working buffer, 100 μl per well, were then added, and the plates were incubated (2 h). Unbound antitoxin was removed by aspiration of the wells and washed five times with working buffer. Goat anti-mouse immunoglobulin G or M (100 μl of a 1:50,000 dilution in working buffer or 1:1,000 dilution, respectively) was then added to appropriate wells, incubated (2 h), and washed. Rabbit anti-goat gamma globulin fraction-alkaline phosphate conjugate (1:1,000) was added to wells, incubated (2 h), and washed with substrate buffer (0.05 M sodium carbonate–0.001 M $MgCl_2 \cdot 6H_2O$, pH 9.8). Finally, 100 μl of *p*-nitrophenylphosphate (1 mg/ml; Sigma) in substrate buffer was added to each well. After 1 h of incubation, the reaction was terminated with the addition of 100 μl of 2 N NaOH per well, the contents of each well were diluted 1:5, using Titertek transfer frames (Flow Laboratories, Inc., Alexandria, Va.), and absorbance was determined at 400 nm. Antitoxin activity was expressed as the optical density of a 1:1,000 dilution of serum.

Immunochemical reagents were obtained from Cappel Laboratories (Cochranville, Pa.), and immunoglobulin-alkaline phosphatase conjugates were obtained through the courtesy of G. Dasch and S. Halle.

A passive hemagglutination assay (PHA) (35) using sheep erythrocytes from a single donor, and a cytotoxicity assay (21, 32) were done as previously described. Results are expressed as the reciprocal of the highest serum dilution ($\log_{10}$) agglutinating the treated erythrocytes.

The adenosine diphosphate-ribosyltransferase (ADPR) assay previously described (9) was used to determine the enzymatic activity of exotoxin A and the residual activity of the toxoids.

Serum enzyme assays, aspartate aminotransferase (SGOT) and alanine aminotransferase (SGPT), were determined on a Gilford 3500 computer-directed analyzer (Gilford Instrument Laboratories, Inc., Oberlin, Ohio).

Guinea pig skin reactions were determined as previously described (35). A checkerboard pattern of 2-cm squares was drawn on each guinea pig back, and 0.1 ml of exotoxin A, pyrogen-free saline, toxoid, or BSA control toxoid was injected intradermally. The skin reactions were scored at 24, 48, and 72 h.

Pathological examinations. Mice were killed by cervical dislocation, and organs (liver, kidneys, spleen, lungs, and heart) were immediately excised and placed in Telley fixative (70% ethanol–Formalin–glacial acetic acid, 20:1:1). Sections were stained with hematoxylin and eosin. The slides were examined without reference to the experimental protocol.

Statistical analysis. Survival rates of test animals within an experiment were compared by using the Wilcoxon rank sum test as described by Bradley (2). The *P* values were computed by using normal approximation with continuity correction. The *P* values are consistent with those drawn from available Wilcoxon rank sum test tables. The LD_{50} was calculated by the Spearman-Karber method described by Finney (12). The LD_{50} values and survival between groups were compared by the two-tailed Student *t*-test.

Reagents. The synthetic glycopeptide MDP was obtained from Calbiochem-Behring Corp. Gentamicin was obtained from Schering Corp., Kenilworth, N.J.

RESULTS

Characteristics of f-TXD. Since the exotoxin A inactivated by the procedure described by Abe et al. (1) on several occasions reverted to some toxicity after storage, a few minor modifications were introduced into the procedure. The length of Formalin treatment was increased from 48 to 72 h at 37°C, and then the treated culture was dialyzed for an additional 48 h at 23°C. Also, the toxoid was chromatographed and stored in the presence of 0.2 M L-lysine–saline. No reversion of the toxoid was observed after 3 months of storage at −20°C.

The Formalin treatment of exotoxin A resulted in a decrease of some of its biological properties (Table 1) without any appreciable loss in its PHIA (9, 42), ELISA, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Fig. 1), and Ouchterlony immunodiffusion (Fig. 2) activity. Whereas the LD_{50} of exotoxin A was 80 ng, no mortality or toxic effects were seen with the largest dose of toxoid (40 μg) tested (Table 1). A 15-fold reduction in ADPR transferase activity (18) was observed between the intact exotoxin A molecule and the toxoid. The toxoid also showed a reduction at least 2,000-fold in the necrotizing activity of guinea pig skin (Table 1). The minimal detectable reaction (3 mm) was seen with 20 μg of toxoid as compared with 3 to 5 mm with 0.005 μg of exotoxin A. In our cytoxicity assay, the LD_{50} for exotoxin A was about 0.5 ng/ml for L cells, but no cytoxic effects were observed with the highest dose (100 ng/ml) of toxoid tested (Table 1).

