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Role of Exotoxin and Protease as Possible Virulence Factors in Experimental Infections with *Pseudomonas aeruginosa*

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Evidence is presented which suggests that both the proteases and the exotoxin produced by *Pseudomonas aeruginosa* multiplying in situ in a burned mouse model are virulence factors. A 50% decrease in functional elongation factor 2 (EF-2) was seen 16 h postinfection in the liver of mice infected with the toxigenic, protease-producing *P. aeruginosa* strain M-2; at the time of death EF-2 was depleted by 80%. This correlates with a reduction in the level of protein synthesis in the liver of infected animals. Treatment with specific antitoxin extended the mean time to death and blocked depletion of EF-2. Administration of gentamicin 24 h after infection caused rapid clearance of bacteria and extended the mean time to death, but all animals treated with either antitoxin or gentamicin eventually died. In contrast, treatment with both antitoxin and gentamicin provided virtually complete protection. Infection of mice with *P. aeruginosa* WR5 (protease-producing, nontoxigenic) or with *P. aeruginosa* PA103 (toxigenic, slow protease producer) required several logs more bacteria and did not result in the same extensive depletion in EF-2 content. When challenge with PA103 was supplemented by injection of purified *Pseudomonas* protease, the mean time to death was shortened and significant reduction in liver EF-2 was observed. It is suggested that both toxin and proteases are required for the full expression of virulence in *Pseudomonas* infections.

Pseudomonas aeruginosa is an opportunistic pathogen which causes fatal infections in compromised hosts, such as burn patients (3, 18). Several extracellular enzymes of *P. aeruginosa* are implicated in its pathogenicity; these include a heat-labile exotoxin (11, 13) and several proteases (5, 10, 17) and hemolysins (26). *Pseudomonas* exotoxin is toxic for several cell lines in culture (15, 19). At the biochemical level, it inhibits protein synthesis in mice (7, 20) and in cell-free protein-synthesizing systems (6). *Pseudomonas* exotoxin, like fragment A of diphtheria toxin, transfers the adenine diphosphoribose (ADP-ribose) portion of nicotinamide adenine dinucleotide into covalent linkage with elongation factor 2 (EF-2). The ADP-ribosylation reaction depletes functional EF-2 and stops protein synthesis.

Knowledge of the biochemical mode of action of *Pseudomonas* exotoxin in vitro (6) suggests that the levels of functional EF-2 in mammalian tissues may be used to monitor the activity of *Pseudomonas* exotoxin in vivo. A biochemical lesion (inactivation of EF-2) identical to that induced by toxin in vitro probably occurs in mice infected with toxigenic strains of *P. aeruginosa*. Previous studies in this laboratory with a burned mouse model (24; D. D. Stieritz and I. A. Holder, J. Med. Microbiol., in press) provided evidence for the production of *Pseudomonas* exotoxin by bacteria multiplying at the burned site; the evidence included detection of ADP-ribosylation activity in the sera of infected burned mice and a significant reduction in the EF-2 content of liver tissue from these animals. Other evidence that *Pseudomonas* exotoxin is produced during *Pseudomonas* infections is the demonstration of neutralizing (22) and hemagglutinating (23) antibodies against *Pseudomonas* exotoxin in sera from individuals recovering from infections with *P. aeruginosa*. This report describes experiments designed to compare the amounts of functional EF-2 in tissues of infected burned mice with the amounts in normal tissues. Levels of active EF-2 in several tissues are correlated with the progression and eventual outcome of *P.*

aeruginosa infections. The therapeutic value of antitoxin and antibiotic treatment in infected burned animals is also assessed.

