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PcrV Immunization Enhances Survival of Burned *Pseudomonas aeruginosa*-Infected Mice

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Burned *Pseudomonas aeruginosa*-infected mice immunized against PcrV, a type III virulence system translocating protein, showed significantly enhanced survival compared to controls. Survival was non-O serotype specific and correlated with a reduced systemic microbial load. Infection with a high-level toxin A-producing strain required supplemental antitoxin treatment to enhance survival.

A recently described set of virulence-associated proteins—the type III system—has been found to be associated with many gram-negative bacteria (6, 7). Type III-mediated intoxication is the product of three functional sets of genes encoding secretion and chaperone proteins, proteins involved in the delivery or translocation of effectors to the cytoplasm of eukaryotic cells, and effector or toxic proteins (6, 7).

Current studies reveal that *Pseudomonas aeruginosa* produces a type III-mediated secretion-intoxication system (3, 13, 14). Translocation of type III effector proteins depends upon a functional secretion-translocation complex (1). The PcrV protein plays a unique role in translocation of the effectors. In a study using PcrV protein immunization in a mouse lung infection model, protection against lethal lung infection, lung injury, and cellular toxicity appeared to be mediated by PcrV antibodies (10). In a burned-mouse infection model, mutants deficient in type III effector protein production remained virulent but a translocating protein-deficient mutant lost its virulence (5), suggesting that the type III system plays a role in *P. aeruginosa* burn infections. Thus, immunization with a purified type III translocating protein, such as the PcrV protein used in the lung infection model, might enhance survival in mice burned and infected with virulent *P. aeruginosa* strains. Results of such immunization studies are presented in this report.

Immunogen and immunization procedures. PcrV was produced as a lipopolysaccharide-free histidine-tagged infusion protein in pET16b and was purified by nickel chromatography as described previously (10). On day 0, groups of 10 female CF-1 mice weighing 22 to 25 g were immunized intramuscularly in the hind leg (10 μg of immunogen in 0.1 ml of incomplete Freund's adjuvant), followed by a booster dose (10 μg in saline) without adjuvant on day 14. On day 21, mice were bled via the retro-orbital sinus and the sera were separated and titered for anti-PcrV antibody (see below). On day 28, mice were burned and challenged with *P. aeruginosa*. Controls were similarly immunized without PcrV protein immunogen. Antisera were titered by standard enzyme-linked immunosorbent assay (10). Mean antibody titers for immunized and control mice are reported as the dilution of serum required to achieve an absorbance reading at 405 nm of 0.1. Antibody titers from 26 mice ranged from 2,000 to 256,000. Titers from nonimmunized controls were 0.

Burned-mouse model and immunization protection studies. The burned-mouse model described in 1975 (12) and modified in 1996 (8) was used. In this model, a nonlethal thermal injury of 15% of the body surface area causes host immunosuppression which reduces the 50% lethal dose of *P. aeruginosa* from $>10^6$ CFU to a 90 to 100% lethal dose of 10^2 to 10^3 CFU. Thus, this model is a very stringent test of treatment materials. Three isolates of *P. aeruginosa,* serotyped with sera from Denka Siekin (Accurate Scientific and Chemical Corp., Westbury, N.Y.), were used. Strain M-2 (O serotype B) was originally isolated from a mouse intestine (12). Strains SBI-N and 1071, O serotypes G and B, respectively, were burn patient isolates. Strain 1071 is a high-level exotoxin A-producing strain, producing 200 times larger amounts of toxin than other burn isolates tested (4). No significant protection occurred in mice infected with strain 1071 (see below); however, immunized mice challenged with strains M-2 and SBI-N showed significantly greater survival at 10 days after burning and infection than did mock-immunized controls (Table 1). This protection occurred despite anti-PcrV titers that were quite varied (2,000 to 256,000). These results suggest that high titers of PcrV antibody are not necessary for significant survival enhancement to occur. The fact that these two strains were of

TABLE 1. Effects of PcrV protein immunization on mortality in burned *P. aeruginosa*-infected mice

Challenge strain	O serotype	Group[a]	No. of dead mice/total no. of mice on day[b]:						
			1	2	3	4	5	6	10
M-2	B	C	0/10	6/10	10/10				
		E	1/10	2/10	2/10	2/10	2/10	2/10	2/10*
SBI-N	G	C	0/10	10/10					
		E	0/10	1/10	2/10	3/10	4/10	4/10	4/10*
1071	B	C	0/10	9/10	10/10				
		E	0/10	2/10	5/10	5/10	5/10	6/10	6/10

[a] C, control; E, experimental.
[b] For the three separate experiments represented here, significant differences in mortality were determined by chi-square analysis at day 10 after burning and challenge. Differences were considered significant at P of <0.05 (*).

