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Passive Protection by Antitoxin in Experimental *Pseudomonas aeruginosa* Burn Infections

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The protective effect of intravenously administered rabbit antitoxin serum was studied in lethal *Pseudomonas aeruginosa* burn infections in mice. Survival after infection with 2 median lethal doses of a toxigenic, low-protease-producing strain (PA103) was enhanced in antitoxin-treated mice, as compared with controls that had received anti-bovine serum albumin serum ($P = 0.0004$). Survival time was prolonged in other antitoxin-treated mice infected with toxigenic, high-protease-producing strains (PA86 and PA220, $P = 0.0003$ and $P = 0.01$, respectively). In contrast, antitoxin had no protective effect in mice challenged with a nontoxigenic strain (WR 5, $P = 0.57$). There were fewer viable bacteria in blood and liver of antitoxin-treated mice than in those of anti-bovine serum albumin-treated controls after infection with toxigenic organisms, whereas there were no significant differences between the two groups after challenge with the nontoxigenic strain. These data suggest that *P. aeruginosa* exotoxin A contributes to lethality in this burn infection model, and this effect is diminished by passive immunization with antitoxin.

Exotoxin A (4, 5, 12) is produced in vitro by most clinical isolates of *Pseudomonas aeruginosa* (2, 19). The purified exotoxin is lethal for various mammalian species (1, 10) and has been shown to be cytotoxic in vitro (13, 14). At the subcellular level exotoxin inhibits protein synthesis (15) as a result of the inactivation of elongation factor 2 (6, 7). Despite this accumulating information about the biological activities of exotoxin and the demonstration that pseudomonas infection in humans induces an antibody response to exotoxin A (19), its role in infection is unclear. In addition, although exotoxin is neutralized by antitoxin in vitro (5), the protective effect of antitoxin in experimental infection has not been adequately investigated. Although Liu and Hsieh (11) demonstrated protection in mice injected intraperitoneally with antitoxin against lethal doses of live *P. aeruginosa*, because of the relatively low virulence of this bacterium for normal mice, an unrealistically large inoculum was required to produce lethal disease in this model.

In this study, we used a *P. aeruginosa* mouse burn infection model developed by Stieritz and Holder (20), which closely parallels human burn wound sepsis. In this model infection is readily and reproducibly caused by a small inoculum. By examining the protective effect of administering antitoxin serum in this model, we were able to assess indirectly the pathogenic role of exotoxin as well as to evaluate the immunoprophylactic potential of antitoxin.

MATERIALS AND METHODS

Organisms. *P. aeruginosa* strains were obtained from the following sources: PA103 from Pinghui V. Liu, University of Louisville School of Medicine, Louisville, Ky.; PA86 and PA220 from the National Naval Medical Center, Bethesda, Md.; WR-5 from the Walter Reed Army Hospital, Washington, D.C. The clinical source, immunotype, and other properties of these strains are listed in Table 1. Strain WR-5 was identified by Bjorn et al. (2) as a nontoxigenic strain.

Challenge inoculum. Although it is not known whether exotoxin is an inducible enzyme, the microorganisms were grown under conditions that permitted optimal exotoxin production and then were washed repeatedly after harvesting to remove extracellular products, including exotoxin.

A Trypticase soy agar (BBL, Cockeysville, Md.) slant was inoculated from a frozen vial of stock culture and incubated overnight at 37°C. The organisms were then suspended in 25 ml of Trypticase soy broth dialysate to achieve an optical density at 660 nm (OD_{660}) of 0.15 (4), and this was placed in a baffled 300-ml nephelometer flask (Bellco Glass, Vineland, N.J.) to increase aeration. The flask was incubated at

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32°C on an oscillating shaker (Psycrotherm, model G-26, New Brunswick Scientific Co., New Brunswick, N.J.) set at 200 oscillations/min until the OD_{660} of the culture reached 0.60. A sufficient volume (3 to 5 ml) of the log-phase culture was used to inoculate another nephelometer flask containing 60 ml of Trypticase soy broth dialysate to achieve an OD_{660} of 0.05. The culture was incubated as above until the OD_{660} reached 0.20. The organisms were then washed three times with sterile phosphate-buffered saline (PBS), pH 7.4, and suspended in PBS to achieve an OD_{660} of 0.60. The suspension was diluted with sterile PBS to the desired concentration and used to infect mice. When harvested under these conditions the total number of bacteria, as determined in a Petroff-Hausser bacteria counting chamber (C. A. Hausser & Sons, Philadelphia, Pa.), equaled the viable count.

