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Evidence to Support the Therapeutic Potential of Bacteriophage Kpn5 in Burn Wound Infection Caused by *Klebsiella pneumoniae* in BALB/c Mice

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The emergence of antibiotic-resistant bacterial strains is one of the most critical problems of modern medicine. Bacteriophages have been suggested as an alternative therapeutic agent for such bacterial infections. In the present study, we examined the therapeutic potential of phage Kpn5 in the treatment of *Klebsiella pneumoniae* B5055-induced burn wound infection in a mouse model. An experimental model of contact burn wound infection was established in mice employing *K. pneumoniae* B5055 to assess the efficacy of phage Kpn5 *in vivo*. Survival and stability of phage Kpn5 were evaluated in mice and the maximum phage count in various organs was obtained at 6 h and persisted until 36 h. The Kpn5 phage was found to be effective in the treatment of *Klebsiella*-induced burn wound infection in mice when phage was administered immediately after bacterial challange. Even when treatment was delayed up to 18 h post infection, when all animals were moribund, approximately 26.66% of the mice could be rescued by a single injection of this phage preparation. The ability of this phage to protect bacteremic mice was demonstrated to be due to the functional capabilities of the phage and not due to a nonspecific immune effect. The levels of pro-inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-10) were significantly lower in sera and lungs of phage-treated mice than phage untreated control mice. The results of the present study bring out the potential of bacteriophage therapy as an alternate preventive approach to treat *K. pneumoniae* B5055-induced burn wound infections. This approach not only helps in the clearance of bacteria from the host but also protects against the ensuing inflammatory damage due to the exaggerated response seen in any infectious process.

Keywords: Bacteriophage, burn wound infection, *Klebsiella pneumoniae*, inflammatory cytokines

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Burns provide a suitable site for bacterial multiplication and are a persistent source of infection than surgical wounds, mainly because of the larger area involved and longer duration of patient stay in the hospital [9, 22, 23]. Acute burn wounds cause a breach in the protective skin barrier and suppress the immune system, rendering the patients highly susceptible to colonization by opportunistic organisms of exogenous and endogenous origins [33]. Skin burns are highly susceptible to microbial infections, primarily by bacteria. As a result, microbial infections remain a leading cause of death among patients who are hospitalized for burns [12, 21]. *K. pneumoniae* is an opportunistic pathogen and mainly attacks immunocompromised individuals. It is the leading pathogen in Gram-negative bacillary invasive burn wound infections [24].

The rising prevalence of antibiotic resistance in burn wound bacterial pathogens represents a serious therapeutic challenge for clinicians attending to burn patients. At the same time, the pace of development of new antibiotics has been inadequate, resulting in a shortage of novel classes of antibacterial agents to eliminate multidrug-resistant pathogens [17, 26]. This dramatic situation has created an urgent need for developing alternative anti-infectives [7] for controlling such infections, especially in burn patients who do not respond to conventional antibiotic therapies. Bacteriophages, or simply phages, can be the best answer to antibiotic resistance in treatment of bacterial infections [5, 8, 11, 15, 19, 29, 32]. However, there are conflicting reports available in the literature on the efficacy of phages in different infections [25, 28]. Hence, there is a need to evaluate their potential in different disease models.

Earlier, we had confirmed the therapeutic potential of five different *K. pneumoniae* B5055-specific bacteriophages in treating burn wound infection in mice [14]. Among all the phages, phage strain Kpn5 was found to be the most effective in its action. In the present study, we provide additional evidence to prove its suitability for treating burn wound infection.

Materials and Methods

Bacterial Strain and Growth Media

K. pneumoniae B5055 obtained from Dr. Matthias Trautmann, Department of Medical Microbiology and Hygiene, Ulm University Hospital, Steinhövelstrasse 9, D-89075 Ulm, Germany and maintained in the laboratory was used in this study. The strain was maintained on nutrient agar slants at 4°C.

Effect of Phage Kpn5 Against *K. pneumoniae* B5055 *In Vitro*

A bacterial suspension (10^8 CFU/ml) in 10 ml of nutrient broth was incubated with phage Kpn5 (10^7 PFU/ml) at an MOI of 0.1, in shaking culture. To determine the numbers of viable bacteria, portions of the suspensions were serially diluted and plated onto nutrient agar at different time points. The agar plates were incubated at 37°C, overnight.

