Immunity to Lysostaphin and Its Therapeutic Value for Ocular MRSA Infections in the Rabbit

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PURPOSE. To determine the effects of immunization against lysostaphin on the bactericidal action of lysostaphin in ocular tissue and the possible induction of allergic reactions.

METHODS. Rabbits were immunized against lysostaphin by subcutaneous, intranasal, or topical routes. Anti-lysostaphin antibody titers were determined by ELISA and by neutralization of lysostaphin. Methicillin-resistant Staphylococcus aureus was intrastromally or intravitreally injected into rabbit eyes. Eyes were treated either topically with drops of lysostaphin (0.3%) or with a single intravitreal injection (0.1 mL) of lysostaphin (0.1%). At the time of death, corneas or vitreous humors were cultured to determine the number of colony forming units (CFU).

RESULTS. Rabbits in keratitis experiments that were immunized subcutaneously, intranasally, or topically had serum antibody titers of 10,240, 187, and 1,867, respectively, and neutralization titers of 8 or less. In both normal and immunized rabbits with keratitis, lysostaphin significantly reduced the log CFU to less than 1 log, whereas the untreated eyes contained more than 106 CFU/cornea (P ≤ 0.0001). Rabbits that were subcutaneously or topically immunized for endophthalmitis experiments had serum antibody titers of 1636 or 137, respectively, and neutralization titers of 2 or less. A single intravitreal injection of lysostaphin (0.1%) sterilized all eyes of immunized and nonimmune rabbits with endophthalmitis. No adverse effects were observed with the administration of lysostaphin to either normal or immunized rabbit eyes.

CONCLUSIONS. Lysostaphin treatment of immunized rabbits was effective in treating S. aureus-infected eyes, despite the presence of anti-lysostaphin antibody. No adverse reactions were produced by administration of lysostaphin to immunized rabbits. (Invest Ophthalmol Vis Sci. 2002;43:3712–3716)

Staphylococcus aureus is a major cause of bacterial ocular infections in the United States.1–3 S. aureus is a leading cause of bacterial keratitis and patients with epithelial trauma caused by contact lens wear or foreign bodies are susceptible to S. aureus keratitis.4,5 S. aureus keratitis can cause severe inflammation, pain, corneal perforation, scarring, and loss of visual acuity.6 Staphylococcus epidermidis and S. aureus are responsible for half of all endophthalmitis cases,4,7 and approximately 70% of cases occur as a result of intraocular surgery.7 With every intraocular surgery, there is a risk of introducing microorganisms into the eye that cause endophthalmitis.8–10 S. aureus infections are becoming increasingly more difficult to treat because of changes in the frequencies of isolation, distribution in the population, and cell wall properties of antibiotic-resistant strains. Antibiotic resistant forms of S. aureus (methicillin-resistant S. aureus, MRSA) represent an increasingly major cause of nosocomial infections worldwide.11 Of notable concern is the increased isolation of MRSA strains from patients with no history of hospitalization or antibiotic usage.12 Furthermore, the increasing incidence of fluoroquinolone-resistant S. aureus strains has resulted in more frequent use of vancomycin therapy.13–17 Because of the prevalence of antibiotic-resistant strains, vancomycin has emerged as the preferred drug for empiric therapy for staphylococcal ocular infections.18–20 Vancomycin, however, is a slow-acting antibiotic that has significant adverse ocular effects.21,22 There is further concern regarding the emergence of S. aureus strains in Japan and in the United States that are described as being vancomycin intermediate-resistant (VISA).19 Infections by such atypical strains cannot be effectively treated with vancomycin alone.19,20,25 New treatments are needed to compensate for the broadening distribution of MRSA in the nonhospitalized population and for the increasing antibiotic resistance of these strains.