Mouse burn infection model. The burned mouse model developed by Stieritz and Holder (20) was used with some modifications. Female Swiss white mice, NIH/Nmri CV strain, weighing 20 ± 2 g, were anesthetized with methoxyflurane (Penthrane, Abbott Laboratories, North Chicago, Ill.) and subjected to a 10-s, 2.5- by 2.5-cm flame burn (15% of total body surface) as previously described (20). Viable bacteria suspended in 0.5 ml of PBS at the desired concentration were immediately injected subcutaneously in the burn site.

Representative mice from the colonies used in the study were monitored by passive hemagglutination assay (18) for antibodies to exotoxin, which were absent in all cases.

Quantitation of bacteria in blood and liver. Mice were killed by cervical dislocation, and blood was obtained by cardiac puncture. The liver was perfused, excised, rinsed in PBS, diced, and homogenized in cold PBS, using a Sorvall Omnimixer (Ivan Sorvall, Inc., Norwalk, Conn.). The number of colony-forming units of *P. aeruginosa* per milliliter of blood or per total liver was determined by serial dilution plate counts done on Trypticase soy agar plates, and the results from three animals were averaged.

Antisera. Antitoxin and anti-bovine serum albumin (BSA) were prepared in male New Zealand rabbits weighing 3 to 5 kg. The immunization schedule for preparation of antitoxin has been described previously (5). Anti-BSA serum was prepared by two intravenous injections of 4 mg of BSA (Miles Laboratories, Inc., Kankakee, Ill.) separated by 5 days and one subcutaneous injection of 2 mg of BSA in Freund incomplete adjuvant (Difco Laboratories, Detroit, Mich.) on day 28.

Antisera (antitoxin and anti-BSA) were heated at 56°C for 30 min and absorbed with whole cells of the appropriate challenge strains to eliminate possible antibodies against cell wall components. The procedure was as follows. Bacteria were grown overnight in complete Trypticase soy broth at 37°C with minimal aeration to reduce toxin production. The culture was treated with Formalin in a final concentration of 0.25%, incubated at 37°C for 30 min, and held at 6°C for approximately 48 h. The cells were then washed four times with PBS. The bacterial pellets were mixed with antisera, and the suspensions were held for 2 h at 37°C and overnight at 6°C and centrifuged at 15,000 × *g* for 30 min. The absorption was repeated once more as described. After absorption, antisera were negative for hemagglutinating antibodies to type-specific lipopolysaccharide of challenge strains; these assays were kindly performed by G. C. Cole and M. W. Fisher (Parke-Davis Co., Detroit, Mich.). In addition, antisera were not bactericidal for any of the four pseudomonas challenge strains used in the study.

The antisera were also checked for the presence of antibodies against pseudomonas protease. The purified protease with elastase activity (21) and its antiserum (21) were kindly provided by B. Wretlind (Karolinska Hospital, Stockholm, Sweden). Anti-BSA, antitoxin, and antiprotease sera were incubated with the purified enzyme for 30 min at 23°C. The suspensions were then assayed for protease activity according to the procedure of Kunitz (8) with minor modifications (21). The results indicate that neither anti-BSA nor antitoxin serum contained detectable protease antibodies. On the other hand, antiprotease serum completely inhibited protease activity at a 1:50 dilution. In addition, protease antibodies were not detected in anti-BSA and antitoxin sera by the Ouchterlony test.

Statistical analysis. Survival distributions were compared by using the Wilcoxon Rank Sum Test as described by Bradley (3). The *P* values were computed by using a normal approximation with continuity correction (3). These *P* values are consistent with those drawn from available Wilcoxon Rank Sum Test tables.

RESULTS

Burn infections. Subcutaneous injection of viable *P. aeruginosa* in burn sites resulted in lethal infections associated with bacteremia and systemic invasion. Each of the four challenge strains produced a lethal infection at a dose characteristic for the individual strain (Table 1). In the case of the toxigenic strains, the burn injury reduced the minimal lethal inoculum by as much as three logs (Table 1). An inoculum equivalent to 2 median lethal doses (LD_{50}) usually resulted in 100% mouse mortality.

Although organisms were recovered from blood and liver 20 h or more after inoculation, at necropsy there was evidence of abscess formation only at the injection site. The only consistent histological finding outside the burn area was minimal to moderate hepatocellular necrosis indistinguishable from that described in mice treated with purified toxin (16). These findings were not present in mice infected with the nontoxigenic WR-5 strain.