No pathological changes were seen in organs (liver, kidneys, spleen, lungs, and heart) of mice given 10-μg doses (i.v.) of toxoid at four weekly intervals or a single dose of 40 μg of toxoid. In contrast, i.v. injection of mice with 40 ng of exotoxin A resulted in complete necrosis of the liver in about 48 h (data not shown; 31). It has been shown previously (31) that injection of exotoxin A results in a rapid increase in serum enzyme (SGOT and SGPT) levels. No significant increase in SGOT and SGPT activity ($P > 0.1$) was observed after the i.v. injection of 20 μg of toxoid into mice as compared with normal, untreated mice (Table 2). On the other hand, the injection of 40 ng exotoxin A resulted in high serum enzyme activity.

On the basis of the above standard tests used

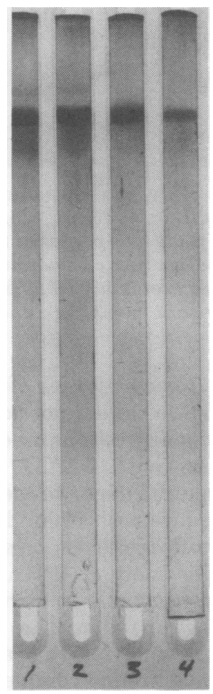

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of exotoxin A and f-TXD. (1) Starting material exotoxin A; (2) after 24 h of Formalin-lysine treatment; (3) before PD-10 column chromatography; (4) final product, f-TXD. Electrophoresis was from top (cathode) to bottom (anode).*

TABLE 1. *Characteristics of exotoxin A and f-TXD*

Sample	ADPR transferase activity (cpm)	L-cell cytotoxicity (ng)	Guinea pig skin necrotizing activity[a] (μg)	Mouse LD_{50} (μg)	Antigenicity	
					PHIA[b]	ELISA (OD_{400})[c]
Exotoxin A before toxoiding	2,500	0.5	0.005	0.08	1,000	1.16
f-TXD	150	>100[d]	>20.0[d]	>40.0[d]	512	1.36

[a] Highest dose tested producing a reaction of 3 to 5 mm in 48 h.

[b] Hemagglutination inhibition assay (PHIA) (10, 40). Twenty-five microliters of 1:64,000 sheep antitoxin serum was incubated at 37°C for 1 h with serial twofold dilutions of exotoxin A or f-TXD before addition of exotoxin-coated sheep erythrocytes. The highest dilution of antigen that inhibited hemagglutination was designated the endpoint.

[c] Optical density at 400 nm.

[d] Highest dose tested.

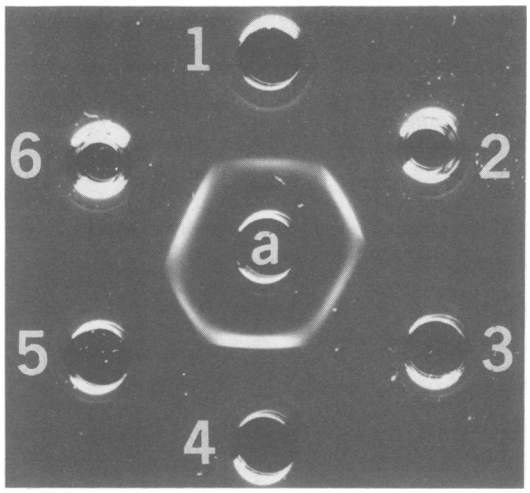

FIG. 2. *Ouchterlony gel diffusion pattern: (a) control well, sheep antitoxin (40); (1, 3, 5) exotoxin A; (2, 4, 6) toxoid. The plate was prepared with 0.75% agarose and 0.2% sodium azide in normal saline. Wells contained 20 μl of exotoxin (200 μg/ml); well 2 contained 20 μl of toxoid (80 μg/ml), and wells 4 and 6 contained 40 μl.*

to evaluate the toxic activities of exotoxin A, it appears that the toxoid did not adversely affect the host at the concentrations used in this study.

