AAC Accepts, published online ahead of print on 26 March 2007
Antimicrob. Agents Chemother. doi:10.1128/AAC.01028-06

Title: Phage therapy of *Pseudomonas aeruginosa* infection in the burn mouse model.

Running title: Phage Therapy of burn wounds

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ABSTRACT

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

Introduction

Pseudomonas aeruginosa plays a prominent role as an etiological agent of serious infections in burn patients. 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 rapid proliferation within the damaged tissues often leads 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 burn patients is needed.

An alternative or supplement to antibiotic therapy, which is currently being re-examined, is the use of bacterial viruses (phage/bacteriophage) to target bacterial infections, i.e. phage therapy (13, 16-18, 22, 27, 29-31). 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 out of seven cases, while untreated grafts failed uniformly, suggesting that phage might be useful in preventing *P. aeruginosa* infections in 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, 14, 19, 23-26, 32-34), little documentation exists with regards to treatment of burn-wound infections (2). In the following study we have employed the thermally injured mouse model (28) to examine the efficacy of

phage therapy in abrogating fatal *P. aeruginosa* infections. These studies include examining different routes of phage administration.

Methods and Materials

Bacterial strains, Bacteriophages and culture conditions.

PAO1^{Rif} is a rifampicin-resistant derivative of a virulent *P. aeruginosa* strain PAO1 (11) kindly provided by Abdul Hamood (12) and was grown in Luria-Bertani (LB) media supplemented with rifampicin (80ug/ml), 1mM $MgSO_4$ and 1mM $CaCl_2$, in a gyratory shaker at 250 rpm at 37^{o}C.

P. aeruginosa (Pa) phages were plaque-purified subcultures of phages that had been purchased from the American Type Culture Collection (ATCC catalogue, "Bacteria and Bacteriophages 18th edition") (ATCC, Manassas, VA). Monophage preparations were propagated on their respective hosts growing in LB media at 37^{o}C in a gyratory shaking water bath at 250 rpm. Phage lysates were centrifuged (5000 X g for 15 minutes) to remove cellular debris, filter sterilized (Millipore, MA, 0.22 μm pore size) and stored over a drop of chloroform at 4^{o}C in amber bottles. Phage preparations to be used therapeutically were passed through a column containing Detoxi- Endotoxin Removing Gel (Pierce, Rockford, IL) as recommended by the manufacturer (Pierce instructions http://www.piercenet.com) and eluted with pyrogen-free water. The eluted phages were diluted to the appropriate titer with filter sterilized phosphate-buffered saline (pH 7.2) prior to administration to the mice. Phage titers were determined by serial dilution and plaque assays employing the soft overlay technique (1). This entailed adding 100μl of the different phage dilutions to 100 μl of an overnight culture of their host strain, allowing the mixture to stand at room temperature for 5 minutes for phage adsorption, after

which 3ml of soft agar (0.7% LB agar maintained at 48^{o}C) was added and the mixture poured over an LB plate. The soft agar overlay was allowed to solidify and the plates were incubated overnight at 37^{o}C and the plaques 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 PA01^{Rif}. Of those, two formed hazy plaques, suggesting that they may be lysogenic. To determine relative virulence of the remaining 5 phage we developed an *in vitro* virulence test in which we determined the MIC required to clear (lyse) an exponentially growing culture of PAO1^{Rif} (~10^{7}/ml) over a given period of time (5 hours). Those with the lowest MIC 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 their surface to which phage bind (phage receptors). In an attempt to decrease the likelihood of the emergence of phage-resistant PA01^{Rif} strains, we selected phages that utilized different phage receptors for infection. To identify such phages we isolated phage-resistant PA01^{Rif} mutants to each of the 5 different phages being analyzed and determined the sensitivity of these mutants to the other phages being analyzed. We assumed that a phage to which a PA01^{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 employed in this study contained approximately 10^8 plaque forming units (pfu)/100µl inocula of each of the following phage: Pa1 (Phage ATCC No. 12175-B1); Pa2 (Phage ATCC No. 14203-B1) and Pa11 (Phage ATCC No. 14205-B1) (Catalogue of Bacteria & Bacteriophages, 18th Ed. 1992).

Animals.

Adult female ND4 Swiss Webster mice (weighing between 20-24 grams) were used for this study. Animals were anesthetized with 0.4 ml of 5% sodium pentobarbital by intraperitoneal injection. The mice were housed in the Texas Tech University Health Sciences Center (TTUHSC) Vivarium. 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, Texas.

Thermal injury model.