Animals

Adult BALB/c mice, six weeks old, weighing 20–25 g, were obtained from Central Animal House, Panjab University, Chandigarh, India. All animals were given antibiotic-free diet (Hindustan Liver limited, Mumbai, India) and water *ad libitum*. Animal study was conducted following protocols approved by the Institutional Animals Ethical Committee. Independent trials were performed three times in each experiment. The error bars in the graphs are representative of the standard deviation in each experiment.

Survival and Stability of Phage Kpn5

The survival and stability of *Klebsiella* phage Kpn5 were measured in mice, according to the method of Cerveny *et al*. [6]. Uninfected mice were injected i.p. with phage preparation (10^8 PFU/ml). At 1, 3, 6, 12, 24, 36, 48, and 72 h, blood was collected in screw-capped vials containing 0.05 M EDTA. Then, mice were sacrificed and the peritoneal fluid, skin, and lungs were aseptically removed and the tissue homogenates were subjected to phage count.

Burn Wound Model

An experimental third-degree burn wound mouse model was established using *K. pneumoniae* B5055, using the method of Dale *et al*. [10], as described in an earlier paper [14].

Optimization of Phage Dose

The effects of different phage doses on the ability of phage Kpn5 to treat burn wound infection induced by *K. pneumoniae* in full-thickness burned mice were evaluated. Animals were divided in 8 groups of mice (10 mice in each). Briefly, a full-thickness burn was induced in all the mice and subsequently challenged with *K. pneumoniae*, as described earlier. All the mice were treated immediately with a single injection of Kpn5 phage administered i.p. at various MOI (0.001, 0.01, 0.1, 1.0, 10, 100, and 200). Burned and infected mice without any phage treatment were kept as controls.

Treatment with Phage Kpn5

The therapeutic potential of phage Kpn5, specific for *K. pneumoniae* B5055, was evaluated for its ability to resolve burn wound infection in mice. The percentage survival and bacterial load in different organs of burned and infected mice following phage treatment were evaluated earlier [14].

Delayed Phage Treatment

The protective ability of delayed administration of phage Kpn5 after bacterial challenge of *K. pneumoniae* B5055 was also studied. Five groups of mice (10 mice in each) were used. In Group I, all the mice were challenged with bacteria only, acted as positive control, and no phage preparation was injected. In Groups II–V, burned and infected mice were injected i.p. with 100 µl of phage preparation (MOI of 1.0), 6, 12, 18, and 24 h, respectively, after bacterial challenge. Animals were monitored for any signs of morbidity and mortality. Survival rates for control and phage-treated groups were recorded at 24, 48, and 72 h.

Effect of Heat-Inactivated Phage

A sample of phage Kpn5 (3×10^8 PFU/ml) was heat inactivated by incubation at 80°C. Phage that had been heated for a total of 25 min, at which time no viable phage was detectable, was used to determine whether phage rescue of mice with burn wound infection requires functional phage or whether the rescue might be associated with nonspecific immune activation. The mice in this study were divided into three groups (10 mice in each). All mice were burned and challenged with *K. pneumoniae* B5055 culture subcutaneously as described earlier. In Group 1, burned/infected mice were treated with a single i.p. injection of viable Kpn5 phage (3×10^8 PFU/ml) immediately after bacterial challenge. Group II was treated with a single i.p. injection of 3×10^8 PFU/ml of heat-inactivated Kpn5 phage immediately after the bacterial challenge. Group III (control) received a single injection of nutrient broth medium instead of phage, administered i.p. immediately after bacterial challenge. Animals were monitored for any signs of morbidity and mortality. Survival rates for control and phage-treated groups were recorded at 24, 48, and 72 h.

Measurement of Cytokine Levels

Since inflammatory cytokines are thought to be good markers of the severity of bacterial infections [34], we measured the levels of IL-1β, TNF-α, and IL-10 in the sera and lungs of burned/infected (untreated) and phage-treated groups. Normal and burned mice were also taken as controls to measure the concentration of different inflammatory cytokines. The concentrations of IL-1β, TNF-α, and IL-10 in sera and lung tissues were measured by using commercial enzyme-linked immunosorbent assay kits (BD Biosciences, Pharmingen, C.A., U.S.A.).

