

Phage Therapy of *Pseudomonas aeruginosa* Infection in a Mouse Burn Wound Model[∇]

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Mice compromised by a burn wound injury and subjected to a fatal infection with *Pseudomonas aeruginosa* were administered a single dose of a *Pseudomonas aeruginosa* phage cocktail consisting of three different *P. aeruginosa* phages by three different routes: the intramuscular (i.m.), subcutaneous (s.c.), or intraperitoneal (i.p.) route. The results of these studies indicated that a single dose of the *P. aeruginosa* phage cocktail could significantly decrease the mortality of thermally injured, *P. aeruginosa*-infected mice (from 6% survival without treatment to 22 to 87% survival with treatment) and that the route of administration was particularly important to the efficacy of the treatment, with the i.p. route providing the most significant (87%) protection. The pharmacokinetics of phage delivery to the blood, spleen, and liver suggested that the phages administered by the i.p. route were delivered at a higher dose, were delivered earlier, and were delivered for a more sustained period of time than the phages administered by the i.m. or s.c. route, which may explain the differences in the efficacies of these three different routes of administration.

Pseudomonas aeruginosa plays a prominent role as an etiological agent of serious infections in patients with burn wounds. Acute burn wounds cause a breach in the protective skin barrier and suppress the immune system, rendering the patients highly susceptible to bacterial infection. *P. aeruginosa* colonization of severe burn wounds and its rapid proliferation within the damaged tissues often lead to disseminated infections, resulting in bacteremia and septic shock (8, 20) and high rates of mortality and morbidity. Treatment of such infections is confounded by the innate and acquired resistance of *P. aeruginosa* to many antimicrobials (8, 15). It has been estimated that at least 50% of all deaths caused by burns are the result of infection (8), and untreatable infections have become a tragically frequent occurrence in patients infected with *P. aeruginosa* (9). Hence, the development of new therapeutic and prophylactic strategies for the control of bacterial infection in patients with burn wounds is needed.

An alternative or supplement to antibiotic therapy, which is currently being reexamined, is the use of bacterial viruses (phage/bacteriophage) to target bacterial infections, i.e., phage therapy (13, 16–18, 22, 29, 30–32). Soothill examined the ability of bacteriophage to prevent the rejection of skin grafts of experimentally infected guinea pigs (27). His findings demonstrated that the phage-treated grafts were protected in six of seven cases, while untreated grafts failed uniformly, suggesting that phage might be useful for the prevention of *P. aeruginosa* infections in patients with burn wounds. However, while multiple studies have demonstrated the benefits of phage therapy

for a variety of bacterial infections in animal model systems (3–7, 10, 14, 19, 23–26, 33–35), little documentation exists with regard to the treatment of burn wound infections (2). In the study described here we used the mouse model of thermal injury (28) to examine the efficacy of phage therapy in abrogating fatal *P. aeruginosa* infections. These studies include the examination of different routes of phage administration.

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MATERIALS AND METHODS

Bacterial strains, bacteriophages, and culture conditions. PAO1^{Rif} is a rifampin-resistant derivative of virulent *P. aeruginosa* strain PAO1 (12), which was kindly provided by Abdul Hamood (11) and which was grown in Luria-Bertani (LB) medium supplemented with rifampin (80 µg/ml), 1 mM MgSO₄, and 1 mM CaCl₂ in a gyratory shaker at 250 rpm at 37°C.

The *P. aeruginosa* phages were plaque-purified subcultures of phages that had been purchased from the American Type Culture Collection (Catalogue of bacteria and bacteriophages, 18th ed., 1992; ATCC, Manassas, VA). Monophage preparations were propagated on their respective hosts growing in LB medium at 37°C in a gyratory shaking water bath at 250 rpm. Phage lysates were centrifuged (5,000 × g for 15 min) to remove cellular debris, filter sterilized (pore size, 0.22 µm; Millipore), and stored over a drop of chloroform at 4°C in amber bottles. The phage preparations to be used therapeutically were passed through a column containing Detoxi-endotoxin removing gel (Pierce, Rockford, IL), as recommended by the manufacturer, and eluted with pyrogen-free water. The eluted phages were diluted to the appropriate titer with filter-sterilized phosphate-buffered saline (PBS; pH 7.2) prior to administration to the mice. Phage titers were determined by serial dilution and plaque assays by the soft overlay technique (1). This entailed the addition of 100 µl of the different phage dilutions to 100 µl of an overnight culture of their host strain. The mixture was then allowed to stand at room temperature for 5 min for phage adsorption, after which 3 ml of soft agar (0.7% LB agar maintained at 48°C) was added and the mixture was poured over an LB agar plate. The soft agar overlay was allowed to solidify, the plates were incubated overnight at 37°C, and the plaques were counted to determine the phage titer.

