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, 1,87, 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.1–5 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.6,7 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,8,9 and approximately 70% of cases occur as a result of intraocular surgery.10 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

Purposes of this study were (1) to determine the effects of immunization against lysostaphin on the bactericidal action of lysostaphin in ocular tissue and the possible induction of allergic reactions, and (2) to determine the effectiveness of lysostaphin treatment of MRSA keratitis and endophthalmitis in rabbits immunized with S. aureus, methicillin-resistant S. aureus (MRSA), and as being essentially free of adverse effects.12

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)

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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 lyse S. aureus by cleaving glycine–glycine bonds, thereby disrupting the peptidoglycan layer of the cell wall.24–51 Lysostaphin was studied in the 1960s and 1970s as a potential therapeutic agent in a number of animal models.25–27,30,32,33 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 antibacterial therapeutic agent, because antibiotic resistance has 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

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Antibody titer was achieved. For intranasal immunization, rabbits re-
mixed with incomplete Freund’s adjuvant buffer (10 mM Na₂CO₃ and 35 mM NaHCO₃ [pH 9]) and placed into
(45 month for 5 months. For topical immunization, rabbits received 1 drop
passages for three consecutive days and were similarly boosted every
last administration of immunogen, and the titers were determined by
for 7 consecutive days. Immunized rabbits were bled 30 days after the
consecutive days, and then, after 30 days, lysostaphin was again ap-
daily for 14 days. After another 30 days, lysostaphin was applied
length of 410 nm.
sorbance of the wells of the microtiter plates were read at a wave-
alkaline phosphatase (Sigma) diluted 1:500 in blocking buffer was
rate sites on the side and back of the rabbit. Rabbits were subsequently
immunizations were performed by injecting 400 μg of lysostaphin
(Sigma) mixed with complete Freund’s adjuvant (Sigma) at four sepa-
ate 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 re-
ceived 0.1 mL of lysostaphin (1000 μg/mL) instilled 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 ap-
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 anti-
body-capture ELISA. Lysostaphin (10 μg/mL) was dissolved in carbon-
ate 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 ab-
sorbance of the wells of the microtiter plates were read at a wave-
length of 410 nm.

Antibody Neutralization Assay
Serum from rabbits immunized with lysostaphin were assayed for neutral-
ization 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 lyed a culture of approximately 10⁷ 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 lys-
ostaphin.
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 (0.1%). Corneas of immune and nonimmune rabbits treated with lysostaphin (0.5%) had less than 1 log CFU compared with the untreated group that had 7.07 ± 0.10 log CFU/cornea (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).

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 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 (≥5 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>0.72 ± 0.37</td>
<td>7/10</td>
</tr>
<tr>
<td>Intranasal</td>
<td>5.13 ± 0.14</td>
<td>0.99 ± 0.80</td>
<td>4/6</td>
</tr>
<tr>
<td>Topical</td>
<td>5.71 ± 0.14</td>
<td>0.50 ± 0.51</td>
<td>4/5</td>
</tr>
<tr>
<td>Normal</td>
<td>5.84 ± 0.21</td>
<td>0.75 ± 0.46</td>
<td>4/8</td>
</tr>
<tr>
<td>Untreated</td>
<td>5.58 ± 0.41</td>
<td>0.54 ± 0.42</td>
<td>0/7</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 = 0.2539).

### 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 (≥5 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>Subcutaneous</td>
<td>2.15 ± 0.29</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Topical</td>
<td>1.25 ± 0.69</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Normal</td>
<td>1.58 ± 0.36</td>
<td>0.0 ± 0.0</td>
<td>4/4</td>
</tr>
<tr>
<td>Untreated</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 1.0% 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.\(^{25-27,30,32,33,35-35,41}\) We have reported lysostaphin to be an effective agent for treatment of experimental MRSA keratitis and endophthalmitis.\(^{38,39}\) Although treatment of these infections with lysostaphin can nearly sterilize or completely sterilize the eye, concerns have been raised regarding the immunogenicity of lysostaphin in terms of its safety and efficacy.\(^{27}\) The present study was undertaken to detect any deleterious immune-mediated effects of repeated application of this foreign protein to the host.

To determine the most effective route for the production of anti-lysostaphin antibodies, specific-pathogen-free rabbits were immunized through three different routes (i.e., subcutaneous, topical, and intranasal). The highest anti-lysostaphin titers were observed in those rabbits immunized subcutaneously. Repeated topical application of lysostaphin produced a moderate antibody titer whereas intranasal immunization, even though it produced low-titered serum and titers were observed in those rabbits immunized subcutaneously, did not produce significant antibody titers.

Previous studies have demonstrated that lysostaphin was well tolerated by rabbits, in some cases with prolonged intravenous treatments for up to 9 weeks.\(^{35}\) Although antibodies developed in the rabbits after prolonged treatment, lysostaphin persisted in exhibiting high levels of bactericidal activity in the serum of treated animals.\(^{35-41}\) Previous studies have shown that only minimal adverse reactions were observed in studies with administration of multiple intravenous doses of lysostaphin.\(^{35,41}\)

The bactericidal activity of lysostaphin on MRSA was inhibited in vitro when sera with high anti-lysostaphin titers were incubated with lysostaphin; however, low-titered serum and preimmune serum did not neutralize lysostaphin. The high ELISA titers yet low neutralization titers of the serum from immunized rabbits may reflect a minimal number of antibody molecules specific for epitopes within or adjacent to the catalytic site of lysostaphin. The low neutralization titers explain the therapeutic effectiveness of lysostaphin in rabbits with high antibody titers. These rabbits had received substantial doses of lysostaphin over a prolonged interval, suggesting that the repeated use of lysostaphin is not likely to induce an antibody capable of neutralizing its bactericidal activity.

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 a 6-log reduction in the CFU per cornea. Similar results were observed in rabbits treated for endophthalmitis, with the vitreous being sterilized after a single lysostaphin treatment regardless of immune status. Although these rabbits received multiple immunizations and achieved substantial serum antibody titers, their anti-lysostaphin antibody titers before and after the last immunization were nearly identical, suggesting that a near-maximal titer had been approached. Thus, the high titer anti-lysostaphin antibody state of the rabbits did not interfere with the therapy afforded by topical or intravitreous administration of lysostaphin.

Although lysostaphin is highly effective in reducing bacterial numbers in the cornea and vitreous, it has not been shown to significantly reduce the disease (as measured by SLE scoring) associated with keratitis or endophthalmitis. However, rabbits immunized and subsequently treated with lysostaphin displayed no observable differences in ocular disease in comparison with nonimmune control animals in either model tested. These results suggest that there was no increase in disease attributable to antibody-antigen reactions involving lysostaphin and the elicited anti-lysostaphin antibodies in these rabbits. These findings could alleviate some concerns involving adverse immune-mediated reactions as a result of therapies involving repeated application of this foreign protein. These data demonstrate that lysostaphin is able to retain its bactericidal activity in vivo, despite the presence of high neutralizing antibody titers without an undesirable immune reaction and thus could become a viable form of therapy in cases of MRSA keratitis or endophthalmitis.

**References**