Statistical Analysis

Data were expressed as means ± standard deviation (SD) of the mean and statistical analysis was performed with Graph Pad Instat Software (Version 3.00, Graph Pad Software, San Diego, California, U.S.A.) using the Student's *t*-test for calculations of mean and standard deviation, and one-way ANOVA followed by the Boneferroni test for multiple comparisons. Differences with $p<0.05$ were considered statistically significant.

Results

Lytic Activity of Kpn5 Phage Against *K. pneumoniae* B5055 *In Vitro*

To evaluate the lytic activity of phage Kpn5 against *K. pneumoniae* B5055 *in vitro*, the host bacterium was mixed with the phage at a MOI of 0.1 and incubated at 37°C

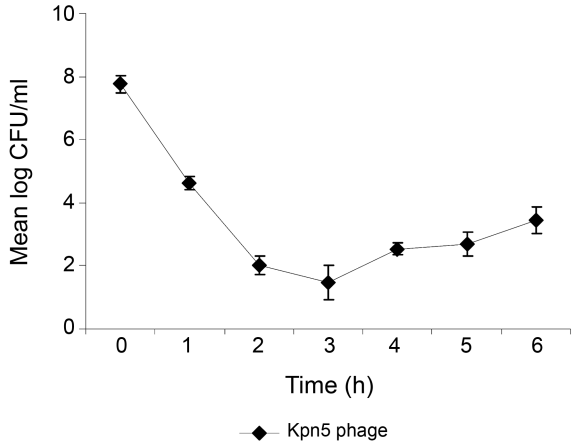

Fig. 1. Lytic activity of phage Kpn5 against *K. pneumoniae* B5055 *in vitro.*
Bacterial suspensions of *K. pneumoniae* B5055 and phage Kpn5 were incubated together in nutrient broth (0.1 MOI). The numbers of viable bacteria were determined and the data represent the means and standard deviations of the means (n=3 at each time point).

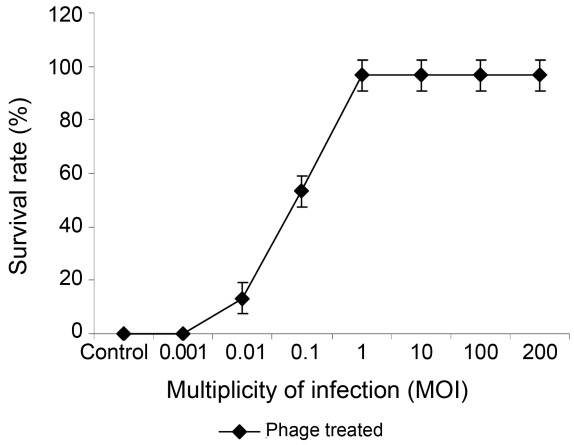

Fig. 3. Dose effect of phage Kpn5 in rescuing mice from lethal burn wound infection caused by *K. pneumoniae* B5055.
After s.c. injection of *K. pneumoniae* B5055 at LD_{100} value into the burn site, phage Kpn5 was injected into the mouse peritoneal cavity at various MOIs, and the percent survival of animals was noted. Burned mice injected only with *K. pneumoniae* B5055 served as controls (MOI of 0). There was a statistically significant difference in survival rates between mice treated with phage Kpn5 at a MOI≥0.1 and untreated control mice ($p<0.001$).

under shaking condition. The number of viable bacteria declined gradually from 10^8 CFU/ml at the start of incubation to 10^4 CFU/ml after 1 h of incubation, demonstrating that phage Kpn5 had potent lytic activity against *K. pneumoniae*. Phage Kpn5 continued lytic activity and the maximum decrease of 6 log cycles was observed at 3 h of incubation (Fig. 1). After this, a gradual increase in bacterial count was observed, possibly as a result of the emergence of bacterial population resistant to Kpn5.