Selection of therapeutic phages. To select for the phages to be used in our phage cocktail, we used two criteria: virulence and host range (i.e., utilization of different phage receptors). Of our 13 ATCC phages, 7 were able to grow on PAO1^{Rif}. Of those, two formed hazy plaques, suggesting that they may be lysogenic. To determine the relative virulence of the remaining five phages, we developed an in vitro virulence test in which we determined the MIC required to

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TABLE 1. Protection studies: efficacy of phage therapy on *P. aeruginosa* infection of burn wounds

Treatment	No. of survivors/total no. of mice (% survival) ^a							
	Expt 1		Expt 2		Expt 3		Combined expt ^b	
	48 hpi	72 hpi	48 hpi	72 hpi	48 hpi	72 hpi	48 hpi	72 hpi
PAO1 only	0/6 (0)	0/6 (0)	1/6 (16.7)	0/6 (0)	2/6 (33.3)	1/6 (16.7)	3/18 (17)	1/18 (6)
Phage by i.p. route only	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	18/18 (100)	18/18 (100)
Phage by i.m. route only	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	18/18 (100)	18/18 (100)
Phage by s.c. route only	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	6/6 (100)	18/18 (100)	18/18 (100)
Phage by i.p. route + PAO1 infection	6/6 (100)	5/6 (83.3)	6/6 (100)	6/6 (100)	5/5 (100)	4/5 (80)	17/17 (100) ^c	15/17 (88) ^c
Phage by i.m. route + PAO1 infection	3/6 (50)	2/6 (33.3)	3/6 (50)	0/6 (0)	4/6 (66.7)	3/6 (50)	10/18 (56) ^d	5/18 (28)
Phage by s.c. route + PAO1 infection	2/6 (33.3)	2/6 (33.3)	3/6 (50)	0/6 (0)	5/6 (83.3)	2/6 (33.3)	10/18 (56) ^d	4/18 (22)

^a The cumulative survival was determined at 48 h and 72 hpi.

^b The average percent survival of animals in three experiments determined at 48 and 72 hpi.

^c The rate of survival for animals receiving the phage cocktail i.p. was significantly greater ($P < 0.0001$) than that for untreated infected control animals at the same time period.

^d The rate of survival for animals receiving the phage cocktail i.p. was significantly greater ($P < 0.01$) than that for untreated infected control animals at the same time period.

^e The rate of survival for animals receiving the phage cocktail i.p. was significantly greater ($P < 0.0026$) than that for untreated infected control animals at the same time period.

clear (lyse) an exponentially growing culture of PAO1^{Rif} (~10⁷/ml) over a given period of time (5 h). Those with the lowest MICs were considered the most virulent. We realize, however, that we are measuring only one virulence parameter and that in vivo and in vitro virulence may not be equivalent and may be determined by other phenotypic traits (6, 21).

Resistance to phage often arises through bacterial mutations that alter receptors on the bacterial surface to which the phage binds (phage receptors). In an attempt to decrease the likelihood of the emergence of phage-resistant PAO1^{Rif} strains, we selected phages that utilized different phage receptors for infection. To identify such phages we isolated PAO1^{Rif} mutants resistant to each of the five different phages being analyzed and determined the sensitivities of these mutants to the other phages being analyzed. We assumed that a phage to which a PAO1^{Rif} phage-resistant mutant was sensitive did not share a common receptor with the phage to which the mutant was resistant.

