Lysostaphin Treatment of Methicillin-Resistant Staphylococcus aureus Keratitis in the Rabbit

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PURPOSE. To determine the efficacy of lysostaphin treatment of methicillin-sensitive and methicillin-resistant Staphylococcus aureus (MRSA) keratitis in a rabbit model.

METHODS. The sensitivity to lysostaphin and vancomycin were compared for 34 MRSA and 12 methicillin-sensitive strains. Methicillin-resistant S. aureus strain 301 (MRSA 301) or a methicillin-sensitive strain of low virulence, ISP546, was intrastromally injected into rabbit corneas. Rabbit eyes were treated topically every 30 minutes from 4 to 9 or 10 to 15 hours postinfection with 0.28% lysostaphin or 5.0% vancomycin. Rabbits were killed and corneas were excised and cultured to determine the number of colony forming units (CFU) per cornea.

RESULTS. Ninety percent minimal inhibitory concentrations were at least 19-fold lower for lysostaphin than for vancomycin. With early therapy (4–9 hours postinfection) lysostaphin sterilized all MRSA 301–infected corneas, whereas untreated corneas contained 6.52 log CFU/cornea (P ≤ 0.0001). Corneas infected with MRSA 301 and treated similarly with vancomycin retained 2.3 ± 0.85 log CFU/cornea, and none were sterile. When therapy was begun later (10–15 hours postinfection) the residual bacteria in lysostaphin-treated eyes were significantly less numerous than in vancomycin-treated eyes (0.58 ± 0.34 vs. 5.83 ± 0.16 log CFU/cornea, respectively; P ≤ 0.0001). Three experiments were performed to demonstrate that lysostaphin penetrated the cornea to kill bacteria in vivo; lysostaphin-treated eyes were found to recover from infection, bacteria that did not cause epithelial defects (ISP546) were susceptible to lysostaphin, and inhibition of lysostaphin when harvesting corneas did not alter the observed therapeutic values of lysostaphin.

CONCLUSIONS. Lysostaphin is very effective in treating keratitis mediated by methicillin-sensitive or methicillin-resistant S. aureus. (Invest Ophthalmol Vis Sci. 2000;41:1432–1437)
found in the cell wall of *S. aureus*. Lysostaphin has a molecular weight of 27 kDa and contains one molecule of zinc per mole of protein. The enzyme is destroyed by pepsin or trypsin and inhibited by Hg²⁺, Cu²⁺, and Zn²⁺ ions.

The use of lysostaphin for chemotherapy was proposed over 30 years ago. Lysostaphin purified from *S. simulans* was found to be effective in treating experimental staphylococcal infections in various nonocular animal models and was once used systemically in a human neutropenic patient to treat staphylococcal abscesses. Lysostaphin was also shown to be effective in reducing the nasal carriage of *S. aureus* in humans.

### MATERIALS AND METHODS

#### Bacteria

MRSA strain 301 used in these studies was isolated from a human corneal ulcer and was previously analyzed in a rabbit keratitis model. *S. aureus* strain ISP546, a methicillin-sensitive (MSSA) strain, is deficient in the accessory global regulator (agr) and does not produce α-toxin, which is the main toxin associated with virulence and mediates corneal erosions.

#### Minimal Inhibitory Concentration

Minimal inhibitory concentrations of lysostaphin and vancomycin were determined by the tube-broth dilution method using Mueller-Hinton broth (Difco, Detroit, MI) supplemented with zinc chloride (200 mM). The number of viable bacteria was graded on a scale of 0 to 4. The parameter grades were (injection, chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon formation, and iritis) were graded on a scale of 0 to 4.

#### Lysostaphin Inhibition Assay

MRSA 301 was grown for 24 hours in tryptic soy broth (TSB; Difco) at 37°C and washed three times in sterile Tris-buffered saline (50 mM Tris, 150 mM NaCl, pH 7.5). Approximately 10⁵ colony-forming units (CFU)/ml of *S. aureus* were added to doubling dilutions of antibiotic and incubated at 35°C for 24 hours. The MIC was designated as the lowest concentration that inhibited growth of *Staphylococcus* as determined by the lack of turbidity. The MIC₉₀ is the lowest concentration that inhibited 90% of the strains tested.