Lysostaphin is a zinc metalloproteinase (27 kDa) extracted from Staphylococcus simulans that lyses S. aureus by cleaving glycine–glycine bonds, thereby disrupting the peptidoglycan layer of the cell wall.24–31 Lysostaphin was studied in the 1960s and 1970s as a potential therapeutic agent in a number of animal models.25,27–30,32,35 Lysostaphin was also shown to be effective in reducing the nasal carriage of S. aureus in humans.24,30,34 Lysostaphin is being reexamined as an antibiotic therapeutic agent, because antibiotic-resistant strains have become prevalent for many S. aureus strains.35–37 Experimental use of lysostaphin as a therapeutic agent in nonocular sites in humans has been described as effective in killing S. aureus35 and as being essentially free of adverse effects.34 Lysostaphin has been shown, in the rabbit model, to be a highly potent therapy for keratitis38 and endophthalmitis39 mediated by MRSA. The major concern regarding the use of lysostaphin is not its effectiveness, but rather the possibility that lysostaphin, as a foreign protein, could induce an immune response, such as harmful hypersensitivity reactions. Another concern is that antibody to lysostaphin could prevent bacterial killing by neutralizing the enzymatic activity of lysostaphin. To address these concerns, the effectiveness and safety of lysostaphin therapy for keratitis and endophthalmitis were studied in rabbits immunized to lysostaphin by three different routes of immunization.

MATERIAL AND METHODS

Rabbits

New Zealand White rabbits (2.0–3.0 kg) were treated and maintained in accordance with the tenets of the ARVO Statement for the Use of...
Animals in Ophthalmic and Vision Research. All rabbits were anesthetized by subcutaneous injection of a 1:5 mixture of xylazine (100 mg/mL; Rompun; Miles Laboratories, Shawnee, KS) and ketamine hydrochloride (100 mg/mL; Ketaset; Bristol Laboratories, Syracuse, NY). Proparacaine hydrochloride (0.5% alcane; Alcon Laboratories, Fort Worth, TX) was topically applied to each eye before intrastromal or intravitreal injections. Pupils were dilated with 1% tropicamide ophthalmic solution (Bausch and Lomb, Inc., Tampa, FL). Rabbits were killed with an overdose of pentobarbital (Sigma, St. Louis, MO).

**Immunization**

Specific pathogen-free New Zealand White rabbits were immunized by three routes: subcutaneous, intranasal, and topical. Rabbits were bled before all immunizations and antibody titers to lysostaphin were determined by enzyme-linked immunosorbent assay (ELISA). Subcutaneous immunizations were performed by injecting 400 μg of lysostaphin (Sigma) mixed with complete Freund’s adjuvant (Sigma) at four separate sites on the side and back of the rabbit. Rabbits were subsequently immunized (boosted) monthly for 5 months with 400 μg lysostaphin mixed with incomplete Freund’s adjuvant (Sigma) until a significant antibody titer was achieved. For intranasal immunization, rabbits received 0.1 mL of lysostaphin (1000 μg/mL) and placed into the nasal passages for three consecutive days and were similarly boosted every month for 5 months. For topical immunization, rabbits received 1 drop (45 μL) lysostaphin (5 mg/mL) applied to their eyes every day for 21 consecutive days, and then, after 30 days, lysostaphin was again applied daily for 14 days. After another 30 days, lysostaphin was applied for 7 consecutive days. Immunized rabbits were bled 30 days after the last administration of immunogen, and the titers were determined by ELISA. For all routes of immunization, boosters were administered until the ELISA titers to lysostaphin no longer increased significantly after the last booster immunization.

**ELISA**

Quantification of IgG antibody to lysostaphin was determined by antibody-capture ELISA. Lysostaphin (10 μg/mL) was dissolved in carbonate buffer (10 mM Na2CO3 and 35 mM NaHCO3 [pH 9]) and placed into a 96-well microtiter plate overnight at 4°C. The plates were then washed with phosphate-buffered saline containing 0.05% Tween 20 (PBST; Sigma) and blocked for 4 hours at room temperature with 5% goat serum (Sigma) in phosphate-buffered saline (blocking buffer). Serial dilutions of sera were added to the plates and incubated at room temperature for 2 hours. The microtiter plates were then washed with PBST and 100 μL anti-rabbit IgG (γ chain specific) conjugated to alkaline phosphatase (Sigma) diluted 1:500 in blocking buffer was added to each well. Microtiter plates were washed in PBST and then developed with para-nitrophenyl phosphate (pNPP, Sigma). The absorbance of the wells of the microtiter plates were read at a wavelength of 410 nm.