Survival and Stability of Kpn5 Phage in Mice

The stability of phage Kpn5 in mice after i.p. injection in various body compartments was measured in terms of phage yield (Fig. 2). The maximum phage counts in blood, peritoneal fluid, lungs, and skin were obtained at 6 h post injection. The phage count in peritoneal fluid was significantly higher ($p<0.001$) in comparison with that obtained in the blood, lungs, and skin at 6 h. Decreased phage counts were obtained in all the samples at 36 h after injection. However, no phage could be isolated in peritoneal lavage fluid, blood, lungs, and skin after 36 h.

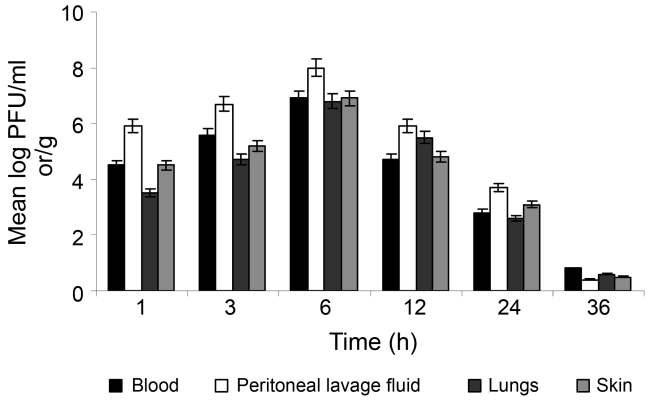

Fig. 2. Phage count in blood, peritoneal lavage fluid, and lung and skin homogenates at varying time periods after phage administration (10^8 PFU/ml) in BALB/c mice.

Determination of Phage Dose for Treatment

A single dose of Kpn5 phage at various MOI (0.001, 0.01, 0.1, 1.0, 10, 100, and 200) was administered immediately after bacterial challenge. A dose-dependent effect on the state of health of infected mice was clearly visible after 24 h. At higher doses of phage (MOI of 1.0, 10, 100, and 200), 96.66% survival with minimal sign of illness (mild lethargy) was seen in the first 24 h (Fig. 3). As the phage dose decreased (0.001, 0.01, and 0.1), the animals became critically ill with 0%, 13.33%, and 53.33% survival, respectively, up to 7 days. Mice injected only with the nutrient broth used to prepare phage suspensions served as controls and showed 0% survival. A statistically significant difference in survival rates of mice treated with phage Kpn5 at MOI of 1.0 and untreated control mice ($p<0.001$) was observed. All the mice that were alive and healthy on day 7 remained so for an additional 20 days, at which point the experiment was terminated.

Effect on Ability of Delayed Treatment with Phage Kpn5 Strain to Treat Mice Burn Wound Infection Caused by *K. pneumoniae*

In this experiment, *K pneumoniae* B5055 at LD_{100} was injected in burned mice s.c. directly under the anterior end

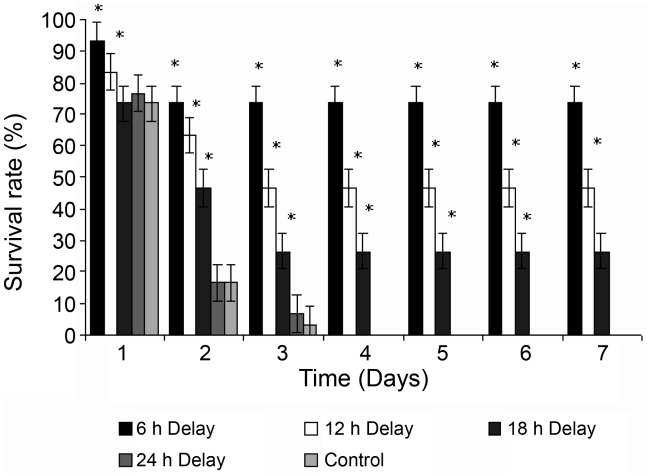

Fig. 4. Protective effect with delayed administration of phage Kpn5.
Purified Kpn5 (MOI 1.0) was administered in burned mice at various time intervals, after challenge with *K. pneumoniae* B5055. Burned mice injected only with *K. pneumoniae* B5055 served as controls. Survival rates were determined up to 7 days. The asterisk indicates statistically significant differences compared with that of the controls: *$p<0.001$.