#### Antibiotic Preparation

Lysostaphin (Sigma, St. Louis, MO) was dissolved in sterile deionized water to a concentration of 2.8 mg/ml (0.28%). Vancomycin (Vancoled; Lederle Pharmaceuticals, Carolina, Puerto Rico) was dissolved in sterile deionized water and further diluted 1:4 in artificial tears (Tears Naturale Free; Alcon, Humacao, Puerto Rico) to a final concentration of 50 mg/ml (5.0%), the concentration recommended for clinical use. The ph of the vancomycin solution was adjusted to 6.5 with HCl before diluting in artificial tears. All antibiotics were prepared immediately before use and kept at 0 to 4°C.

#### Treatment Schedule

Rabbits were topically treated for 5 hours postinfection with a single topical drop (45 μl) every 30 minutes. The treatment schedules were from 4 to 9 or 10 to 15 hours postinfection. Rabbits were killed 1 hour after the last treatment. Rabbits were randomly divided into three groups: group 1 received 0.28% lysostaphin, group 2 received 5.0% vancomycin, and group 3 was untreated.

#### Bacterial Quantification

Corneas were prepared for bacterial quantification as previously described. Briefly, corneas were removed aseptically, dissected, and homogenized in sterile buffered saline using a tissue homogenizer (Tekmar, Cincinnati, OH). Aliquots of corneal homogenates were serially diluted in buffered saline, plated in triplicate on tryptic soy agar plates (TS; Difco), and incubated for 24 hours at 37°C. The optical density was measured at a wavelength of 620 nm. The change in optical density (from 0.21 to 0.00 OD) between bacterial suspensions with lysostaphin and those without lysostaphin was determined as a measure of bacterial cell lysis, resulting from the action of lysostaphin.

#### Rabbis

New Zealand White rabbits (2.0–3.0 kg) were treated and maintained in accordance with the tenets of the ARVO State-
Corneal erosions were detected using fluorescein (Fluor-I-Strip A.T.; Everst Laboratories, Philadelphia, PA); diameters were measured, and values were expressed in millimeters.

**Statistical Analysis**

Data were analyzed using the Statistical Analysis System (Cary, NC) program for personal computers. For CFU determinations, analysis of variance and Student’s *t*-tests between least-squared means from each group showing statistical variances were performed. For SLE scores, nonparametric one-way analysis of variance (Kruskal–Wallis test) and Wilcoxon’s test were used for comparison among groups. P values ≤ 0.05 were considered significant.

**RESULTS**

**Susceptibility of Strains**

MICs of lysostaphin were determined for 34 strains of MRSA and 12 strains of MSSA (Table 1). The MIC$_{90}$ for MRSA were 2-fold lower than that of MSSA. All groups had an equivalent MIC$_{90}$ for vancomycin. The MIC$_{90}$ concentration of lysostaphin was 19.5-fold lower than the MIC$_{90}$ concentration of vancomycin for MRSA and 39-fold lower than the MIC$_{90}$ of vancomycin for MSSA.

**Treatment of Experimental MRSA Keratitis**

With early therapy (4–9 hours postinfection), lysostaphin sterilized all corneas, whereas untreated corneas contained $6.52 \pm 0.10$ log CFU/cornea ($P \leq 0.0001$; Fig. 1A). No eyes treated with vancomycin were sterile, and these eyes had significantly more CFU per cornea than eyes treated with lysostaphin ($P = 0.005$; $2.30 \pm 0.85$ log CFU vs. $0.0$ log CFU per cornea, respectively).

When therapy was begun later (10–15 hours postinfection), lysostaphin reduced the CFU/cornea to $0.85 \pm 0.46$ log CFU compared to $6.59 \pm 0.12$ log CFU/cornea in the untreated eyes ($P \leq 0.0001$; Fig. 1B). In contrast, the number of log CFU per cornea in the vancomycin-treated group was not significantly different from the untreated group ($5.83 \pm 0.16; P = 0.1364$). Lysostaphin therapy late in infection reduced the CFU/cornea approximately 100,000-fold more efficiently than vancomycin therapy.