**Antibody Neutralization Assay**

Serum from rabbits immunized with lysostaphin was assayed for neutralization of lysostaphin activity in vitro. Serum was serially two-fold diluted in Tris-buffered saline (50 mM Tris, 150 mM NaCl [pH 7.5]) in the wells of microtiter plates. Lysostaphin at a concentration that fully lyzed a culture of approximately 10^6 CFU/mL of MRSA strain 301 in approximately 20 minutes was added to each well. Bacteria for the assay were grown overnight, washed three times in Tris-buffered saline, and added to each well. The serum and lysostaphin were allowed to react in each well for 15 minutes before the bacteria were added. Once bacteria were added, the optical densities (570 nm) were determined every 5 minutes for 90 minutes. The highest dilution of serum that prevented a 25% or more decrease in optical density was considered the end point of the antibody assay. Bacteria in buffer with lysostaphin but without serum served as a negative control. Additional controls included bacteria and normal serum, with or without lysostaphin.

**Bacteria for Ocular Infections**

MRSA 301 has been analyzed in rabbit models of keratitis and endophthalmitis. Cultures were grown overnight in tryptic soy broth (TSB; Difco, Detroit, MI). The overnight culture was subcultured (1:100) in fresh TSB and a log phase culture was grown to an optical density of 0.325 at 650 nm. This logarithmic phase culture was serially diluted in TSB before injection. The final bacterial concentration was confirmed by dilution plating in triplicate on tryptic soy agar (TSA, Difco) plates.

**Infection Models**

Keratitis was initiated by intrastromally injecting 10 μL of log phase culture containing approximately 100 CFU, into rabbit corneas with a 30-gauge needle, as previously described.40 Endophthalmitis was initiated by injecting 0.1 mL of log phase culture containing approximately 50 CFU into the midvitreous cavity of rabbit eyes, as previously described.39

**Treatment Schedule**

Lysostaphin was dissolved in sterile deionized water to a concentration of 1 mg/mL (0.1%) for treatment of endophthalmitis and 3 mg/mL (0.5%) for treatment of keratitis. Rabbit eyes with keratitis were topically treated from 10 to 15 hours after infection with a single topical drop (45 μL) of lysostaphin (3 mg/mL) applied every 30 minutes. Rabbits were killed 1 hour after the last treatment. Rabbit eyes with endophthalmitis were injected in the midvitreous cavity with 0.1 mL of lysostaphin (1 mg/mL) at 8 hours after infection and killed at 24 hours after infection.

**Bacterial Quantification**

The number of viable *S. aureus* per cornea was determined by culturing corneal homogenates in triplicate, as previously described.40 Corneas were aseptically removed and homogenized, and the homogenate and dilutions of the homogenate were cultured in triplicate to determine viable bacterial counts. The number of viable *S. aureus* per milliliter of vitreous humor was determined by culturing vitreous and dilutions of vitreous, as previously described.39 Vitreous humor was removed by aspiration with a 1-ml tuberculin syringe. Each vitreous sample was vigorously vortexed and serially diluted in sterile phosphate-buffered saline (PBS) and cultured in triplicate on TSA plates for viable bacterial counts.

**Pathologic Examinations**

Pathologic evaluation for keratitis involved slit lamp examinations (SLE) of rabbit eyes with a biomicroscope (Topcon; Koakui Kikai KK, Tokyo, Japan) by two masked observers. Each of seven ocular parameters (injection, chemosis, corneal infiltrate, corneal edema, fibrin in the anterior chamber, hypopyon formation, and iritis) was graded on a scale of 0 to 4. The parameter grades were totaled to produce a single SLE score ranging from 0 (normal eye) to a maximum of 28 (most severely affected), as previously described.40 Pathologic scoring of endophthalmitis involved grading rabbit eyes from 0 to 3. A score of 0 described a normal eye; 1, an eye with mild vitreous haze and good red reflex; 2, moderate vitreous haze and partial red reflex; and 3, total opacification of vitreous cavity and loss of red reflex.