Based on our virulence and host range (receptor usage) tests, we selected three phages for use in this study. The phage cocktails used in this study contained approximately 10⁸ PFU/100 µl inoculum of each of the following phages: Pa1 (ATCC 12175-B1); Pa2 (ATCC 14203-B1), and Pa11 (ATCC 14205-B1) (ATCC catalogue of bacteria and bacteriophages, 18th ed., 1992).

Animals. Adult female ND4 Swiss Webster mice (weight, 20 to 24 g) were used for this study. The animals were anesthetized with 0.4 ml of 5% sodium pentobarbital by intraperitoneal (i.p.) injection. The mice were housed in the Texas Tech University Health Sciences Center Vivarium. The animals were treated in accordance with protocol no. 96020-06, approved by the Animal Care and Use Committee at Texas Tech University Health Sciences Center in Lubbock.

Thermal injury model. The mouse model of thermal injury of Stieritz and Holder (28), as modified by Hamood (11), was used in this study. Briefly, the hair was clipped from the backs of anesthetized mice, and the area was denuded with a commercially available hair remover. The mice were securely placed into a template with an opening of 4.5 cm by 1.8 cm to expose their shaved backs. A nonlethal full-thickness thermal injury to the skin was induced by placing the exposed back area to 90°C water for 10 s. Fluid replacement therapy consisting of a subcutaneous (s.c.) injection of 0.8 ml of a 9% NaCl solution was administered immediately following the burn. The mice were challenged by s.c. injection of 100 µl of the PAO1^{Rif} inoculum (2×10^2 to 3×10^2 CFU) directly under the anterior end of the burn. The phage cocktail was administered immediately after the *P. aeruginosa* challenge.

During recovery from anesthesia, the mice were kept under warming lights and observation. The cumulative mortality among the treatment groups was recorded at 48 and 72 h postinfection (hpi). Lethargic animals were monitored every hour. The surviving animals were killed at 96 hpi. Liver and spleen tissue samples were removed from the animals, weighed, suspended in 2 ml of filter-sterilized PBS (pH 7.2), and homogenized with sterile mortars and motor-driven Teflon pestles. The numbers of PAO1^{Rif} cells (CFU/g tissue) were determined by serial dilution and plating on LB agar containing 80 mg/ml of rifampin. The titers (PFU/g tissue) of the phages recovered from the tissues of mice were determined by the soft agar overlay technique described above, following the addition of a few drops of chloroform (per ml).