**TABLE 1. Determination of the MIC$_{90}$ of Lysostaphin for S. aureus**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Vancomycin (µg/ml)*</th>
<th>Lysostaphin (µg/ml)*</th>
<th>Vancomycin to Lysostaphin Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. aureus</em></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin-sensitive†</td>
<td>1.2</td>
<td>0.031</td>
<td>39</td>
</tr>
<tr>
<td>(0.6104–1.2207)</td>
<td>(0.0078–0.0625)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methicillin-resistant†</td>
<td>1.2207</td>
<td>0.0625</td>
<td>19.5</td>
</tr>
<tr>
<td>(0.3052–2.4414)</td>
<td>(0.0039–2.5)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Minimal inhibitory concentrations of 90% of isolates tested were determined using the broth dilution method with Mueller–Hinton broth supplemented with 5% sodium chloride. Approximately $10^7$ CFU/ml of bacteria were added to doubling dilutions of antibiotics tested and incubated at 35°C for 24 hours. The MIC$_{90}$ was determined as the lowest antibiotic concentration that completely inhibited growth of 90% of the strains tested as determined by lack of turbidity.

† Twelve strains of methicillin-sensitive *S. aureus* and 34 strains of methicillin-resistant *S. aureus* were used.

**Figure 1.** Lysostaphin treatment of rabbit eyes infected with MRSA 301. Rabbit corneas were infected with approximately 100 CFU of MRSA 301 and treated with vancomycin (50 mg/ml) or lysostaphin (2.8 mg/ml). (A) Rabbit eyes were treated every 30 minutes from 4 to 9 hours postinfection. (B) Rabbit eyes were treated every 30 minutes from 10 to 15 hours postinfection. All rabbits were killed 1 hour after treatment, and corneas were removed aseptically, dissected, and homogenized in sterile phosphate-buffered saline. Aliquots of corneal homogenates were serially diluted, plated in triplicate on tryptic soy agar plates, and incubated for 24 hours at 37°C. The number of viable *S. aureus* per cornea was expressed as base 10 logarithms ± SEM.
Treatment of Experimental Staphylococcus Keratitis without a Corneal Defect

To determine the ability of lysostaphin to penetrate the intact cornea, S. aureus strain ISP546, an agr-deficient mutant lacking the ability to cause corneal epithelial erosion was used.45,46 When therapy of ISP546 infections began at 10 to 15 hours postinfection, lysostaphin penetrated the intact corneal epithelium and significantly reduced the CFU/cornea to 0.58 ± 0.34 log CFU/cornea compared with 5.94 ± 0.24 log CFU/cornea of untreated eyes (P ≤ 0.0001; Fig. 2). Vancomycin treatment of ISP546 keratitis resulted in a value not significantly different from the untreated eyes (5.41 ± 0.11 log CFU/cornea; P = 0.3677). Unlike infections with MRSA 301, no erosions were detectable by SLE with fluoroscein during the course of ISP546 infection.

Evidence of In Situ Killing of S. aureus

To confirm that lysostaphin was penetrating the cornea, an experiment was performed in which the corneas were placed into an inhibitor of lysostaphin (ZnCl) immediately upon harvesting. The ZnCl (200 mM) inhibited potential lysostaphin-mediated killing of Staphylococcus during corneal homogenization and culturing. Lysostaphin was administered 4 to 9 hours postinfection, and the presence of ZnCl did not alter the experimental results compared with previous experiments in which ZnCl was not used. Lysostaphin sterilized 75% of treated corneas (0.33 ± 0.33 log CFU/cornea) compared with the untreated control group where no corneas were sterile (6.48 ± 0.10 log CFU/cornea; P ≤ 0.0001).