MATERIALS AND METHODS

Microorganisms. Four strains of *P. aeruginosa* with different virulence characteristics were used in this study. Two of these strains have high virulence for burned mice. *P. aeruginosa* M-2, originally isolated from the small intestine of CF-1 mice, produces toxin and protease. PA1210, a clinical isolate producing both protease and toxin, was supplied by J. Y. Homma, Institute of Medical Sciences, Tokyo, Japan. Burned mice which received 100 to 200 viable organisms of either M-2 or PA1210 subcutaneously (s.c.) had a mean time to death of 34 to 36 h. Two less virulent strains were also studied. PA103, a toxigenic strain which produces small amounts of protease, was provided by P. V. Liu, Louisville, Ky.; 10^7 organisms of this strain given s.c. resulted in a mean time to death of 52 h in burned mice. WR5, a nontoxigenic, protease-producing strain, was kindly supplied by B. H. Iglewski, Portland, Ore. A mean time to death of 44 h was obtained with s.c. injection of 10^7 organisms in burned mice. Strains M-2 and WR5 are serum resistant and PA103 is serum sensitive. Bacteria were prepared for animal challenge as described previously (24).

Toxin. *Pseudomonas* toxin was prepared and purified as previously described (9). The mean lethal dose (LD_{50}) for mice is 0.5 μg given intraperitoneally (i.p.).

Antitoxin. Heat-inactivated horse serum and antiserum raised in ponies against a purified toxin preparation were adsorbed twice with heat-killed, washed *P. aeruginosa* cells prior to use. This treatment removed the low levels of opsonizing and agglutinating antibodies present in both sera. Adsorbed sera were diluted 1:5 in sterile saline; 0.25 ml per mouse was administered i.p. One microliter of undiluted antitoxin is sufficient to neutralize 1 μg of toxin.

Antibiotics. Gentamicin sulfate (Garamycin; Schering Corp., Kenilworth, N.J.) was diluted in sterile physiological saline. Each mouse received a total of 2.2 mg of gentamicin given i.p. and s.c.

Protease. Crystalline *Pseudomonas* protease prepared from culture filtrates of *P. aeruginosa* IFO3080 was the generous gift of K. Morihara, Osaka, Japan. The protease has a molecular weight of 48,000; it contains proteolytic activity but no elastolytic activity (16; personal communication). The preparation gives a single band upon sodium dodecyl sulfate gel electrophoresis. Protease activity was measured by use of a plate assay with skim milk as substrate. Purified *Bacillus* protease was used to prepare a standard reference curve. Growth of *P. aeruginosa* M-2 in brain heart infusion broth for 24 h resulted in production of 100 μg of protease per ml.

Animal model. The burned mouse model of Stieritz and Holder was used (25). Female CF-1 mice (Carworth Farms, Wilmington, Mass.) weighing 22 to 24 g were anesthetized with Metafan and received a partial thickness alcohol flame burn on the shaved back followed by an i.p. injection of 0.5 ml of sterile saline as fluid replacement therapy. Immediately after being burned, animals received the appropriate s.c. challenge inoculum in 0.1 ml of physiological saline at the burned site.

Quantitation of EF-2. The active EF-2 content of mouse tissues was assayed by the method of Gill and Dinius (4). Tissues from four mice were pooled, rinsed, diced, and homogenized in 3 ml of cold 0.25 M sucrose. Endogenous nicotinamide adenine dinucleotide was removed from homogenates; the homogenates were centrifuged and assayed for active EF-2 in the presence or absence of 100 μg of diphtheria toxin. The reaction mixture contained 0.07 M dithiothreitol, 1 mg of bovine serum albumin per ml, 0.25 M histamine, 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.2), 0.1 mg of diphtheria toxin per ml, and 0.12 μCi of [^{14}C]nicotinamide adenine dinucleotide (uniformly labeled in the adenine moiety; specific activity, 302 mCi/mmol; Amersham/Searle Corp., Des Plaines, Ill.). After 15 min of incubation at 37°C, the reaction was stopped by the addition of an equal volume of 12% trichloroacetic acid. Precipitates were collected and counted as described previously (24). All assays were performed in triplicate. The difference between radioactive counts incorporated with and without diphtheria toxin represents the ADP-ribose-EF-2 complex formed. Data are expressed as counts per minute per milligram of protein. Protein content of tissue homogenates was determined by the method of Lowry et al. (14). The data presented represent the average of two to four separate experiments carried out in triplicate, with four mice per sample.