Immunization with f-TXD. In our initial attempts to immunize mice, we used a previously described procedure with Freund complete adjuvant (1). Significant levels of antitoxin were produced by this method (PHA, $\log_{10}$ 2.57 ± 0.33), but we were unable to elicit a consistent antitoxin production when the adjuvant was omitted. Since it appeared that an adjuvant is required and since Freund complete adjuvant is relatively toxic and thus may contribute to the pathogenicity of the infection, we decided to use a synthetic adjuvant, MDP (5, 6). Although MDP has been shown to have a pyrogenic effect (11), repeated injections into mice (data not shown) did not appear to cause any visual or pathological changes detectable by light microscopy.

Mice were immunized with 1.0, 2.5, 5.0, and 10 μg of f-TXD plus 50 μg of MDP per dose (0.1 ml) in saline on approximately days 0, 14, 28, and 42. Control groups consisted of untreated mice and mice immunized, according to the same protocol, with equivalent amounts of f-BSA and MDP and with MDP alone. Mice immunized with 1.0 or 2.5 μg of f-TXD did not show any antitoxin response, whereas mice injected with 5.0 μg had a variable response (data not shown). The minimal dose in our system appeared to be 10 μg of toxoid. Immunization with this dose gave a significant rise in antitoxin titer as measured by PHA and ELISA (Fig. 3). After the third injection, mice were protected against at least 100 LD_{50} (highest dose tested) of purified exotoxin A given i.v. In all further experiments with f-TXD, unless otherwise noted, 10 μg of formalinized protein plus 50 μg of MDP was administered according to the above schedule.

After immunization was completed, after the third or fourth injection, mice were traumatized and infected with either one of two toxigenic *P. aeruginosa* strains (PA103 or PA220) or with a nontoxigenic mutant of PA103, PA103-29 (Table 3). The survival of f-TXD-immunized mice challenged with the toxigenic strains was 50 to 85%, which was significantly greater than survival of the control mice immunized with f-BSA ($P < 0.01$). No significant differences in survival were seen between the f-BSA group and the other two control groups, i.e., mice treated with MDP alone or untreated (data not shown). When f-TXD-immunized mice were infected with the nontoxigenic strain PA103-29, the survival rate between f-TXD and f-BSA groups was the same ($P > 0.05$; Table 3), indicating that the protection elicited by f-TXD in previous experiments is exotoxin A specific. The deaths of the mice infected with the nontoxigenic strain can probably be attributed to other pseudomonas virulence factors such as endotoxin or slime (23, 39, 45). Regardless of the role of exotoxin A, it seems reasonably clear from work in our as well as other laboratories that it is only one of a number of toxic substances produced by the organism (29). The protective effect of immunization with f-TXD was indicated by another series of experiments. Mice immunized with f-TXD had significantly higher LD_{50} ($P < 0.01$) than the control group (Table 4). Thus, in our system, immunization with f-TXD and MDP resulted in an exotoxin A-specific protection against *P. aeruginosa* infections.

Immunization with g-TXD. We also tested the efficacy of a glutaraldehyde toxoid prepared

TABLE 2. *Enzyme levels in sera of mice given an injection of exotoxin A or f-TXD*[a]

Enzyme	Treatment	Levels of enzyme[b] (mIU/ml)		
		0	16 h[c]	24 h
SGOT	None	37 ± 3[d]		
	Exotoxin A		168 ± 30	218 ± 136
	f-TXD		46 ± 8	50 ± 8
SGPT	None	20 ± 3[d]		
	Exotoxin A		106 ± 35	128 ± 33
	f-TXD		23 ± 3	27 ± 6

[a] Exotoxin A, 40 ng; f-TXD 20 μg; i.v.
[b] Results expressed as mean ± standard deviation.
[c] Hours postinjection.
[d] Probability between untreated mice and f-TXD-treated mice, >0.1; Student *t*-test.