Passive immunization. Twenty hours after receiving intravenous injections of various doses of antitoxin, mice were burned and infected with 2 LD_{50} of strain PA103; controls were given anti-BSA serum in equivalent volumes. A 0.15-ml amount of this lot of antitoxin was required for protection (Table 2). Volumes of antisera larger than 0.2 ml, either antitoxin or anti-BSA, made the mice visibly sick immediately after injection;

TABLE 1. *P. aeruginosa mouse burn infection challenge strains*

Strain	Source	Sero-type[a]	Exo-toxin A	Protease	Serum resistance[b]	LD_{50} (CFU)[c]	
						Normal mice	Burned mice
PA103	Sputum	2	+	−	−	1.8×10^6	1.2×10^3
PA86	Blood	3	+	+	+	2.5×10^6	2.3×10^3
PA220	Blood	1	+	+	+	$>1.4 \times 10^4$	$<1.0 \times 10^1$
WR-5	Blood	7	−	+	+	24.0×10^6	7.5×10^6

[a] Fisher-Devlin type.
[b] Normal human serum.
[c] CFU, Colony-forming units.

volumes of 0.3 ml were often lethal, presumably as the result of volume and/or colloid overload. Based on these considerations, 0.2-ml volumes were administered in all subsequent experiments.

Groups of mice were administered 0.2 ml of antitoxin or anti-BSA serum and then burned and infected 20 h later with 2 LD_{50} of each of the four challenge strains. Results are shown in Fig. 1. Antitoxin conferred complete protection in infections with the low-protease-producing, toxigenic PA103 strain ($P = 0.0004$) and prolonged survival of mice infected with the two high-protease- and toxin-producing strains, PA86 and PA220 ($P = 0.0003$ and $P = 0.01$, respectively). In contrast, antitoxin failed to enhance the survival of mice infected with the nontoxigenic WR-5 strain ($P = 0.57$). All experiments shown in Fig. 1 were repeated, with similar results, although these additional data are not shown.

To substantiate the specificity of protection conferred by antitoxin, mice were administered antitoxin that had been absorbed with purified toxin (1,800 LD_{50}/ml) before burning and infecting with PA103. Control mice received unabsorbed antitoxin or anti-BSA serum. The protective effect of antitoxin was abolished by the absorption procedure, thus confirming that protection was due to toxin-specific antibodies (Fig. 2).

Of additional interest was the observation that microscopic liver necrosis frequently seen in anti-BSA-treated mice 48 h or more after infection with PA103 was not present in mice that had received antitoxin; nor was it seen in mice infected with the nontoxigenic WR-5 strain, as previously mentioned.

Quantitation of bacteria in blood and liver. In a parallel set of experiments, passively immunized mice were infected with the same four challenge strains and sacrificed sequentially 18 to 70 h later, and their blood and livers were cultured quantitatively. The antitoxin-treated mice infected with the toxigenic strains (PA103, PA86, PA220) had consistently lower viable bacterial counts in blood and liver than the anti-BSA-treated mice (Fig. 3 and 4). In contrast, there were no differences in bacterial counts between the two treatment groups after infection with the nontoxigenic WR-5 strain.

TABLE 2. *Mortality of burned mice pretreated with either antitoxin[a] or anti-BSA serum[b] and infected with P. aeruginosa (PA103)*

Serum dose (ml)	Mortality (no. of dead mice/total mice)		
	Antitoxin	Anti-BSA serum	P[c]
0.10	8/10	10/10	>0.05
0.15	0/8	10/10	<0.005
0.20	1/15	15/15	<0.005

[a] Titer by passive hemagglutination = 1:32,000. By mouse neutralization (4), 0.1 ml diluted 1:256 neutralized 5 LD_{50} of purified exotoxin.
[b] No antibodies against exotoxin by passive hemagglutination.
[c] Chi-square test.