In vivo protein synthesis. Protein synthesis in the intact animal was determined by the method of Bonventre and Imhoff (2). Infected and control mice received 150 μCi of [^{3}H]leucine (specific activity, 58 Ci/mmol; Amersham/Searle Corp.) i.p. 30 h after infection and burning. After 90 min, animals were sacrificed, and tissues were removed, quick frozen on dry ice, and stored at −70°C until processed. Organs from each animal were processed individually. Data are expressed as percent inhibition of leucine incorporation in tissues of infected animals as compared with uninfected control animals.

Bacterial quantitation. Organs of infected animals were homogenized in sterile saline. The number of viable bacteria in homogenates was determined by serial dilution plating and is expressed as the number of *P. aeruginosa* cells per gram (wet weight) of tissue.

RESULTS

Since we have previously established (24) that a reduction occurs in the level of active EF-2 in the liver of mice infected with the M-2 strain (protease-producing, toxigenic) of *P. aeruginosa*, it was considered of interest to examine the effect of infection with strains of *Pseudomonas* differing in exotoxin and protease production. Mice were burned and infected with four different strains of *P. aeruginosa* (Table 1). Mice infected with *P. aeruginosa* M-2 or PA1210, strains which produce both toxin and protease, showed a marked depletion of EF-2 in the liver. Some reduction was seen in other organs, but not to the same extent as in the liver.

TABLE 1. *Reduction of functional EF-2 in tissues of burned mice after infection with various strains of P. aeruginosa*

Strain	Percent reduction[a]			
	Liver	Kidney	Spleen	Heart
M-2[b] (toxigenic, protease-producing)	83	26	37	0
PA1210[c] (toxigenic, protease-producing)	67	29	29	ND
PA103[d] (toxigenic, non-protease-producing)	49	0	23	ND
WR5[e] (nontoxigenic, protease-producing)	0	16	0	22

[a] ND, Not determined.
[b] Animals infected with 2×10^2 bacteria; sacrificed at 32 h.
[c] Animals infected with 2×10^2 to 4×10^2 bacteria; sacrificed at 30 h.
[d] Animals infected with 2×10^7 bacteria; sacrificed at 34 h.
[e] Animals infected with 4×10^7 bacteria; sacrificed at 46 h.

Strain PA103, which produces *Pseudomonas* exotoxin and small amounts of protease, and strain WR5, which produces protease but synthesizes no toxin, were less virulent and required significantly higher inocula to kill a burned mouse than did strains producing both toxin and protease. There was no reduction of EF-2 in the livers of burned mice infected with the nontoxigenic strain WR5, even in the terminal stage of infection (46 h). A reduction in liver EF-2 was seen late in infection with PA103. Thus, experimental infections with protease-producing, toxigenic strains of *P. aeruginosa* result in a marked reduction of EF-2 content in the liver, whereas infections with the nontoxigenic strain have no significant effect on liver EF-2.

More extensive analysis of EF-2 levels in burned, infected mice was carried out with strain M-2. The time course of EF-2 reduction is presented in Fig. 1. Content of functional EF-2 in the liver was markedly reduced as infection with *P. aeruginosa* M-2 progressed. Levels were reduced by 60% as early as 24 h and by 83% when animals appeared moribund. Thus, it appears that at the time of death almost no functional EF-2 remains in the liver of infected animals. Levels of active EF-2 in kidney, spleen, and heart were only slightly reduced at all time periods examined. Depletion of functional EF-2 can be correlated with an inhibition in protein synthesis, as shown in Table 2. Protein synthesis was inhibited in the liver of infected animals by

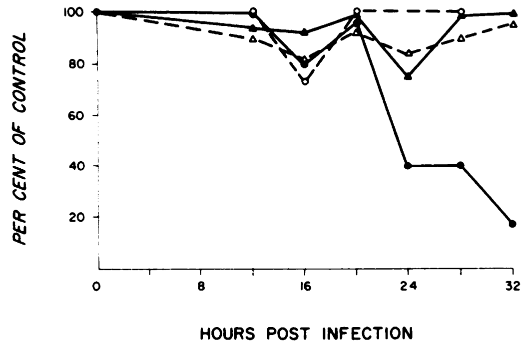

FIG. 1. *Kinetics of EF-2 depletion after infection of mice with P. aeruginosa M-2. Mice were burned and infected with 1.5 × 10² bacteria. Levels of EF-2 were determined at indicated times. Data represent an average of two separate experiments. (●) Liver, (○) heart, (△) kidney, (▲) spleen.*

