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Protamine Sulfate Reduces the Susceptibility of Thermally Injured Mice to *Pseudomonas aeruginosa* Infection[1]

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Background. **In this study, we investigated the ability of protamine sulfate, at sub-bactericidal dosing, to interfere with the *in vivo* virulence of *Pseudomonas aeruginosa* (PAO1) during burn wound infection.**

Materials and methods. **The study was conducted using the murine model of thermal injury. Preliminary experiments determined a protocol for administration of protamine sulfate that had no *in vivo* bactericidal effects. Based on this, the effect of local injection of protamine sulfate on the *in vivo* virulence of PAO1 was assessed using these parameters: (1) the percent mortality among PAO1-infected, thermally injured mice; (2) the local proliferation and spread of PAO1 within the infected burned tissue; (3) the systemic spread of PAO1 within thermally injured/infected mice; and (4) the local cytokine response elicited by PAO1 thermally injured/infected mice.**

Results. **Injection of protamine sulfate into the thermally injured tissue of PAO1-infected/thermally injured mice significantly decreased the percent mortality and inhibited the systemic dissemination of PAO1 microorganisms to the liver and spleen. It had no effect, however, on the ability of the bacteria to proliferate and spread within the thermally injured tissue. It also was determined that protamine sulfate was ineffective at preventing mouse death at the dose administered if injected intramuscularly instead of directly into burned tissue. Protamine sulfate reduced the expression of the proinflammatory cytokines IL-6 and LIF in the injured/infected tissue. Heparan sulfate given in conjunction with protamine sulfate returned mortality levels to those of untreated mice.**

Conclusions. **Our results suggest that: (1) local injection of sub-bactericidal doses of protamine sulfate reduces the virulence of *P. aeruginosa*; (2) this effect is due to interference with the systemic rather than local spread of *P. aeruginosa*; and (3) local application of protamine sulfate may have potential as supportive therapy for prevention of systemic *P. aeruginosa* infection in severely burned patients.**

Key Words: **Burn wound, *Pseudomonas aeruginosa*, *in vivo* virulence; protamine sulfate.**

INTRODUCTION

Infection is the leading cause of morbidity and mortality in the setting of thermal injury [1, 2]. The loss of skin barrier with exposure of the moist, nutrient-rich connective tissue underneath and the overall dysregulation of immunity provide conditions ideal for the proliferation and spread of bacteria within the injured tissue and their subsequent dissemination throughout the immunocompromised host.

The Gram-negative bacterium *Pseudomonas aeruginosa* is one of the most virulent and most commonly encountered pathogens in burn wound infection [3–6]. The extensive damage induced during *P. aeruginosa* infection is attributable to the ability of the microorganism to produce numerous virulence factors [3]. Establishment of *P. aeruginosa* infection within the burned tissue is usually followed by sepsis and results in death more than 50% of the time [3, 6].

Because of the significant associated morbidity and mortality, prevention and early treatment are critical

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in the management of *P. aeruginosa* burn wound infection. One of the mainstays of this is aggressive antimicrobial therapy. However, one major concern is that currently available antibiotics will be rendered ineffective in the near future because of the increasing prevalence of multiresistant *P. aeruginosa* strains.

The majority of currently available antimicrobial therapies function by attempting to completely eradicate pathogens from the host organism, either by killing the microorganisms directly or by inhibiting their growth [7]. It is now known that such action also selects for populations of inherently resistant organisms, rendering therapy ineffective [8], [9]. This has stimulated the search for alternative therapies that do not directly target bacterial viability but instead attenuate the pathogen's ability to cause significant damage to the host organism, most specifically sepsis and death [10, 11]. It is thought that such therapies would place less selective pressure on the global bacterial population and limit the emergence of strains resistant to the therapy. Examples of such therapies recently evaluated that have shown promise include both those directed at improving host immune response [9, 12, 13] and those directed at inhibiting the coordinated production and action of bacterial virulence factors [14–17].

Current research suggests that protamine sulfate has potential as such a therapy in the setting of *P. aeruginosa* infection. Protamine sulfate is a cationic polypeptide derived from salmon sperm [18]. Its most-recognized use is reversal of heparin anticoagulation, particularly after cardiopulmonary bypass. While not used as an antimicrobial, it is known to be bactericidal at high concentrations (MIC 10 mg/mL) [18]. In a recent study, Park *et al.* [19] demonstrated an alternative activity. At sub-bactericidal concentrations, protamine sulfate significantly decreased the incidence of sepsis and mortality in a murine model of *P. aeruginosa* lung infection [19]. In this study, we attempted to determine whether the same effect is achievable in the mouse model of thermal injury and to define the molecular mechanism of protamine sulfate antibacterial activity in this setting.