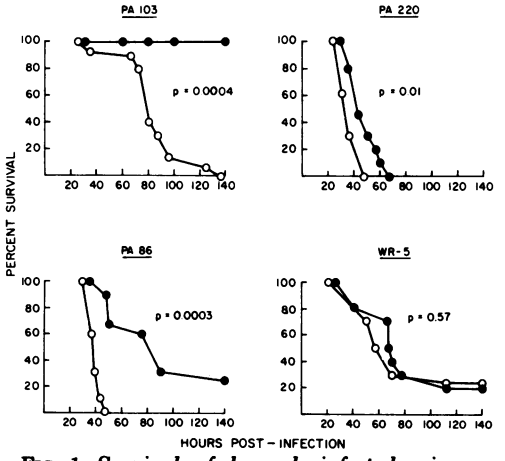

FIG. 1. *Survival of burned, infected mice pretreated with rabbit antitoxin serum (●) or anti-BSA serum (○). Number of mice in antitoxin and anti-BSA treatment groups, respectively: PA103 = 8, 11; PA86 = 9, 10; PA220 = 11, 11; WR-5 = 10, 10. Results of duplicate experiments (not shown) were similar. A 0.1-ml amount of antitoxin serum, diluted 1:256, neutralized 5 LD_{50} of purified toxin.*

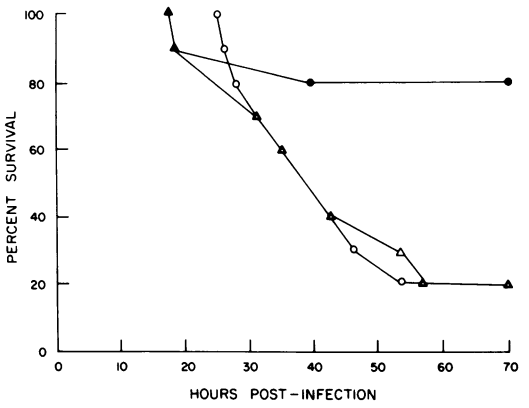

FIG. 2. *Survival of burned, infected (PA103) mice pretreated with rabbit antitoxin serum (●), anti-BSA serum (○), or antitoxin serum absorbed with exotoxin (△). Number of mice used in each treatment group = 10. A 0.1-ml amount of unabsorbed antitoxin, diluted 1:64, neutralized 5 LD_{50} of purified toxin.*

DISCUSSION

Exotoxin is produced in vitro by most clinical strains of *P. aeruginosa* (2, 19), and its in vivo release during human infections has been demonstrated indirectly by serological means (17). Its known biological activities include lethality for a wide range of animal species (1, 10, 14, 16) as well as toxicity for a variety of cells grown in culture (13, 14). At the subcellular level, exotoxin is a potent inhibitor of protein synthesis (15) by a mechanism identical to that for diphtheria toxin (6, 7). These observations suggest that exotoxin may play a pathogenic role in pseudomonas infections.

The primary goal of this study was to relate what is already known about the biological activities of purified exotoxin to its potential role in infections. In attempting to do this, we examined the protective effect of antitoxin in pseudomonas burn infections.

The mouse burn infection model of Stieritz and Holder (20) appeared suitable for our purposes since it is analogous to clinical pseudomonas burn sepsis and requires realistically small infecting doses; this latter characteristic of the model avoided the injection of experimental animals with significant amounts of preformed toxin or other bacterial products.

The different levels of protection conferred by antitoxin serum on groups of mice infected with three different toxigenic strains (Fig. 1) may reflect different biological and biochemical properties of the organisms. One of the toxigenic strains, PA103, was serum sensitive and produced little or no protease, whereas PA86 and PA220 were serum-resistant protease producers. In addition, all three organisms represented different Fisher-Devlin immunotypes. It is tempting to speculate that the inability of PA103 to produce protease, itself a presumed virulence factor also known to inactivate exotoxin in vitro, may have enhanced or unmasked the protective effect of antitoxin. Consistent with this hypothesis was the more modest protection afforded by antitoxin in infections by the relatively low-protease-producing strain PA86 and the lesser, although still highly significant, protection conferred by antitoxin in infections caused by PA220, the most potent protease producer and most virulent of the challenge strains, as judged by LD_{50}. Variations in the protective action of antitoxin may also have been due to other factors, such as peculiarities of in vivo as compared with in vitro exotoxin production and release, or the production of other extracellular toxins such as phospholipase and hemolysin. Since the antisera were extensively absorbed with the challenge organism, it is unlikely that differences in protection among mice infected with different strains were due to antibodies directed against cell wall lipopolysaccharide.

Despite these variations, significant protection was demonstrated in infections caused by all three toxin-producing strains, and not in the case of the single nontoxigenic organism. Since this protection was antitoxin specific, we may conclude that exotoxin contributed directly or indirectly to mouse lethality in this burn sepsis model, although the underlying mechanism(s) is still unknown.