**Statistical Analysis**

Data were analyzed on computer, as previously described22,38–40 (SAS, Cary, NC). For CFU determinations, analysis of variance and protected Student’s tests between least-square means from each group were performed. For clinical scores, nonparametric one-way analysis of variance (Kruskal-Wallis test) and Wilcoxon’s test were used for comparison among groups. By conventional standards, the type I error is 0.05 and type II error is 0.20.
RESULTS

Antibody Titers for the Keratitis Model

Rabbits that were subcutaneously, intranasally, or topically immunized for the keratitis experiments produced average anti-lysostaphin antibody titers of 10,240 ± 0, 187 ± 44, and 1,867 ± 102, respectively (Table 1). Sera from immunized rabbits were assayed to determine antibody potency in neutralizing lysostaphin. Sera from subcutaneous immunized rabbits had a lysostaphin neutralization titer of 8 (Table 1). Sera from intranasal or topically immunized rabbits did not demonstrate any inhibition of lysostaphin activity. These findings suggest that only sera with high antibody titers to lysostaphin, as determined by ELISA, were able to neutralize lysostaphin activity, in vitro.

Lysostaphin Therapy for Keratitis in Normal and Immune Rabbits

Immune and nonimmune rabbits were challenged with MRSA 301 and treated topically every 30 minutes with lysostaphin (0.3%). The vitreous humor of eyes of immune rabbits was sterile after treatment, whereas the vitreous humor of untreated eyes contained 6.82 ± 0.09 log CFU/mL (P = 0.0001; Table 2). No significant adverse events or inflammation (allergic reactions) were observed during treatment of the immune rabbits with lysostaphin compared with the nonimmune and untreated rabbits. SLE scores of immune rabbit eyes treated with lysostaphin were not significantly different from those of the untreated group at 16 hours after infection (P = 0.2359).

Table 1. Anti-Lysostaphin Antibody and Neutralization Titers for the Keratitis Model

<table>
<thead>
<tr>
<th>Route of Immunization</th>
<th>Antibody Titer*</th>
<th>Lysostaphin Neutralization Titer†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>10,240</td>
<td>8</td>
</tr>
<tr>
<td>Intranasal</td>
<td>160</td>
<td>0</td>
</tr>
<tr>
<td>Topical</td>
<td>1,280</td>
<td>0</td>
</tr>
<tr>
<td>Normal Sera</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Median anti-lysostaphin antibody from immunized and normal rabbits (≥3 rabbits per group), as determined by antibody-capture ELISA.
† Antibody neutralization of lysostaphin was determined by incubating sera from immune or normal rabbits with lysostaphin before the addition of S. aureus strain MRSA 301. Titer were determined by the lowest dilution of sera that inhibited ≥75% of the lysis of S. aureus.

Table 2. Lysostaphin Treatment of MRSA Keratitis in Normal and Lysostaphin-Immunized Rabbits

<table>
<thead>
<tr>
<th>Route of Immunization</th>
<th>Slit Lamp Score*</th>
<th>Log CFU/Cornea†</th>
<th>Sterile Eyes/Total Eyes (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10 Hours‡</td>
<td>16 Hours‡</td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>5.56 ± 0.37</td>
<td>9.25 ± 0.27</td>
<td>0.72 ± 0.37</td>
</tr>
<tr>
<td>Intranasal</td>
<td>5.13 ± 0.14</td>
<td>8.35 ± 0.36</td>
<td>0.99 ± 0.80</td>
</tr>
<tr>
<td>Topical</td>
<td>5.71 ± 0.14</td>
<td>9.23 ± 0.48</td>
<td>0.50 ± 0.51</td>
</tr>
<tr>
<td>Normal</td>
<td>5.84 ± 0.21</td>
<td>10.55 ± 0.42</td>
<td>0.75 ± 0.46</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.58 ± 0.44</td>
<td>8.75 ± 0.62</td>
<td>0.70 ± 0.10</td>
</tr>
</tbody>
</table>

* Rabbit eyes (n ≥ 6 per group) were examined at 10 and 16 hours after infection by slit lamp examination for pathologic changes.
† Rabbit corneas were treated every 30 minutes with a single drop of lysostaphin (0.3%) from 10 to 15 hours after infection. Corneas were harvested at 16 hours after infection and cultured to determine log CFU/cornea. Corneas of immune and nonimmune rabbits treated with lysostaphin (0.3%) had significantly less CFU compared with the untreated group (P ≤ 0.0001).
‡ Slit lamp examination scores of rabbit eyes before treatment with lysostaphin were not significantly different at 10 hours after infection (P = 0.1412).
§ Scores of immune rabbit eyes treated with lysostaphin were not significantly different than those of the untreated group at 16 hours after infection (P = 0.2359).