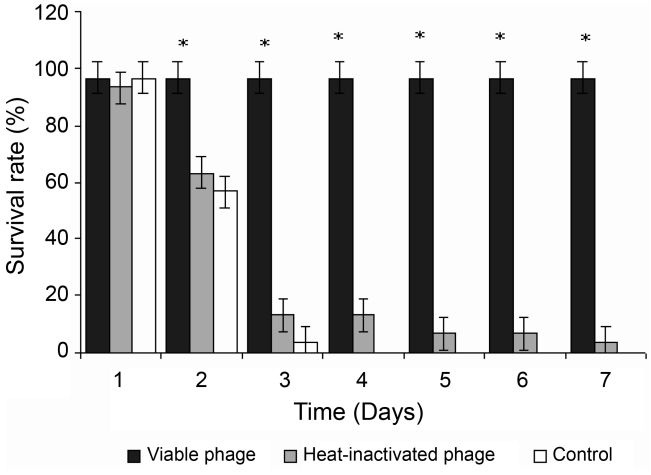

Fig. 5. Comparison of the ability of active phage and non-functional heat-inactivated phage to rescue burned and infected mice.
One group of 10 mice was treated with functional (plaque-forming) phage Kpn5, and the other two groups were treated either with no phage or heat-inactivated phage. Of the bacteremic mice treated with functional phage, 96.66% survived, whereas only 3.33% of the mice in the heat-inactivated group survived, and none of the control group survived. The animals were monitored for 20 more days, and no changes in their states of health were noted.

of the burn on the back of mice. At various time intervals thereafter, ranging from 6 to 24 h, the mice received a single i.p. injection of the phage dose at an MOI of 1.0. The results of this experiment are presented in Fig. 4. It was observed that a single i.p. injection of phage could rescue 73.33% of the animals, even when treatment was delayed up to 6 h after burn/bacterial challenge. If treatment was delayed beyond that point, morbidity increased and mortality began to appear. However, even with delays of 12 and 18 h, at which point all the mice were moribund, 46.66% and 26.66%, respectively, of the animals could be rescued and went on to recover completely. With 24 h delay of phage treatment, 6.66% of the mice were rescued and the survival rate was almost similar to that of untreated control mice with a 3.33% rate of survival ($p>0.05$). The survival rates among mice treated with phages at 6, 12, and 18 h were significantly higher that those of the 24 h delay group and untreated control groups ($p<0.001$).

Effect of Heat-Inactivated Phage on Survivability of Burned Mice

Heating at 80°C for 5 min decreased the phage titer by 1,000-folds and no viable phage was detected after heating for 25 min. As illustrated in Fig. 5, only mice inoculated with plaque-forming (viable) phage showed enhanced survival, with 96.66% survival, whereas 0% of nutrient broth-treated control mice and 3.33% of mice injected with heat-inactivated phage survived on the 7th day post phage treatment. These results were statistically significant ($p<0.001$). The animals were observed for an additional 20 days, and no changes in their state of health were noted.

Cytokine Levels in Serum Samples and Lungs of Phage-Treated Burned and Infected Mice

The levels of the pro-inflammatory cytokines (IL-1β and TNF-α) and anti-inflammatory cytokines (IL-10) in serum samples and lungs of normal, burned, Kpn5 phage-treated (*via* i.p. route), and untreated burned infected mice were determined at 24, 48, and 72 h post infection. The results presented in Fig. 6 indicate low levels of different cytokines in the sera and lungs of naïve mice. When observed in only burned mice, a gradual increase in the level of cytokines in both types of samples was observed ($p<0.001$). Infection in burned mice led to further increase in the levels of inflammatory mediators. A gradual increase in the level of cytokines in sera and lungs of phage-untreated burned infected mice was observed at 24, 48, and 72 h post phage inoculation. A significant decrease in the levels of IL-1β, TNF-α, and IL-10 in sera and lungs was detected ($p<0.001$) in phage-treated mice 72 h post infection and treatment.