It is clear from this and other studies (O. R. Pavlovskis, B. H. Iglewski, and M. Pollack, manuscript in preparation) that certain effects produced by purified exotoxin after injection in mice, such as hepatic necrosis (16), inhibition of protein synthesis (15), and depression of elongation factor 2 in liver and other tissues (7), also occurred in this murine infection model when toxigenic *P. aeruginosa* strains were used. The absence of these phenomena in antitoxin-treated mice, and in mice infected with the nontoxigenic WR-5 strain, helps implicate exotoxin in their causation.

Adding to the complexity of the question regarding the exact pathogenic role of toxin is the observation that mice that received antitoxin had lower viable bacterial counts in blood and liver than control animals (Fig. 3 and 4). These data suggest that the toxin may either enhance the invasiveness of pseudomonas, thus increasing bacteremia, or interfere with normal clearance mechanisms. Hypothetically, exotoxin could disturb the host's immune system by contributing to the breakdown of normal anatomical

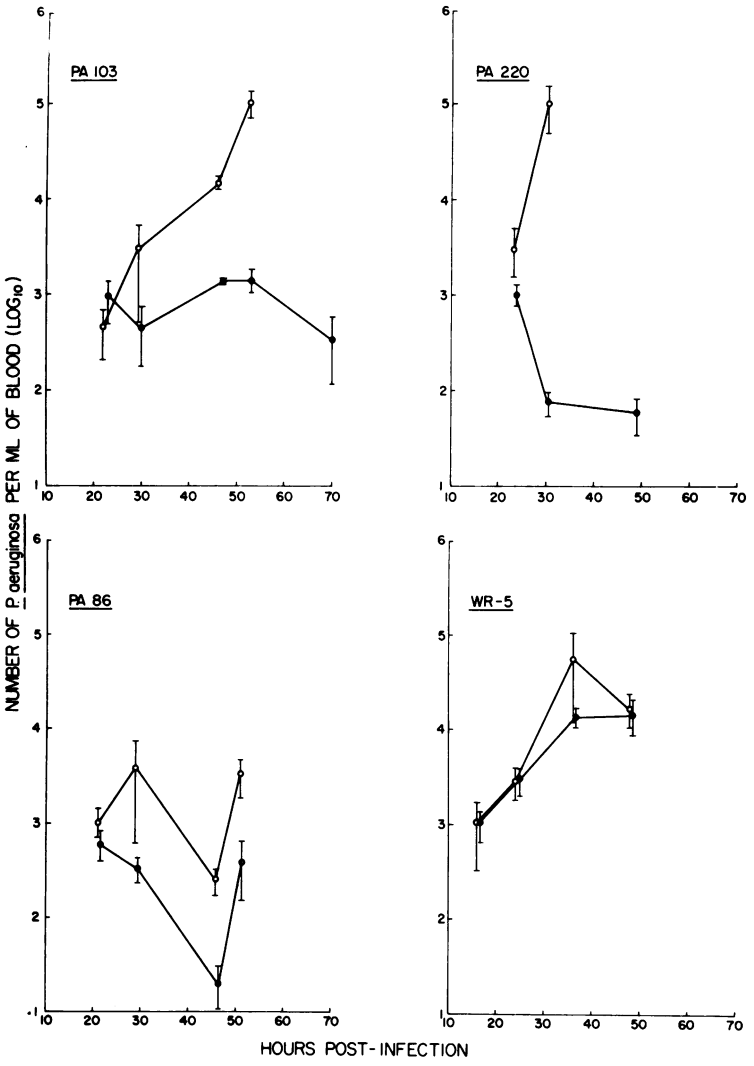

FIG. 3. *Number of pseudomonas colony-forming units ± standard error of the mean per milliliter of blood of burned, infected mice pretreated with antitoxin serum (●) or anti-BSA serum (○). The potency of antitoxin was the same as shown in Fig. 1.*

defense barriers or by interfering with reticuloendothelial and/or leukocyte function. That exotoxin can cause tissue destruction is suggested by its ability to produce dermonecrosis in guinea pigs (19). That reticuloendothelial function might be adversely affected is suggested by the ability of toxin to produce hepatocellular necrosis and to inhibit protein synthesis in the livers and spleens of intoxicated (15, 16) as well as infected mice (Pavlovskis et al., manuscript in preparation; K. Snell, A. Holder, and C. B. Saelinger, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B116, p. 34). Finally, that exotoxin might interfere with leukocyte function is suggested by in vitro data showing that toxin is lethal for human macrophages derived from peripheral blood monocytes (M. Pollack and S. E. Anderson, unpublished data; K. K. Yamada, J. C. Sadoff, and G. H. Lowell, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B110, p. 33).