TABLE 2. *Inhibition of protein synthesis in tissues of mice which have been burned and infected with P. aeruginosa M-2[a]*

Tissue	Inhibition (%)[b]
Liver	67
Kidney	24
Spleen	83
Heart	23

[a] Mice were challenged with 1.3×10^2 *P. aeruginosa* M-2 cells and were sacrificed 28 to 34 h after infection.
[b] Data represent an average of three separate experiments and are expressed as percent reduction of protein synthesis in infected animals as compared to burned controls.

67% and in the spleens by 83% at later stages of infection. Inhibition of protein synthesis in the kidney and heart was minimal.

Effect of protease. In view of the fact that the low protease-producing strain PA103 was not as virulent as the actively protease-producing strains, the effect of injecting purified *Pseudomonas* protease together with bacteria was examined (Table 3). Mice were burned and infected with approximately 100 viable cells of *P. aeruginosa* PA103; at the time of infection and at 8 and 24 h thereafter, mice received 10 μg of protease s.c. at the burn site. This regimen has been found to reduce the LD_{50} for strain PA103 by a factor of 1,000 (I. A. Holder and C. G. Haidaris, Abstr. Annu. Meet. Am. Soc. Microbiol. 1977, B118, p. 35). Fifty percent of the mice receiving both protease and bacteria (100 organisms) were dead at 80 h, whereas mice receiving bacteria or protease alone survived until the end of the observation period (day 7). If the protease was heat inactivated (30 min, 85°C) prior to administration, no potentiation of infection with

TABLE 3. *Reduction of functional EF-2 in mice treated with purified protease and P. aeruginosa PA103*

Treatment	Tissue	EF-2 (cpm/mg)	Depletion (%)
Burned control	Liver	10.8×10^3	—
	Kidney	7.1×10^3	—
	Spleen	16.6×10^3	—
PA103[a]	Liver	10.4×10^3	4
	Kidney	9.6×10^3	0
	Spleen	16.3×10^3	2
Protease[b]	Liver	9.0×10^3	17
	Kidney	8.4×10^3	0
	Spleen	17.6×10^3	0
PA103 + protease[c]	Liver	6.3×10^3	42
	Kidney	7.2×10^3	0
	Spleen	12.1×10^3	28

[a] Mice challenged with 85 viable cells of *P. aeruginosa* PA103 alone; sacrificed at 65 h.

[b] Mice given protease at time of burning and at 8 and 24 h; sacrificed at 65 h.

[c] Mice were burned and challenged with 100 cells of *P. aeruginosa* PA103; at the time of infection and at 8 and 24 h, mice received 10 μg of purified *Pseudomonas* protease at the burn site. Mice were sacrificed at 65 h.

PA103 was observed (unpublished data). When the level of functional EF-2 was measured, depletion was seen only in animals receiving combined treatment; reduction was observed primarily in the liver. Normal levels of EF-2 were present in the mice challenged only with 85 *P. aeruginosa* PA103 cells or with protease.

Antitoxin, antibiotic treatment. The effect of antitoxin, gentamicin, or combined antitoxin-antibiotic therapy on the course of *Pseudomonas* infection was examined in the burned mouse model. Mice were burned and infected and then received: (i) no treatment, (ii) antitoxin at the time of infection, (iii) gentamicin 24 h after infection, or (iv) horse serum at the time of infection and gentamicin 24 h later (Fig. 2). All animals in the untreated group died within 36 h. Treatment with either antitoxin or gentamicin alone increased the survival times, but all animals in these two groups succumbed by 84 h after initiation of infection. Combined therapy with both antitoxin and gentamicin provided virtually complete protection. Treatment with normal horse serum and gentamicin was included as a control, and survival rates in this group were comparable to those in the group receiving gentamicin alone. Therefore, antiserum and antibiotic therapy offers long-term protection not afforded by either specific antitoxin or gentamicin alone.