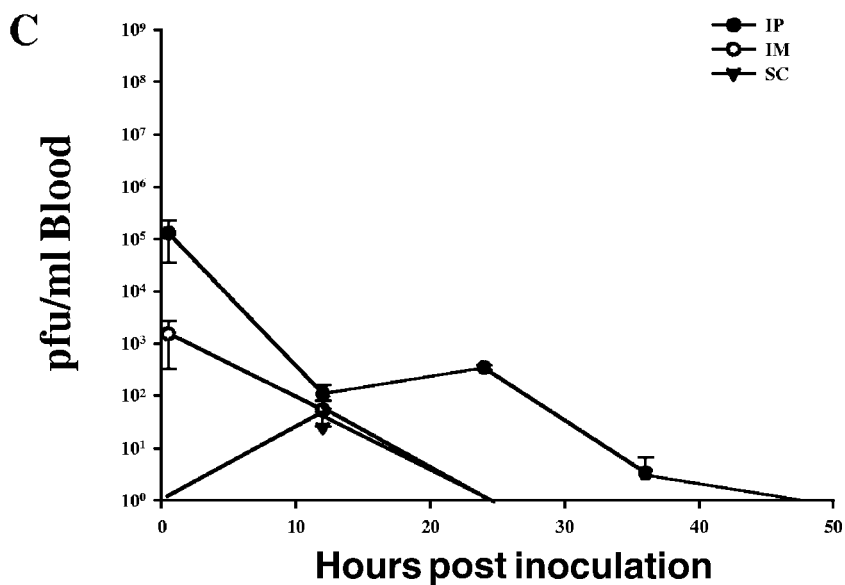
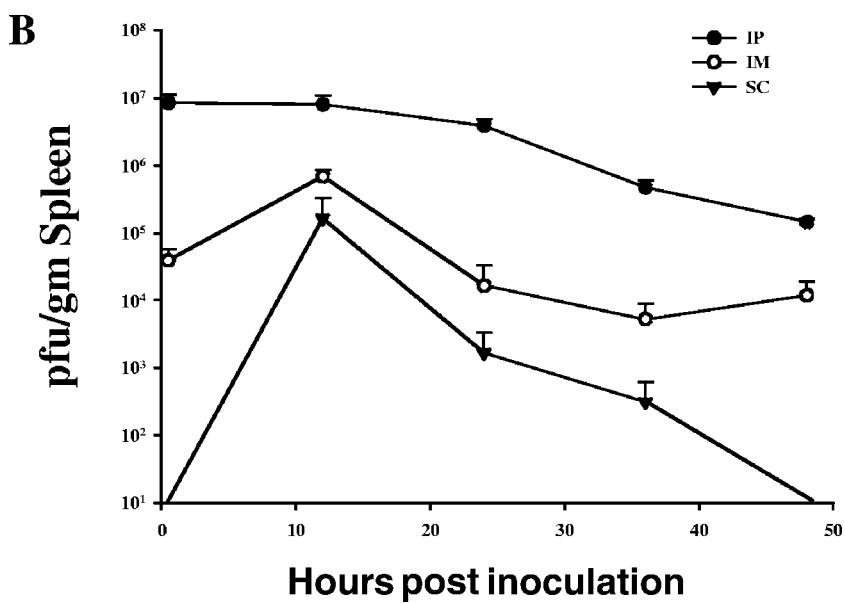
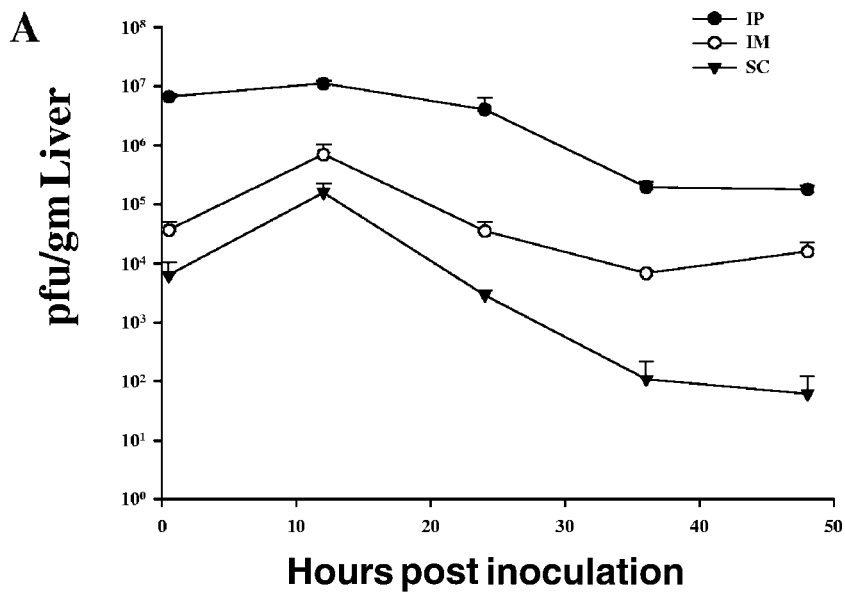
Pharmacokinetic studies. A phage cocktail inoculum (~3 × 10⁸/100 µl per inoculum) was administered to unwounded, noninfected mice i.p., intramuscularly (i.m.), or s.c. Three animals from each treatment group were killed at 0.5, 12, 24, 36, and 48 h following phage injection. Briefly, blood, obtained by cardiac puncture from anesthetized animals, was collected in tubes containing EDTA. After blood collection, the anesthetized mice were euthanized by injection of Fatal Plus (sodium pentobarbital), followed by cervical dislocation. Liver and spleen tissue samples were removed from the animals. The tissues were weighed, suspended in 2 ml of filter-sterilized PBS, and homogenized with sterile mortars and motor-driven Teflon pestles. The phages in the tissues were enumerated as described above and are expressed as PFU/gram of tissue.