Discussion

The research to evaluate the potential of phage therapy, because of its various advantages over antibiotic therapy, has gained momentum in the recent years. This strategy is considered advantageous, especially in situations where organisms are multiple antibiotic resistant. *K. pneumoniae* is one such nosocomial pathogen of which multiple

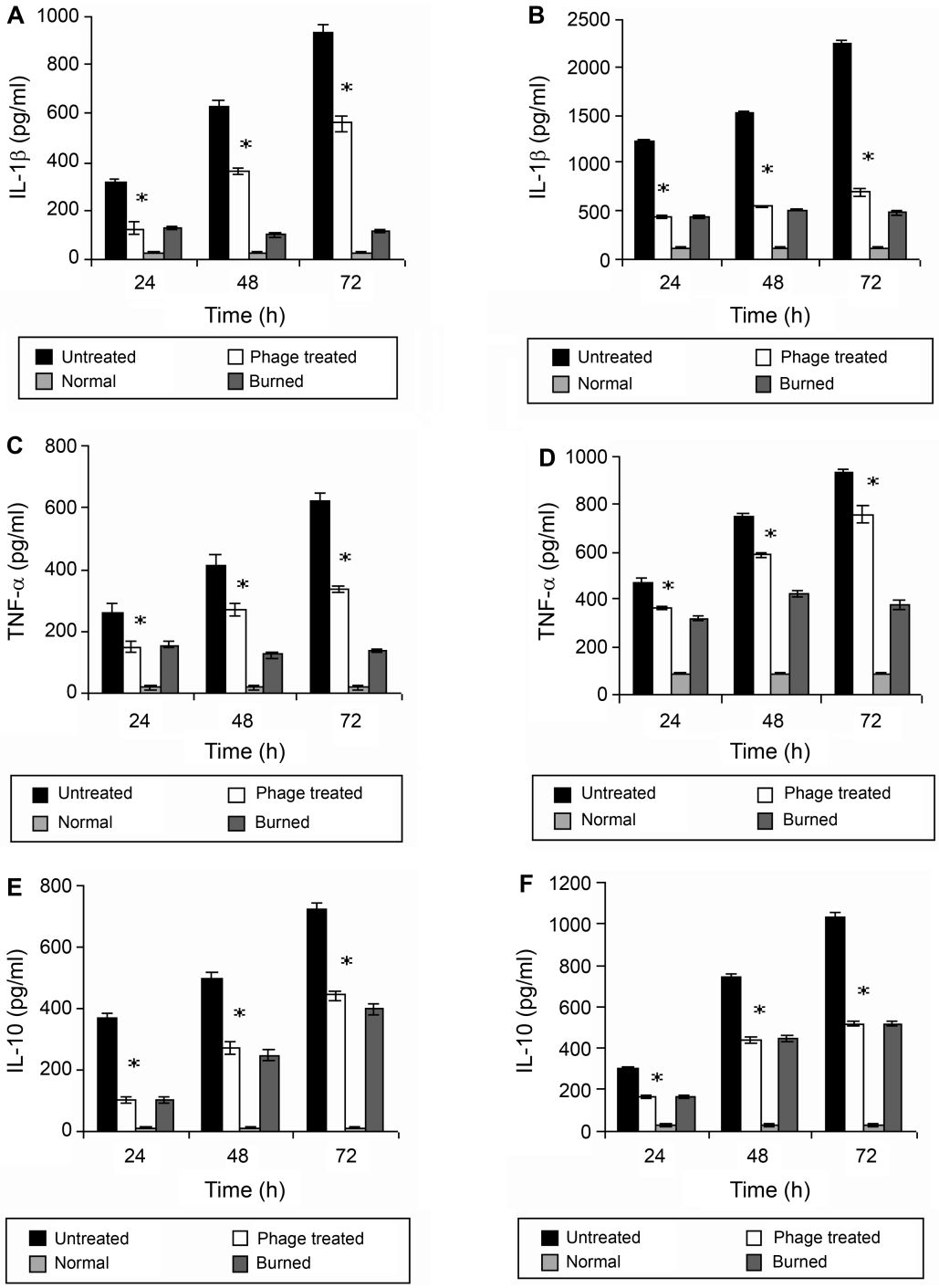

Fig. 6. Cytokine levels in sera and lungs following burn wound infection in mice.
The levels of IL-1β, TNF-α, and IL-10 in sera (A, C, E) and lungs (B, D, F) of normal, burned, phage-treated, and phage-untreated (burned infected) mice were determined. Asterisks indicate statistically significant differences of phage-treated mice compared with that of other groups: *$p<0.001$.