Another possible explanation for the lower viable bacterial counts in the antitoxin-treated group is that exotoxin arrayed on the surface of invading bacteria enables antitoxin to act as an opsonin. This seems unlikely, however, in the light of two observations. First, the failure of extensive absorption of antitoxin serum with whole bacterial cells to reduce the antitoxin titer

of that serum suggests that no significant exotoxin is arrayed on the cell surface where it would be available to antibody as a basis for opsonization. Second, in a separate experiment (data not shown) preincubation of live bacteria of the challenge PA103 strain with antitoxin or anti-BSA serum before inoculation did not affect the viable bacterial counts in the blood of infected animals at 24 and 48 h. This, too, suggests that our antiserum has no opsonizing effect on infecting bacteria.

Whatever the role of exotoxin in pseudomonas infections, it seems reasonably clear that it is only one of a number of pathogenic factors. This study in the mouse burn sepsis model, as well as other data cited, suggests that exotoxin does contribute to the pathogenesis of these infections, that it may interfere with host immunity, and that its effects after in vivo release may be blocked by passive immunization with antitoxin. What the implications of these observations are for the immunoprophylaxis or treatment of human pseudomonas infections remain to be determined.

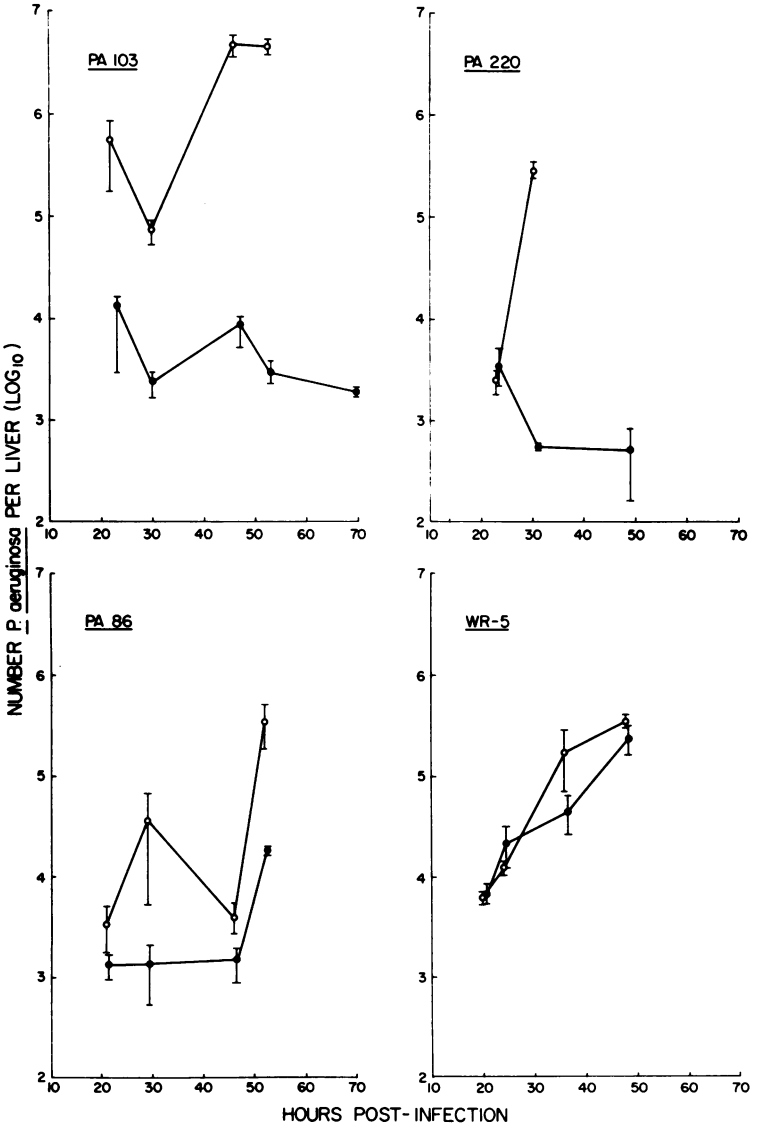

FIG. 4. *Number of pseudomonas colony-forming units ± standard error of the mean per liver of burned, infected mice pretreated with antitoxin serum (●) or anti-BSA serum (○). The potency of antitoxin was the same as shown in Fig. 1.*

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