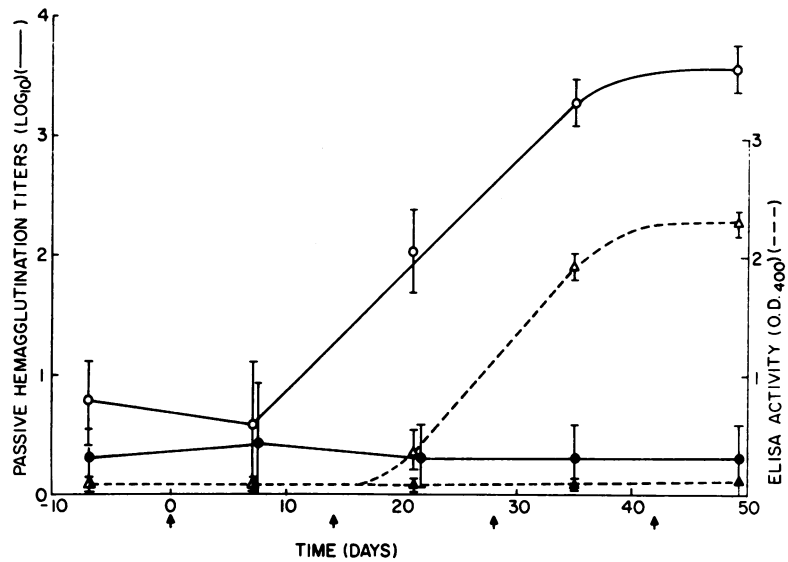

FIG. 3. *Serum PHA antitoxin titers* (——) *and ELISA activity* (- - - -) *of mice immunized at 14-day intervals Symbols:* (○, △) *mice immunized with f-TXD;* (●, ▲) *control mice immunized with f-BSA;* (↑) *time of immunization.* OD_{400}, *optical density at 400 nm.*

TABLE 3. *Survival of mice immunized with f-TXD and infected with P. aeruginosa*

Infecting strain	Expt no.	Immunization schedule (day)	Day of infection	Mortality[a]		P
				f-BSA (control)	f-TXD	
PA103	1	0, 14, 28, 42	56	12/15	3/14	<0.01
	2	0, 14, 27, 42	56	14/15	1/7	<0.01
PA103-29	3	0, 14, 28	43	15/15	15/15	>0.05
PA220	4	0, 14, 40	52	12/13	7/14	<0.01
	5	0, 14, 24, 37	49	14/15	3/9	<0.01
	6	0, 14, 28, 42	56	13/15	7/15	<0.01

[a] Number of dead mice/number of total mice.

by Leppla et al. (Abstr. Annu. Meet. Am. Soc. Microbiol. 1978, B96, p. 29) in our system. Mice were given, as previously established by Leppla et al. and Walker et al. (44), either three or four g-TXD injections (10 μg) at approximately 2-week intervals. A rise in PHA titers and ELISA activity above preimmunization levels was seen after the third injection (Table 5). Approximately 15 days after the final immunization, mice were traumatized and infected with strain PA103 or PA220. The LD_{50} was slightly higher for mice immunized with g-TXD than for the control mice immunized with g-BSA ($P < 0.02$; Table 4). The results of eight experiments indicated that g-TXD-immunized mice survived consistently longer (average, 14.9 ± 7.4 h) than g-BSA-immunized mice ($P < 0.001$). However, the survival of the number of g-TXD-immunized mice was increased only between 0 and 30% (average, 14.4 ± 11.9%; $P < 0.02$) above control animals (g-BSA). The overall results with g-TXD, even though statistically significant results ($P < 0.05$) may have been obtained, suggest that the toxoid offers only a very limited protection against experimental *P. aeruginosa* infections. Similarly, Walker et al. (44) using a rat model could not demonstrate a significant protection with g-TXD against pseudomonas infections. Since the results with g-TXD were not as encouraging as those with f-TXD, g-TXD was not as extensively examined for toxicity or characterized as f-TXD.

Treatment of immunized mice with gentamicin. Since the survival of mice immunized with toxoid was enhanced, an attempt to increase ultimate survival was made by treating mice with gentamicin after infection (6, 42). Immunized mice (f-TXD) and their respective controls were given a single dose (intraperitoneally) of gentamicin (2 mg) 18 to 24 h after trauma and infection. Although there was no difference in survival between gentamicin-treated and un-

TABLE 4. *Mean lethal doses for immunized[a] mice infected with P. aeruginosa*

Immunogen	Infecting strain	Day of infection	Mean lethal dose (log_{10})	
			Control group	Immunized group
f-TXD[b]	PA103	56	4.75[c]	5.55
		59	4.73[c]	5.93
	PA220	50	0.23[c]	2.03
		56	1.76[c]	3.36
g-TXD[d]	PA103	53	4.45[e]	4.85
		54	4.78[e]	4.98
	PA220	48	1.68[e]	2.08
		50	1.51[e]	1.71

[a] Mice immunized on days 0, 14, 28, and 42.
[b] Control mice immunized with f-BSA.
[c] $P < 0.01$; two-tailed Student *t*-test.
[d] Control mice immunized with g-BSA.
[e] $P < 0.02$; two-tailed Student *t*-test.