Statistical analysis. Fisher's exact test (Statview for Windows; SAS Institute, Cary, NC) was used to determine the significance of the differences in survival among the treatment groups and the differences in the distributions of the phages to the tissues.

RESULTS

Protection studies. The ability of the *P. aeruginosa*-specific phages to prevent *P. aeruginosa* infections was examined by use of a modified mouse model of thermal injury, as described above. A phage cocktail containing 1 × 10⁸ PFU of each of three different phages (3.0 × 10⁸ PFU total) was administered i.p., i.m., or s.c. to infected and uninfected wounded animals. As a control for the virulence of the PAO1^{Rif} inoculum, another group of mice was injected with the bacterial inoculum only (no phage). To examine the toxicity of the phages in compromised animals, the wounded but noninfected groups were injected with the phage cocktail (but not *P. aeruginosa*). Animal deaths were recorded at 48 and 72 hpi.

The results of these experiments are presented in Table 1. All of the thermally injured mice that were not infected with PAO1^{Rif} but administered the phage cocktail survived, indicating that the phage cocktail was not toxic to traumatized mice. In the absence of phage there was a 94% rate of mortality in the wounded infected mice in the first 72 hpi. When the phages were administered i.m. or s.c., the rates of mortality were reduced to 72% and 78%, respectively; and by sharp contrast the rate of mortality was reduced to 12% when the phages were delivered i.p. These results demonstrate that the parenterally administered phages significantly increased survival in infected and wounded mice and that the relative pro-



tection afforded by the different routes of phage administration was i.p. > i.m. or s.c.

Of the wounded and infected animals receiving the phages i.p., only 2 of the 17 animals had died by 72 hpi (at 53 hpi and 64 hpi, respectively). The surviving animals were killed at 96 hpi, and the numbers of PAO1^{Rif} organisms detected from the tissues of surviving animals was compared to the numbers from the tissues of animals that died from the *P. aeruginosa* infection. The mean bacterial counts in the tissues of animals that died were 1.53×10^9 CFU/g liver and 6.68×10^7 CFU/g spleen. The mean bacterial counts in the tissues following successful i.p. phage therapy were 5.26×10^2 CFU/gram liver and 2.93×10^2 CFU/gram spleen. These results suggest that the cause of death was the result of systemic *P. aeruginosa* infection and that, as one might expect, successful phage therapy correlates with a reduction in the PAO1^{Rif} burden.

To determine if phage-resistant derivatives of PAO1^{Rif} emerged from the infected mice, we analyzed the PAO1^{Rif} isolates from the tissues of those mice that had died by 48 hpi for their phage sensitivities. All isolates tested (>100) were sensitive to each of the phages in the cocktail (data not shown). Hence, the death of these animals was not the result of the emergence of a phage-resistant derivative of the PAO1^{Rif} strain. The numbers of phages in the liver and spleen of animals that succumbed to *P. aeruginosa* infection in the first 48 hpi were also enumerated to determine if the phages had multiplied. Considering the average weight of these organs (4.6 g and 2.27 g of liver and spleen, respectively) and assuming 100% phage recovery, each mouse harbored a minimum of 7.5×10^9 to 10×10^9 phages. This represents an increase of approximately 20-fold over that administered to these mice ($\sim 3 \times 10^8$ /mouse), indicating that the phages multiplied in vivo, although obviously not to levels that were enough to save the animal.

Pharmacokinetic studies. In an attempt to determine why delivery of the phages by the i.p. route was more efficacious than delivery of the phages by the i.m. or the s.c. route for the treatment of infected animals, we examined the pharmacokinetics of the phages introduced by the i.m., s.c., or i.p. route in uninjured, uninfected animals. Three animals each from groups receiving the phage cocktails i.p., i.m., or s.c. were killed at 0.5, 12, 24, 36, and 48 hpi. The numbers of phages detected per gram of liver and spleen and per milliliter of blood are shown in Fig. 1. In each tissue examined, a consistent pattern of the relative PFU levels after the administration of the phages by the different routes was observed: i.p. > i.m. > s.c.