The number of bacteria present in tissues of mice treated as described above was determined 24, 28, and 32 h after infection (Table 4). Antitoxin had no effect on bacterial growth in vivo since numbers of viable bacteria in all tissues of animals receiving antitoxin alone were similar to those in animals receiving no treatment. These data provide additional evidence that the adsorbed antitoxin used here did not contain significant amounts of opsonizing antibody. Fur-

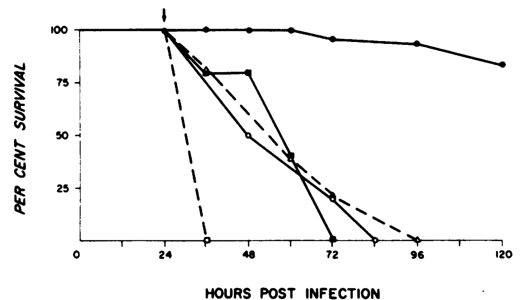

FIG. 2. *Effect of combined antitoxin and gentamicin therapy on survival of burned, infected mice. Mice were injected with 50 to 100 cells of P. aeruginosa M-2. Antitoxin or horse serum was administered at time of infection and gentamicin was given 24 h later.* (□) *No treatment,* (■) *antitoxin,* (○) *gentamicin,* (△) *horse serum and gentamicin,* (●) *antitoxin and gentamicin.*

TABLE 4. *Viable bacteria in tissues from Pseudomonas-infected mice, treated with antitoxin or antitoxin and gentamicin*[a]

Tissue	Time postburn	Treatment: None	Treatment: Antitoxin	Treatment: Antitoxin and gentamicin
Skin	8	4.3[b]	4.3	4.2
	24	7.7	7.4	7.4
	28	6.9	7.3	5.1
	32	7.7	7.6	4.0
Liver	24	4.8	5.0	5.0
	28	6.3	6.4	2.1
	32	6.7	6.9	2.0
Kidney	24	4.5	4.6	4.6
	28	4.3	4.8	1.0
	32	4.6	4.1	1.0
Spleen	24	4.8	4.4	4.4
	28	6.7	5.5	1.0
	32	6.4	4.9	1.0

[a] Mice challenged with 12 viable cells of *P. aeruginosa* M-2; they were given antitoxin at time of infection and gentamicin 24 h later.

[b] Log_{10} of viable bacteria per gram of tissue.

thermore, this experiment suggests that the production of toxin does not promote increased bacterial replication through impairment of host defense mechanisms, at least during the time period examined. In contrast, gentamicin very effectively reduced the number of bacteria in all tissues; highly significant and equivalent decreases were found in the groups treated with antitoxin and gentamicin (Table 4) or with gentamicin alone (data not shown). At 4 h after administration of gentamicin, the bacterial concentrations had fallen 10^2 in skin and 10^4 in the other tissues examined.

The effect of antitoxin and gentamicin therapy on tissue EF-2 levels was also examined. Data in Table 5 show the effect of specific antitoxin treatment on EF-2 content. Burned, infected mice were sacrificed 32 h after infection; mice treated with antitoxin were sacrificed 32 and 46 h after infection. As in previous experiments, the level of active EF-2 in the liver of infected mice was markedly depleted 32 h after infection; in contrast, EF-2 content in all tissues from antitoxin-treated, infected mice was the same as that in the noninfected animals. A single dose of specific antitoxin administered at the time of burning extends the mean time to death of mice infected with *P. aeruginosa* M-2; however, it does not provide full protection. Although the antitoxin-treated mice eventually died, no reduction of EF-2 levels was noted in moribund mice. This suggests that the activity of the toxin produced in vivo can be neutralized by specific antitoxin, but that this does not alter the eventual fatal outcome of the infection. Therefore, it appears that *Pseudomonas* exotoxin is not the sole virulence determinant expressed in fatal *Pseudomonas* infections.