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TABLE 2. Quantitative culture data for mice immunized with PcrV and then burned and challenged with *P. aeruginosa* strain M-2

Immunization	Median CFU/g of indicated tissue[a]	
	Eschar	Liver
Freund's adjuvant control	2.62×10^8 (8.96×10^7, 4.72×10^8)	6.52×10^6 (4.77×10^5, 1.46×10^7)
Freund's adjuvant + PcrV	1.12×10^8 (3.05×10^7, 3.03×10^8)	4.07×10^2 (1.03×10^2, 1.05×10^3)*

[a] Data are from six mice; values in parentheses (left to right) are 25th and 75th percentile confidence limits. *, significantly fewer *P. aeruginosa* CFU than in other groups (Mann-Whitney rank sum test [$P = 0.002$]).

different O serotypes indicated that PcrV immunization protection was not O serotype specific.

Quantitative tissue cultures. Additional groups of immunized and mock-immunized controls were burned and challenged with strain M-2. At 24 h after burning and infection, these mice were sacrificed and quantitative bacterial counts of the eschars and livers were performed (Table 2). While the counts in the local, burned, infected sites (eschars) were the same in both groups, a significant reduction in hepatic counts was observed in the immunized groups compared to the mock-immunized controls.

Thus, protection appeared to be related to the ability of the immunized mice to reduce the microbial load. The findings that the numbers of bacteria were the same in eschars of both immunized and control mice but were significantly lower in the livers of immunized mice suggested that the mechanism(s) for microbial load reduction in the immunized mice was a systemic rather than a local (eschar) event.

Effects of PcrV immunization plus antitoxin treatment on burned 1071-infected mice. To determine whether adjunctive antitoxin treatment would further enhance survival in PcrV-immunized mice infected with *P. aeruginosa* strain 1071, groups of immunized and control mice were treated passively (150 μl of antitoxin plus 350 μl of saline administered intraperitoneally immediately after burning and infection) with antiserum to exotoxin A (List Biological Laboratories, Inc., Campbell, Calif.). Antitoxin treatment alone provided no long-term survival advantage compared with survival of the mock-immunized, untreated control group (Table 3). However, it increased the mean time to death. Others have also reported that administration of antitoxin alone to burned *P. aeruginosa*-infected mice increases mean time to death but not long-term survival (2, 9, 11). Our results are concordant with those reports. In 1071-infected mice, only active PcrV immunization plus antitoxin treatment caused a significant increase in survival over mock-immunized mice on day 2; the survival advantage was lost thereafter. However, the combined immunization group maintained a longer mean time to death even compared to mock-immunized plus antitoxin-treated mice and, while not significant, a larger number of survivors remained from day 2 to day 10 in this group than in all other groups. Long-term survival may have improved with additional antitoxin treatment.

TABLE 3. Effects of active PcrV protein immunization plus passive antitoxin A treatment on mortality in burned mice infected with the highly toxigenic *P. aeruginosa* strain 1071

Treatment	No. of dead mice/total no. of mice on day[a]:							
	1	2	3	4	5	8	9	10
Mock (untreated control)	0/10	7/10	8/10	8/10	8/10	8/10	8/10	8/10
Mock + antitoxin	0/10	3/10	5/10	6/10	6/10	6/10	6/10	6/10
PcrV	0/10	1/10	3/10	4/10	5/10	5/10	5/10	5/10
PcrV + antitoxin	0/10	0/10*	1/10	2/10	2/10	4/10	4/10	4/10

[a] Overall significant differences in mortality were determined at day 2, followed by pairwise chi-square comparisons. Because the four groups allowed six possible pairwise comparisons, our required level of significance was adjusted to P of <0.0083 (*) for these comparisons.

It is not surprising that PcrV immunization alone did not provide long-term protection to burned mice infected with the highly toxigenic strain 1071. Previously, it was shown that *P. aeruginosa* hyperimmune-globulin immunotherapy did not reduce mortality in 1071-infected, burned mice, but significant protection occurred with supplemental antiexotoxin A therapy (4).

Conclusions. We found that (i) active immunization using the purified type III translocating protein PcrV induced variable rises in mouse antibody titers, (ii) immunization provided significantly enhanced survival for mice burned and infected with *P. aeruginosa* strains that do not produce large amounts of exotoxin A, (iii) protection appeared to be non-O serotype specific and correlated with decreased systemic microbial load, and (iv) ancillary antitoxin treatment enhanced significant short-term protection and increased mean survival time in immunized mice burned and infected with a high-level exotoxin A-producing strain.

PcrV immunization provides protection both in the burned, immunosuppressed mouse infection model and in the chronic mouse lung model (10); thus, PcrV immunization, with and without supplementary antitoxin treatment, should be investigated further as a means of protecting against a variety of *P. aeruginosa* infections.

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