DISCUSSION

In the mouse model of thermal injury, 2×10^2 to 3×10^2 PAO1^{Rif} injected at the burn site resulted in 83 to 100% mortality by 48 hpi. Rumbaugh et al. have shown that the *P. aeruginosa* organisms in such an infection proliferate and

spread systemically from skin to underlying tissues and that within 24 hpi as many as 10^4 PAO1^{Rif} CFU per gram of tissue was detected in the liver and spleen (21). In this study we have demonstrated that a single dose of a phage cocktail can effectively decrease the rate of mortality due to *P. aeruginosa* infection of burn wounds in the mouse model of thermal injury. This protection was shown to be the result of a significant decrease in the numbers of *P. aeruginosa* organisms found in the successfully treated animals, indicating that the bacterial viruses used were able to locate and kill PAO1^{Rif} before the animal succumbed to bacteremia and septic shock. However, it was also found that not all infected animals which were treated with the phages survived and that the route of phage administration was particularly important to the efficacy of the treatment, with the i.p. route providing the most significant protection (87%) of the routes tested (Table 1).

It was also found that the *P. aeruginosa* phages had multiplied in mice that had died of infection and that phage-resistant *P. aeruginosa* strains were not recovered from these animals. These results suggest that the use of a phage cocktail containing phages that use different receptors may have prevented the emergence of phage-resistant mutants and that the therapeutic phages had found their host (PAO1^{Rif}) and multiplied, but apparently not in sufficient time and/or in sufficient numbers to prevent mortality. Hence, the differences in the efficacies of the different routes of phage administration may be due to the rate and dose of phage delivery to their targets. This explanation is somewhat supported by the observation that the *P. aeruginosa* phages administered by the i.p. route were delivered at a higher dose, were delivered earlier, and were delivered for a more sustained period of time to the examined tissues of a mouse (Fig. 1) than the phages delivered by the s.c. or i.m. route.

It should finally be pointed out that the mouse model of thermal injury is a very stringent test of phage therapy for a systemic infection. Highly virulent PAO1^{Rif} cells injected s.c. beneath the burn wound, allowing the organisms to proliferate very quickly and spread systemically to cause septic shock and death in a relatively short period of time (24 to 48 h). By comparison, the rates of proliferation and systemic dissemination from natural infections acquired at burn wound surfaces are usually much slower. Furthermore, in most human burn wounds, infection occurs after admission to a hospital or burn ward and is often caused by a hospital-associated strain. Theoretically, such infections could be more conducive to phage therapy/prophylactics than would *P. aeruginosa* infections in the animal model tested here because of the chronic nature of the infection. On the other hand, some chronic wounds are populated by biofilms, which may be more difficult to treat with phage and/or may require different types of therapeutic phage than that used for the treatment of systemic infections. Chronic infection may require prolonged treatment with phage, which may in turn select for phage resistance and in-

FIG. 1. Pharmacokinetics of the phages in noninfected mice. A cocktail containing $\sim 3 \times 10^8$ phage was injected i.p., i.m., or s.c. into uninjured, uninfected mice. Groups of mice ($n = 3$) were killed at 0.5 h hpi, 12 hpi, 24 hpi, 36 hpi, or 48 hpi. Tissues were removed and the numbers of PFU were determined by serial dilution and plating on PAO1^{Rif}. (A) PFU/gram liver; (B) PFU/gram spleen; (C) PFU/ml blood. Values are means \pm standard errors of the means.

duce an immune response, which could reduce the therapeutic value of phage treatment, although phages, unlike antibiotics, evolve with their host(s). Obviously, more detailed studies examining the effect of the phage dosage, the routes and timing of phage administration, the pharmacokinetics and tissue tropism of the phages used, as well as determination of the phenotypic traits of the most effective therapeutic phages for particular types of infections will be needed to determine if phage therapy will provide a much needed alternative/supplement for the treatment of bacterial infections. However, with that said, recent, well-controlled animal studies, which have successfully applied phage therapy to multiple types of bacterial infections, have spawned new enthusiasm for an old idea (3–7, 10, 14, 19, 23–26, 33–35), and the FDA has recently approved phase I trials for the use of phage to treat bacterial infections of diabetic foot ulcers (R. Wolcott personal communication and <http://sanjose.bizjournals.com/sanjose/stories/2007/01/08/story4.html?t=printable>).

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