The effect of gentamicin treatment on the EF-2 content of tissues from infected mice was determined (Table 6). In this situation, EF-2 content of the liver was reduced 70% after 42 h in mice treated with gentamicin alone. However, mice receiving both antitoxin and antibiotic possessed normal levels of functional EF-2. Although not shown here, the content of EF-2 in kidney, heart, and spleen was normal in mice receiving gentamicin alone or gentamicin and antitoxin. Treatment with antibiotics, while extending the mean time to death, does not alter the pattern of inhibition of protein synthesis.

Activity of purified *Pseudomonas* toxin. Finally, the consequence of administering purified toxin s.c. to burned animals was examined. Administration of 10 μg of purified toxin s.c. at the burned site resulted in 100% mortality within 48 h. The EF-2 levels in tissues of these mice were examined 38 h after burn and toxin injection (Table 7). Functional EF-2 in the livers of these animals was markedly depleted. Reductions were also seen in kidney and heart tissues. These results are similar to those obtained in normal (nonburned) mice injected i.p. with pur-

TABLE 5. *Effect of antitoxin treatment on EF-2 levels in Pseudomonas-infected mice*[a]

Experimental group	Reduction of EF-2 content (%)	
	32 h[b]	46 h
Burned, infected		
Liver	81	D[c]
Kidney	0	D
Spleen	76	D
Heart	28	D
Antitoxin-treated		
Liver	4	0
Kidney	0	6
Spleen	2	9
Heart	12	23

[a] All mice were burned and challenged with 5×10^3 cells of *P. aeruginosa* M-2. One group received antitoxin at the time of infection.
[b] Time postinfection.
[c] D, Burned, infected animals dead at 34 h.

TABLE 6. *Effect of combined antibiotic-antitoxin treatment on EF-2 levels in livers of mice infected with P. aeruginosa* M-2.

Treatment	EF-2 content (cpm/mg)
Day 2	
None[a]	10.7×10^3
Gentamicin[b]	3.4×10^3
Gentamicin + AT[c]	13.4×10^3
Day 5	
None[a]	10.2×10^3
Gentamicin + AT[c]	11.2×10^3

[a] Mice were burned, not infected.
[b] Mice were burned, infected with 200 cells of *P. aeruginosa* M-2, and given gentamicin 24 h postinfection. All mice in this group were dead by day 4.
[c] Mice were burned, infected and given antitoxin at time of infection and gentamicin 24 h later. Mice survived at least to day 7.

TABLE 7. *Reduction of tissue EF-2 after subcutaneous challenge of burned mice with purified Pseudomonas exotoxin*[a]

Tissue	EF-2 (cpm/mg of protein)		Reduction (%)
	Control	Toxin-treated	
Liver	11.18×10^3	0.18×10^3	98
Kidney	10.57×10^3	4.03×10^3	62
Spleen	12.84×10^3	9.87×10^3	23
Heart	6.44×10^3	2.27×10^3	65

[a] Mice received 10 μg of purified toxin subcutaneously at the burned site and were sacrificed 38 h later.

ified *Pseudomonas* exotoxin (7). Specific antitoxin is protective when given at the time of toxin administration. However, if antitoxin is given to toxin-treated animals 8 h after toxin injection, no protection is afforded (unpublished data).

DISCUSSION

Although *P. aeruginosa* is known to be a frequent complication in debilitated or immunocompromised patients (18), the factors contributing to its virulence have not been established. It has been suggested that *Pseudomonas* exotoxin may be an important virulence factor (13). Bjorn and co-workers (1) found that most strains of *P. aeruginosa* are toxigenic, whereas other species of *Pseudomonas* are not. Production of toxin at the burn site during the course of experimental *Pseudomonas* infections in mice has been documented (24; Stieritz and Holder, in press). Presence of antibody to *Pseudomonas* exotoxin in sera of human patients recovering from *Pseudomonas* infection (22, 23) suggests that toxin is also made in significant amounts in humans.