The thermal injury mouse model of Stieritz and Holder (30) as modified by Hamood (12) was employed in this study. Briefly, hair was clipped from the backs of anesthetized mice, and the area denuded with a commercially available hair remover. The mice were securely placed into a template with a 4.5 cm by 1.8 cm opening to expose their shaved backs. A non-lethal 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 was immediately administered with a subcutaneous injection of 0.8 ml of 9% NaCl solution immediately following the burn. Mice were challenged by subcutaneous injections of 100 µl of the $PA01^{Rif}$ inoculum, 2-3 × 10^2 colony-forming units (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. Cumulative mortality among treatment groups was recorded at 48 and 72 hr. post infection (hpi). Lethargic animals were followed every hour. Surviving animals were sacrificed at 96 hpi. Liver and spleen tissue samples were removed from the animals, weighed, suspended in 2 ml of filter sterilized phosphate-buffered saline (PBS) (pH 7.2) and homogenized using sterile mortars and motor-driven Teflon pestles. The numbers of $PAO1^{Rif}$ (cfu/gm tissue) were determined by serial dilution and plating on LB agar containing 80 mg/ml of rifampicin. Phage titers (pfu/gm tissue) recovered from the tissue of mice were determined as described above following the addition of a few drops of chloroform (per ml) using the soft agar overlay technique.

Pharmacokinetic studies.

A phage cocktail inoculate (~ 3×10^8/100 µl per inoculum) was administered to unwounded, non-infected mice intraperitoneally (IP), intramuscularly (IM) or subcutaneously (SC). Three animals from each treatment group were sacrificed at 0.5, 12, 24, 36, and 48 hr. following phage injection. Briefly, blood, obtained by cardiac puncture from anesthetized animals, was collected in tubes containing Ethylene diamine tetra-acetate (EDTA). After blood collection, anesthetized mice were euthanized by Fatal Plus® injection, followed by cervical dislocation. Liver and spleen tissue samples were removed from the animals. Tissues were weighed, suspended in 2 ml of filter sterilized PBS and homogenized using sterile mortars and

motor-driven Teflon pestles. Phage in tissues were enumerated as described above and expressed as pfu/gram of tissue.

Statistical analysis.

Fisher's exact test (Statview for Windows, SAS Institute, Cary, NC) was used to determine significant differences in survival among treatment groups and differences in distribution of phage to tissues.

Results.

Protection studies. The ability of *P. aeruginosa*-specific phage to prevent *P. aeruginosa* infections was examined in the modified thermal injury mouse model as described above. A phage cocktail containing 1 X 10^8 pfu of each of 3 different phages (3.0 X 10^8 pfu total) was administered IP, IM or SC to infected and uninfected wounded animals. As a control for virulence of the PAO1^{Rif} inoculum, another group of mice were injected with the bacterial inoculum only (no phage). To examine the toxicity of the phage in compromised animals, wounded but non-infected groups were injected with the phage cocktail (no *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 phage, survived, indicating that the phage cocktail was not toxic to traumatized mice. In the absence of phage there was a 94% mortality in the wounded infected mice in the first 72 hpi. When phage were administered IM or SC, mortality was reduced to 72% and 78% respectively and by sharp contrast, mortality was reduced to 12% when phage were delivered IP. These results demonstrate that parenterally

administered phage significantly increased survival in infected and wounded mice and that the relative protection afforded by differing routes of phage administration was IP>IM or SC.

Of the wounded and infected animals receiving phage IP, only two of the 17 animals had died by 72 hpi. (53hpi and 64hpi). The surviving animals were sacrificed at 96 hpi. and a comparison of numbers of PAO1^{Rif} detected from tissues of surviving animals to those of that died from *P. aeruginosa* infection. The mean bacterial counts in the tissues of animals which died were 1.53 X 10^9 cfu/gm liver and 6.68 X 10^7 cfu/gram spleen. The mean bacterial counts in the tissues following successful IP phage therapy were 5.26 X 10^2 cfu/gram liver and 2.93 X 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 PAO1^{Rif} burden.

To determine if phage-resistant derivatives of PA01^{Rif} emerged from the infected mice, we analyzed the PA01^{Rif} isolates from the tissues of those mice that had died by 48hpi for phage sensitivities. All tested isolates (>100) were sensitive to each of the phage of 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 phage in the liver and spleen of animals that succumbed to *P. aeruginosa* infection in the first 48hpi were also enumerated to determine if the phage had multiplied. Considering the average weight of these organs (4.6 gm and 2.27 gm of liver and spleen, respectively) and assuming a 100% phage recovery, each mouse harbored a minimum of 7.5-10 X 10^9 phage. This represents an increase of approximately 20 fold over that administered to these mice (~ 3 X10^8/mouse), indicating that phage multiplied *in vivo*, although obviously not enough to save the animal.