TABLE 5. *Serum PHA antitoxin titers and ELISA activity of mice immunized with g-TXD[a]*

Day of sample	PHA antitoxin titer ± SD[b] (log_{10})	ELISA activity ± SD (OD_{400})[c]
Preimmunization	0	0.08 ± 0.01
7	0.4 ± 0.1	ND[d]
14	0.9 ± 0.2	0.18 ± 0.05
28	ND	1.70 ± 0.53
42	3.4 ± 0.1	1.67 ± 0.45
55	3.6 ± 0.1	2.02 ± 0.22
62	3.7 ± 0.5	1.82 ± 0.53
70	3.1 ± 0.4	1.83 ± 0.29

[a] Mice immunized on days 0, 10, 21, and 48.
[b] SD, Standard deviation.
[c] Optical density at 400 nm.
[d] ND, Not done.

treated control (f-BSA) mice, a consistent and significant increase in survival of gentamicin-treated, immunized (f-TXD) mice over the immunized but untreated mice was seen (Fig. 4). Virtually 100% survival was obtained with gentamicin treatment of f-TXD-immunized mice. Similar results were obtained with g-TXD-immunized mice (Fig. 5). The increased survival of gentamicin-treated, g-TXD-immunized over gentamicin-treated, g-BSA-immunized mice again suggests that some protection as a result of g-TXD immunization does occur. It is obvious that immunization with exotoxin A toxoids not only increases survival, but also greatly enhances the effectiveness of chemotherapy.

DISCUSSION

The efficacy of two exotoxin A toxoids prepared by either Formalin-lysine or glutaraldehyde treatment was examined against experimental *P. aeruginosa* infections in mice. As demonstrated by its ability to elicit antibody production in experimental animals (4) and in patients with *P. aeruginosa* infections (8, 36), native exotoxin A appears to be a good immunogen. However, the Formalin-lysine treatment apparently reduces its immunogenicity since no antitoxin response was elicited by the toxoid alone. To stimulate antitoxin production, both high doses (10 μg) and an adjuvant (MDP or Freund complete adjuvant) were required. After either three or four injections of f-TXD and MDP, a significant protection against *P. aeruginosa* infections was demonstrated. The survival rate of f-TXD-immunized mice was about 50 to 85% greater than the survival of control mice immunized with f-BSA ($P < 0.01$), and the LD_{50} of immunized (f-TXD) mice was significantly higher than that of controls ($P < 0.01$). The protective immunity was exotoxin A specific.

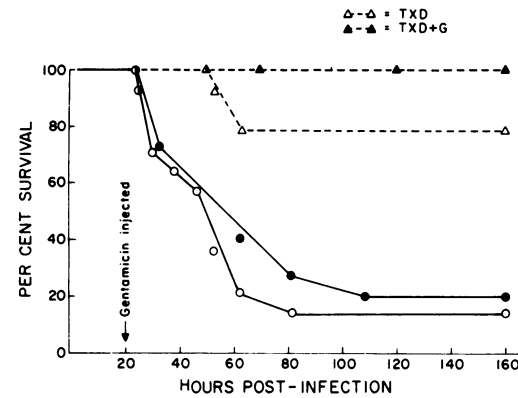

FIG. 4. *Survival of burned, infected (PA103) mice immunized with f-TXD (-----) or f-BSA (——) and treated with gentamicin (●, ▲) or not treated (○, △); 15 or 14 mice per group.*

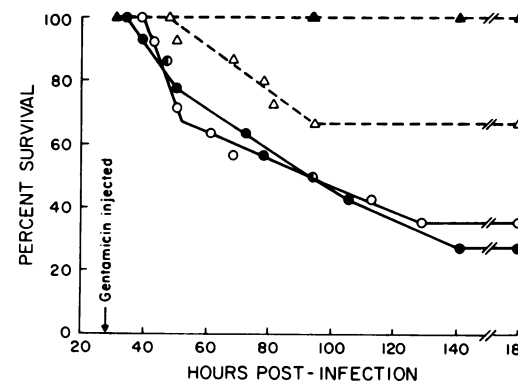

FIG. 5. *Survival of burned, infected (PA103) mice immunized with g-TXD (-----) or g-BSA (——) and treated with gentamicin (●, ▲) or not treated (○, △); 15 or 14 mice per group.*

First, parallel injections of equivalent amounts of Formalin-lysine-treated BSA and MDP or MDP alone did not result in protection against *P. aeruginosa* infections, nor did the results differ from those obtained with untreated mice. Second, immunization with f-TXD did not afford any degree of protection against infection with a nontoxigenic strain.