In biochemical terms, *Pseudomonas* exotoxin is known to inactivate functional mammalian EF-2 (6), which is precisely the same biochemical lesion induced by diphtheria toxin. Saelinger et al. reported a reduction of liver EF-2 in burned mice infected with a toxigenic, protease-producing strain of *P. aeruginosa* (24). In addition, reduction of EF-2 content has been observed in livers of normal mice receiving purified toxin parenterally (7). In the present paper, an analysis of infection with four strains of *P. aeruginosa* which vary with respect to toxin and protease production has illustrated that only the toxigenic strains are capable of inducing the EF-2-related biochemical lesion in the liver. The fact that specific antitoxin blocks EF-2 depletion is further evidence that the effect is a consequence of toxin activity. Histopathology and elevated serum enzyme levels in mice receiving *Pseudomonas* toxin intravenously (21) also suggest that the primary target of toxin action is the liver. The reason for the liver's sensitivity to this toxin is not known. Secondarily, EF-2 depletion results in subsequent inhibition of protein synthesis in the liver. The protein-synthesizing capacity of spleens from infected animals varies, as does the reduction of EF-2 in this organ. Whether these variable results are a consequence of toxin inactivation or are caused by still other extracellular products of *P. aeruginosa* remains speculative.

Experiments with purified *Pseudomonas* toxin injected at the burned site confirm that the enzymatic activity of the toxin measurable in vitro also occurs in specific tissues in vivo. Again, the liver appears most susceptible to purified toxin, but other tissues are involved to a lesser extent. The present experiments also suggest that toxin produced in the skin at the burned site reaches the circulation and is delivered to internal tissues. The same amount of toxin administered s.c. to a burned mouse and a normal, intact mouse is more rapidly lethal for the intact mouse (unpublished data). Poor circulation in burned tissue may result in less adequate adsorption of toxin from burned skin. Other experiments not described here have shown that the LD_{50} of *Pseudomonas* exotoxin administered i.p. to burned mice is not significantly different from that found with intact mice. Therefore, the enhanced virulence of *P. aeruginosa* for burned mice cannot be attributed directly to an increased sensitivity to exotoxin.

Protease production by various *P. aeruginosa* strains also relates to the virulence of the infecting organism. PA103, a toxigenic strain producing low levels of protease, is less virulent in the burned mouse model than toxigenic strains producing higher levels of protease. Injection of purified protease together with PA103 results in a 1,000-fold reduction in LD_{50} values, as cited above. Simultaneous injection of protease and strain PA103 also alters the pattern of depletion of functional EF-2 so that it more closely resembles that seen with toxigenic, high protease-producing strains of *P. aeruginosa*. Protease production at the site of infection may aid in systemic dissemination of the infecting organisms and/or toxin. Alternatively, the role of protease may be additive or synergistic with toxin or still unidentified bacterial products or may be unrelated to toxin production or activity. Our results are in contrast with those of Liu and Hsieh (12), who found that strains of *P. aeruginosa* which produce high levels of protease were relatively avirulent in comparison to strains which produced low levels of protease. One reason for the different results could be the animal models used; Liu challenged normal intact mice i.p. with viable bacteria.

The complete protection afforded *Pseudomonas*-infected mice by treatment with both antitoxin and gentamicin suggests that combined therapy may be useful in clinical situations. Gentamicin therapy significantly reduces the number of bacteria in skin and internal organs of infected animals. Passive protection with specific antitoxin neutralizes the effect of toxin produced in vivo. Neither treatment alone provides total immunity to mice infected with

protease-producing toxigenic *P. aeruginosa* but each does prolong survival. Since specific antitoxin is not fully protective, exotoxin is probably not the sole virulence factor expressed in burned animals. Further evidence for this conclusion is provided by the fact that active immunization with a glutaraldehyde-prepared toxoid resulted in an extension of the mean time to death, but did not provide full protection (unpublished data). In experiments with neutropenic dogs, Kazmierowski et al. (8) showed that combined treatment with gentamicin and type-specific immunoglobulin G antibody resulted in a prolongation of time to death, but did not provide full protection.

Our observations suggest that the pathological sequelae of *P. aeruginosa* infections are the result of several bacterial products, which undoubtedly include *Pseudomonas* exotoxin and one or more proteases. Only recently has the mechanism of one of these virulence factors, exotoxin A, been more clearly defined. Because of the diversity of extracellular products in the *Pseudomonas* armamentarium, it is still premature to assign any one the primary role in pathogenesis of natural infection.

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