Pharmacokinetic studies. In an attempt to determine why phage delivered via IP were more efficacious than those delivered IM or SC in treating infected animals, we examined the pharmacokinetics of phage introduced via IM, SC or IP route in uninjured, uninfected animals. Three animals each from groups receiving phage cocktails IP, IM, or SC were sacrificed at 0.5, 12, 24, 36 and 48hpi. The numbers of phage detected per gm of liver and spleen and per ml of blood are shown in Figure 1. In each tissue examined a consistent pattern of relative pfu levels of IP>IM>SC, was observed.

Discussion

In the thermally injured mouse model, 2-3 X 10^2 PA01^{Rif} injected at the burn site results in 83-100% mortality by 48 hours post infection. Rumbaugh et. al. have shown that the *P. aeruginosa* 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 mortality due to *P. aeruginosa* infection of burn wounds in the thermally injured mouse model. This protection was shown to be the result of a significant decrease in *P. aeruginosa* found in the successfully treated animals, indicating that the bacterial viruses employed were able to locate and kill PA01^{Rif} before the animal succumbed to bacteremia and septic shock. However, it was also found that not all infected animals which were treated with phage survived and that the route of phage administration was particularly important to the efficacy of the treatment, with the IP route providing the most significant (87%) protection of the routes tested (see table 1).

223 It was also found that the Pa phage had multiplied in mice that had died of infection and
224 that phage-resistant *P. aeruginosa* strains were not recovered from these animals. These results
225 suggest that the use of a phage cocktail, containing phage that utilize different receptors, may
226 have prevented the emergence of phage-resistant mutants and the therapeutic phage had found
227 their host ($PA01^{Rif}$) and multiplied, but apparently not in sufficient time and/or numbers to
228 prevent mortality. Hence, the differences in the efficacy of the different routes of phage
229 administration may be due to the rate and dose of phage delivery to their targets. This
230 explanation is somewhat supported by the observation that Pa phage administered by the IP route
231 were delivered at a higher dose, earlier and for a more sustained period of time to the examined
232 tissues of a mouse (figure 1) than were phage delivered by the SC or IM routes.
233 It should finally be pointed out that the thermally injured mouse model is a very stringent
234 test of phage therapy for a systemic infection. Highly virulent $PAO1^{Rif}$ were injected
235 subcutaneously beneath the burn wound, allowing organisms to proliferate very quickly and
236 spread systemically to cause septic shock and death in a relatively short period of time (24-48
237 hrs). By comparison, proliferation and systemic dissemination from natural infections acquired at
238 burn wound surfaces are usually much slower. Furthermore, in most human burn wounds,
239 infection occurs after admission to a hospital or burn ward and often by a hospital associated
240 strain. Theoretically, such infections could be more conducive to phage therapy/prophylactics
241 than would *P. aeruginosa* infections in the animal model tested here because of the chronic
242 nature of the infection. On the other hand, some chronic wounds are populated by biofilms,
243 which may be more difficult to treat with phage, and/or require different types of therapeutic
244 phage than those used for systemic infections. Chronic infection may require prolonged
245 treatment with phages, which may in turn select for phage resistance and induce 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 phage dosage, routes and timing of phage administration, the pharmacokinetics and tissue tropism of employed phage as well as the determination of phenotypic traits of the most effective therapeutic phage 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, 14, 19, 23-26, 32-34) 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/o1/08/story4.html?t=printable)

Acknowledgments.

The authors wish to thank Dr.s Abdul Hamood and Kendra Rumbaugh for their moral and technical support and Dr. John Griswold for providing space and equipment to accomplish thermal injuries and Ben Burrows and Dr. Probhjit Chadha-Mohanty for the critical reading of this manuscript. This work was supported in part by a Texas Tech University School of Medicine Seed Grant (JAF) and from the Texas Tech University Biology department (MV) and the Texas Tech University Graduate School (MV) and comprised part of Marisela Velasquez's requirements for the Masters of Science degree.