Variable results were obtained with g-TXD. The survival time of infected mice immunized with g-TXD was consistently longer than the survival of control mice immunized with g-BSA ($P < 0.001$). The LD_{50} values of the former group were also higher than those of the controls ($P < 0.02$). Nonetheless, the overall survival rate of g-TXD-immunized mice could not be significantly and repeatedly increased. Depending on the experiment, the survival of immunized mice (g-TXD) varied from 0 to 30% above that of the controls (g-BSA). Thus, the efficacy of g-TXD does not appear to be as good as that of f-TXD.

The observation that the survival of mice immunized with g-TXD was not as satisfactory as that of mice immunized with f-TXD, even though both groups had similar antibody levels, is somewhat puzzling. It may be, of course, that PHA titers or ELISA activity do not represent protective antibodies, although this seems unlikely since it was demonstrated that antitoxin PHA titers in patients with *P. aeruginosa* septicemia were associated with survival (30). Several other explanations may be possible. Since the exotoxin A used for g-TXD and f-TXD was produced in different laboratories (3, 4, 23) under somewhat different procedures, they may vary in their structure or in the degree to which they may be denatured and thus elicit antibodies of different affinity or avidity. For example, we have not been able, using our f-TXD, to produce significant levels of antibodies in mice without the use of adjuvant. However, using the same protocol but formalinized toxoid prepared by Cryz et al. in B. H. Iglewski's laboratory (S. J. Cryz, Jr., O. R. Pavlovskis, and B. H. Iglewski, *in* J. Robbins and J. C. Hill, ed., *Seminars in Infectious Diseases*, in press), we were able to elicit antibodies without the use of an adjuvant. Whereas our f-TXD gave a line of identity in an immunodiffusion test, Cryz et al. reported that the above toxoid gave a line of partial identity with their exotoxin A preparation used in the toxoiding. Also, since two different procedures have been used to inactivate the toxin, it is probable that different antigenic sites may have been altered or destroyed, and thus antibodies with different specificities were produced. A more likely explanation may be that MDP sensitizes the host to a more rapid or more effective defensive response to in vivo exotoxin production than g-TXD. The observation that only survival time of g-TXD-immunized mice was increased and not the survival suggests that the host is initially capable of neutralizing exotoxin, but as the infection progresses the defense mechanisms are overwhelmed by exotoxin A or some other virulence factor(s). This is further supported by the data on the gentamicin treatment. Survival of mice immunized with either toxoid could be increased by single injection of gentamicin at 20 h postinfection. It appears that during the early phase of the infection the immunized host effectively neutralizes the deleterious effects of exotoxin A. Thus, if at 20 h postinfection the organisms are eliminated and in vivo exotoxin A production ceases, the host's survival is increased. However, if the organisms are not eliminated either by the host or chemotherapy, in vivo antitoxin levels will be reduced, and the host will be overwhelmed by the toxic effects of exotoxin A and die. In contrast, in control, nonimmune infected animals, exotoxin A has not been neutralized and thus has been able to exert its toxic action from the start of the infection. By 20 h postinfection, irreversible damage has occurred and the host succumbs to the infection in spite of chemotherapy (42).

A number of exotoxin A toxoids (1; Cryz et al., in press) as well as vaccines from other pseudomonas virulence factors (10, 14, 17, 39) or cellular products (33) have been prepared and tested in vivo in various laboratories with positive results. In a collaborative effort with Cryz and co-workers (in press), we were able to show protection of mice immunized with a different formalinized toxoid. Abe et al. (1) was able to protect mice against infection of at least 50 LD_{50} of purified exotoxin A. Although the present data regarding an exotoxin A toxoid vaccine indicates that it offers a considerable protection against pseudomonas infections, the role and efficacy of such a single component vaccine in protection of patients are still unclear. Existing data indicate that possibly proteases (7, 15, 17, 20, 32) or other pseudomonas virulence factors or products such as polysaccharides or pili need to be included in an exotoxin A vaccine in order to increase its efficacy to 100% and bypass the use of toxic adjuvants. Our studies suggest that the primary function of a single component-exotoxin vaccine is to delay the onset of the toxic effects of exotoxin A and, as a result of this, the effectiveness of chemotherapy is enhanced. Such a vaccine should be an important adjunct to means used to prevent and treat *P. aeruginosa* infections.

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