antibiotic-resistant strains are frequently encountered in clinical practice. In our earlier studies, phage Kpn5 was found to be most effective in treating burn wound infection caused by *K. pneumoniae* B5055 in mice [14]. The nontoxic nature of this phage, as revealed in this study, as well as its ability to survive over a period of 36 h, makes it an ideal candidate for phage therapy. Although phage entered into the blood stream after 3 h, it achieved the maximum count at 6 h post injection. In earlier studies, it has been observed that phages take a maximum of 2–4 h

to reach their peak in the blood stream and approximately 6–8 h to reach their peak in various internal organs [3, 4, 8]. The phage count showed a significant decrease at 12 h and no phage was detected at 48 h post injection in this study.

The possibility to prevent *K. pneumoniae* infection was examined in a mouse model of thermal injury in terms of survival rate and bacterial counts in different organs at 24, 48, and 72 h [14]. The results showed that a single i.p. injection of phage Kpn5 at an MOI of 1.0 was enough to effectively decrease the rate of mortality with an overall survival rate of 73.33% in burned and infected mice, even if the treatment was delayed up to 6 h. Moreover, even when phage treatment was delayed until all animals were moribund, 16.66% of the animals could be rescued and subsequently went on to recover completely. Similar results have been obtained in earlier studies where phages were used to treat *Escherichia coli* or vancomycin-resistant *Enterococcus faecium* (VRE)-induced bacteremia in mice [1, 2]. This has been explained on the basis of a population dynamics model, which demonstrates that the exponential growth in number of phage particles due to their self-replicating nature enabled a single injection of phage to be superior to multiple injections of antibiotics [16].

The possibility of bacterial resistance to phage is unquestionably an obstacle in the development of an effective phage therapy system [18]. Indeed, we noted the emergence of mutants of *K. pneumoniae* B5055 that were resistant to Kpn5 phage *in vitro*. Smith and colleagues [30, 31] previously showed that infections produced by phage-resistant mutants of an enteropathogenic strain of *E. coli* and their parents could be successfully controlled with mutant phage derived from phage that had been active against the parent bacteria. Similarly, in our laboratory, we also noted the sensitivity of phage mutants to the innate immune system *in vivo* [27]. Even if bacteria acquire phage resistance, a new mutant phage having lytic activity against these bacteria can be isolated [20]. In our opinion, to overcome such a situation, it would be desirable to use a cocktail of different phages active against the parent as well as mutant strains.

The ability of the phage to rescue bacteremic animals was a result of phage function and not nonspecific immune response activation, since reduced survival was not observed in the group of animals treated with heat-inactivated phage. The bacterial counts in blood and other organs decreased following phage treatment, proving their lytic activity. This experiment demonstrated the requirement for an active phage, that is, phage capable of growing in and lysing the infecting bacterial strain. This may be the main mechanism responsible for the protective effect, as the bacteriophage used was able to locate and kill *K. pneumoniae* before the animal succumbed to bacteremia and septic shock. In fact, the decrease in bacterial numbers in the blood and organs correlated with decreased morbidity and mortality.

The level of inflammatory cytokines, IL-1β, TNF-α, and IL-10, produced during infection in sera and lungs of mice treated with phage Kpn5 was much lower than in the sera and lungs of phage-untreated mice. Since IL-1β and TNF-α are predominately pro-inflammatory cytokines, phage therapy not only helped in the clearance of bacteria from the body, but it also protected the host from ensuing inflammatory damage because of their decreased levels. Although the precise mechanism of anti-inflammatory effects of phage treatment need to be investigated, it may possibly be due to the lowering of bacterial load in the affected organs of phage-treated mice, as reported earlier [14]. In a recent study from our laboratory, decreased lung inflammation has been correlated with decreased bacterial numbers owing to increased alveolar macrophage activity on exposure to clarithromycin [13]. It needs to be investigated whether such a mechanism is established on exposure to phages as well.

These results prove the potential of phage therapy in situations like burn wound infection for which few alternatives are available today for treatment. This study raised certain issues that need to be answered before instituting this form of therapy.

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