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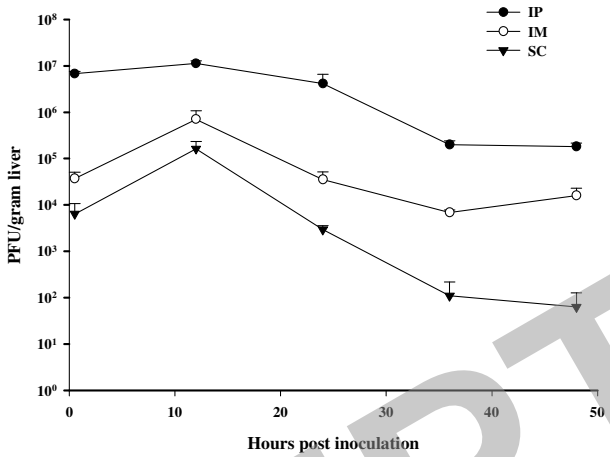
FIGURE LEGENDS

FIG. 1. Pharmacokinetics of phage in non-infected mice. A cocktail containing ~3 $X10^8$ phage was injected intraperitoneally (IP), intramuscularly (IM) or subcutaneously (SC) into uninjured, uninfected mice. Groups of mice (n=3) were sacrificed at 0.5 hours post inoculation (hpi), 12 hpi, 24 hpi, 36 hpi, or 48 hpi. Tissues were removed and numbers of pfu determined by serial dilution and plating on $PAO1^{Rif}$. (A) pfu/gram liver. (B) pfu/gram spleen. (C) pfu/ml blood. Values are means ± standard error of the mean

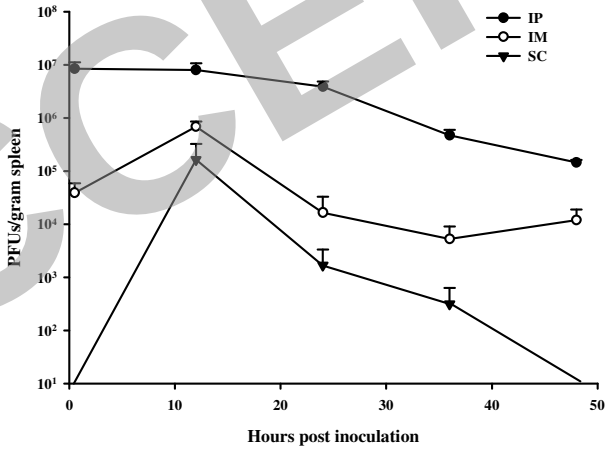
379 Figure #1. Pharmacokinetics of phage introduced IP, IM, or SC to unwounded uninfected mice.
380
381 A. Liver

pfu/gm



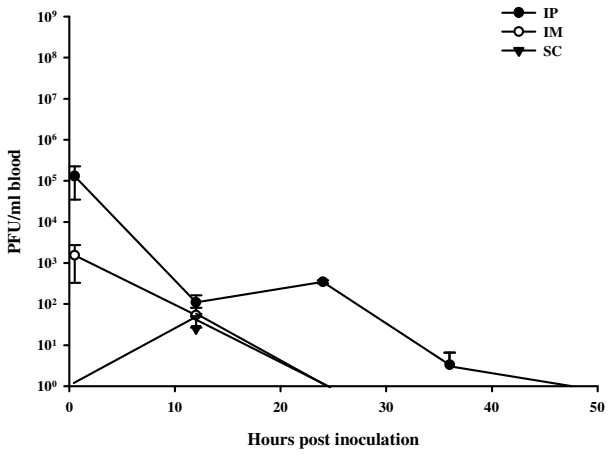
382
383 B. Spleen

pfu/gm



384
385 C. Blood

pfu/ml



386

387

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

Treatment	Number of survivors/total mice (% survival)[1]							
	Experiment 1		Experiment 2		Experiment 3		Combined Experiments[2]	
	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 IP 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 IM 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 SC 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 IP + PAO1	6/6 (100)	5/6 (83.3)	6/6 (100)	6/6 (100)	5/5 (100)	4/5 (80)	17/17 (100)[3]	15/17 (88)[5]
Phage IM + PAO1	3/6 (50)	2/6 (33.3)	3/6 (50)	0/6 (0)	4/6 (66.7)	3/6 (50)	10/18 (56)[4]	5/18 (28)
Phage SC + PAO1	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)[4]	4/18 (22)

389

390 [1] The cumulative survival was determined at 48 hours and 72 hours post infection.

391 [2] The average percent survival of animals in three experiments determined at 48 and 72 hours post infection.

392 [3-5] Survival of animals receiving phage cocktail IP significantly greater than untreated infected controls at same time period.

393 [3] $p<0.0001$, [4] $p=0.01$, [5] $p=0.0026$.