Antibody Titers for the Endophthalmitis Model

Rabbits in the endophthalmitis experiments that were immunized subcutaneously had average antibody titers of 1636 ± 213 and those immunized topically had average titers of 137 ± 18 (Table 3). None of the sera from these rabbits had a detectable neutralization titer for lysostaphin activity.

Lysostaphin Therapy for Endophthalmitis in Normal and Immune Rabbits

Rabbits immunized against lysostaphin were challenged with MRSA 301 and treated with a single intravitreous injection of lysostaphin (0.1%). The vitreous humor of eyes of immune rabbits were sterile after treatment, whereas the vitreous humor of untreated eyes contained 6.82 ± 0.09 log CFU/mL (P = 0.0001; Table 4). Pathology scores were not significantly different between the immune rabbits and those of the nonimmune or untreated groups (P = 0.1591; Table 4). No significant

Table 3. Anti-Lysostaphin Antibody and Neutralization Titers for the Endophthalmitis Model

<table>
<thead>
<tr>
<th>Route of Immunization</th>
<th>Antibody Titer*</th>
<th>Lysostaphin Neutralization Titer†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>2048</td>
<td>2</td>
</tr>
<tr>
<td>Intranasal</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Topical</td>
<td>128</td>
<td>0</td>
</tr>
<tr>
<td>Normal Sera</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

* Median anti-lysostaphin antibody from immunized and normal rabbits (≥3 rabbits per group), as determined by antibody-capture ELISA.
† Antibody neutralization of lysostaphin was determined by incubating sera from immune or normal rabbits with lysostaphin before the addition of S. aureus strain MRSA 301. Titer were determined by the lowest dilution of sera that inhibited ≥75% of the lysis of S. aureus.
‡ Not determined.

Table 4. Lysostaphin Treatment of MRSA Endophthalmitis in Normal and Lysostaphin-Immunized Rabbits

<table>
<thead>
<tr>
<th>Route of Immunization</th>
<th>Clinical Score*</th>
<th>Log CFU/Cornea†</th>
<th>Sterile Eyes/Total Eyes (n)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Subcutaneous</td>
<td>2.15 ± 0.29</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Intranasal</td>
<td>1.25 ± 0.69</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Topical</td>
<td>1.58 ± 0.36</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Normal</td>
<td>0.85 ± 0.64</td>
<td>6.82 ± 0.09</td>
<td>0/4</td>
</tr>
</tbody>
</table>

* Rabbit eyes (n ≥ 4 per group) were examined at 24 hours after infection for pathologic changes. Clinical pathology scores of immune rabbit eyes treated with lysostaphin were not significantly different from those of the untreated group at 24 hours after infection (P = 0.1591).
† Rabbits with endophthalmitis were injected at 8 hours after infection with lysostaphin (0.1 mL of a 1% solution). Vitreous humor was collected at 24 hours after infection and cultured to determine log CFU per milliliter. Eyes of immune and nonimmune rabbits treated with lysostaphin (0.1%) had significantly less CFU compared with the untreated group (P ≤ 0.0001).
adverse events were observed after treatment of the immune groups compared with the nonimmune groups.

**Discussion**

The increasing incidence of nosocomial and community-acquired infections attributable to MRSA and quinolone-resistant *S. aureus* strains has prompted the need for continued development of new antistaphylococcal therapies. Lysostaphin has been shown to be an effective therapeutic agent for the treatment of staphylococcal infections. However, rabbits immunized with lysostaphin by any of the routes tested and subsequently challenged with MRSA in the keratitis model responded well to lysostaphin treatment, as evidenced by approximately a 6-log reduction in the CFU per cornea. These findings could alleviate some concerns involving adverse immune-mediated reactions as a result of therapies involving repeated application of this foreign protein.

Treatment of MRSA in Lysostaphin-Immune Rabbits

16. Smith MA, Sorenson JA, Lowy FD, Shakin JL, Harrison W, Jakobic FA. Treatment of experimental methicillin-resistant *Staphylococ-