MATERIALS AND METHODS

Bacterial Strains, Growth Medium, and Chemicals

The *P. aeruginosa* strain used in this study, PAO1, originally was isolated from a wound infection [20]. It has been used in different animal models, including the thermal injury mouse model and the acute and chronic murine lung infection models [21, 22]. We previously determined that at a dose of 10^{3-4} colony forming units (cfu), PAO1 produced more than 90% mortality among thermally injured/infected mice. PAO1 was routinely grown in Luria Bertani (LB) broth [23]. The protamine sulfate (Sigma-Aldrich, St. Louis, MO) was dissolved in PBS at a concentration of 1 mg/mL.

Animals

Adult female Swiss Webster (outbred) mice weighing 20–24 g were housed under controlled environmental conditions at the Texas Tech University Health Sciences Center (TTUHSC) Laboratory Animal Resource Center. The animals were given food and water *ad libitum*. When necessary, mice were euthanized by intracardial injection of 0.2 mL of Sleepaway (sodium pentobarbital–7.8% isopropyl alcohol euthanasia solution; Fort Dodge Laboratories, Inc., Fort Dodge, IA). Animals were treated humanely and in accordance with the protocol approved by the Animal Care and Use Committee at TTUHSC in Lubbock, Texas.

The Thermally Injury Mouse Model, Protamine Sulfate Treatment, and Determination of Percent Mortality

We used the previously described murine thermal injury model with minor modifications [22, 24]. In summary, adult female Swiss Webster mice were anesthetized, shaved, and secured in a template that exposes 15% of the total body surface area. The exposed area of skin was placed in 90°C water for 10 s to induce the thermal injury. After a subcutaneous injection of 0.8 mL of 0.9% saline (as fluid replacement), the mice were injected with 10^{3-4} cfu of PAO1 in 100 μL of PBS into the posterior aspect of the eschar. Approximately 20 min after that, the mice were injected with protamine sulfate at a dose of 5 μg/g mouse weight (in 100 μL of PBS) at the same site. Control mice were thermally injured, injected with 0.8 mL of PBS, and inoculated with PAO1 as above. In place of protamine solution, the mice were injected with a sham dose of 100 μL of PBS into the eschar. All doses of protamine sulfate or PBS administered at later time points were given at the same dose and location as the initial dose. In some experiments, heparan sulfate (Sigma) was given as a one-dose injection of 100 μg. To assess percent mortality, the different groups of thermally injured/infected mice were followed over 7 days, and the number of dead mice in each group was determined at 24 h intervals starting from the time of burn injury/infection.

Determining the Effect of Protamine Sulfate on the Local and Systemic Spread of *P. aeruginosa*

The mice were subjected to thermal injury, inoculated with PAO1, and injected with protamine sulfate solution or PBS solution as described above. At 24 h after burn/infection, the mice were euthanized per protocol. To determine the effect on local spread of *P. aeruginosa*, two 5 × 5 mm sections of burned skin were obtained from the mice, the first at the site of PAO1 and protamine sulfate injection (inoculation site) and the second at a site 15 mm cephalad from the first sample (distant site). To determine systemic spread, the spleen and liver of each animal were obtained. These samples were then weighed and homogenized in 2 mL of PBS (Wheaton Overhead Stirrer, Wheaton Instruments, Millville, NJ). A 0.5-mL sample of the homogenate was serially diluted in PBS, and a 100-μL aliquot of each dilution was plated on nutrient agar and the number of colonies was counted. The number of cfu from each sample was calculated per gram of tissue.

Determining Cytokine Expression

Extraction of total RNA from mouse skin sections was performed by using TRI Reagent-RNA/DNA/Protein isolation reagent per the manufacturers guidelines (Molecular Research Center, Inc., Cincinnati, OH). RNA was DNased to remove any contaminating genomic DNA (RQ1 RNase-Free DNase, Promega, Madison, WI). A total of 20 μg of DNAsed RNA was used to detect the expression of murine cytokine genes using the mck4 Multi-Probe Template Set and the Riboquant Ribonuclease Protection Assay System (PharMingen, San Diego, CA). The probes were hybridized overnight at 56°C with 20 μg of total RNA from either the liver or the skin. After hybridization, the mixture was treated with RNase A+T1 mix (PharMingen) to digest the unhybridized single-stranded RNA. The protected fragments then were purified by phenol/chloroform extraction and ethanol precipitation. The samples were resuspended in 5 μL of loading dye and