In another experiment, rabbit eyes were infected with MRSA 301 and treated with 0.28% solution of lysostaphin every 30 minutes for 5 hours (4 to 9 hours postinfection). These treated eyes were found to develop only limited pathology through 25 hours postinfection (Fig. 3), and these changes diminished with time such that by 36 hours postinfection the lysostaphin-treated eyes had SLE scores of less than 5, indicating minimal evidence of infection. These lysostaphin-treated rabbit eyes were observed for 6 additional days, with the SLE score reaching a value of 0 by day 2. There was no recurrent infection in these eyes over the next 4 days. This is in contrast with untreated rabbits infected with MRSA 301 whose SLE scores exceeded values of 18 by 25 hours postinfection and who had to be killed.

DISCUSSION

The present study demonstrated that lysostaphin is a highly effective therapy for experimental keratitis caused by S. aureus. Lysostaphin appears to be more effective than any other drug tested in the treatment of experimental S. aureus keratitis.48,49 Early therapy with lysostaphin sterilized the infected corneas, including those infected with a MRSA strain. Lyso-
lysostaphin also effectively killed a low-virulence strain of *S. aureus*, indicating that its effectiveness in the rabbit eye was not dependent on extensive erosion of the corneal epithelium. Lysostaphin had MIC values for multi-drug-resistant *S. aureus* that were similar to that of methicillin-sensitive *S. aureus*. Lysostaphin had MIC<sub>90</sub> values 19.5- and 39-fold lower than those of vancomycin for MRSA and MSSA, respectively.

The reduction in bacterial CFU in corneas treated with lysostaphin was shown to be due to in situ killing of bacteria and not the action of residual lysostaphin action in corneal homogenates. Furthermore, infected eyes treated with lysostaphin were observed over an extended period (7 days) and found to become free of discernable pathologic changes.

Lysostaphin was an effective therapy during the late phases of *Staphylococcus* infection when bacterial replication was minimal. This finding agrees with the in vitro studies showing that lysostaphin’s activity is lethal to *S. aureus*, regardless of their metabolic state. The lysing of actively multiplying, resting, or dead *Staphylococcus* is an unusual trait among antibiotics, and such activity evidences the potential of lysostaphin as an ocular antimicrobial therapy. Tobramycin is the only other antimicrobial agent found to maintain its effectiveness in both the early and late phases of experimental *Staphylococcus* keratitis. Tobramycin, however, has far less potency than lysostaphin and is not effective against MRSA strains.

The ability of lysostaphin to penetrate the cornea could be related to its enzymatic activity. Lysostaphin has weak, but significant, proteolytic activity on mammalian tissue. Lysostaphin was shown to be effective in degrading elastin, which has a high glycine content. This proteolytic action could augment lysostaphin's penetration through the epithelial barrier of the cornea. Such proteolytic action could be particularly important in understanding the effectiveness of lysostaphin in eyes infected with methicillin-sensitive strains of low virulence. The methicillin-sensitive strain was chosen for analysis because it fails to produce any visible defects in the corneal epithelium. The in vivo susceptibility of the low-virulence strain to lysostaphin illustrates the ability of the enzyme to penetrate the cornea.

Previous studies have shown vancomycin to induce conjunctival inflammation and corneal edema. Dissolving vancomycin in artificial tears was shown to significantly reduce, but not eliminate, this irritation. Lysostaphin, however, did not show any irritation, as graded by slit lamp examination. Further studies of ocular lysostaphin administration are needed to determine whether any adverse effects are induced by repeat topical application of this enzyme. Because lysostaphin is a bacterial protein of 27 kDa, it has the potential to induce immunologic reactions.

Lysostaphin has been investigated periodically over the past 30 years as a therapy for humans and as an experimental systemic therapy in an animal model of infection (i.e., endocarditis). Lysostaphin has been applied safely and effectively to human nasal passages of *Staphylococcus* carriers. Although rechallenge of most subjects with a second intranasal application of lysostaphin was accomplished without reaction, further study of the immune response has not yet been performed. The systemic use of lysostaphin in the past has not been encouraged because of the immunogenicity from the previously impure protein. However, the current availability of recombinant lysostaphin may provide an opportunity for a single, continuous, brief course of therapy.

References


