The authors compared 24 doses of hourly topical fortified tobramycin (Group A) therapy with a single subconjunctival administration of multivesicular megaliposome-encapsulated tobramycin (Group B) and free subconjunctival tobramycin (Group C) in treating a rabbit model of keratitis caused by *Pseudomonas aeruginosa*. One cornea each of 50 rabbits was infected with *P. aeruginosa* for 24 hr. The animals then were divided randomly into five groups of ten each. Groups A, B, and C were treated as described. Group D received liposomes without tobramycin and Group E, hourly balanced salt solution. Significantly fewer *Pseudomonas* colonies were present in the corneas of all three drug-treated groups (A, B, and C) compared with the two control groups (D and E) at 24 hr (P < 0.005). Significantly fewer *Pseudomonas* colonies were present in Groups A and B compared with Group C (P < 0.02). No significant difference was noted between Groups A and B (P = 0.30). Tobramycin encapsulated in megaliposomes may be useful in treatment of pseudomonal keratitis. Invest Ophthalmol Vis Sci 32:3216-3220, 1991

Pseudomonal corneal ulcers are rapidly progressive and destructive. Immediate treatment is essential if useful vision is to be retained. Aminoglycosides given topically every 30-60 min commonly are used to treat this condition. However, compliance with such a schedule of frequent dosing may be difficult, particularly in children or elderly or disabled patients.

Liposomes are biodegradable lipid vesicles that may permit less frequent dosing by delaying the release of the encapsulated drug. High levels of gentamicin were found in the cornea when liposomes containing this drug were administered by subconjunctival injection. However, the clinical efficacy of liposome-encapsulated aminoglycosides in treatment of pseudomonal keratitis has not been studied to our knowledge. We compared the efficacy of frequent topical fortified tobramycin with single subconjunctival liposome-encapsulated tobramycin and single subconjunctival tobramycin without liposomes to treat a rabbit model of keratitis caused by *Pseudomonas aeruginosa*.

**Materials and Methods**

One cornea in each of 50 albino New Zealand rabbits (weight range, 1.8–2.0 kg) was infected with *P. aeruginosa*, strain number 3, a clinical blood isolate. The tobramycin minimal inhibitory concentration of this isolate was 1.0 μg/ml. The animals then were placed randomly into five treatment groups of ten rabbits each. Twenty four hours later, treatment was initiated. Group A received an hourly topical 50-μl drop of fortified tobramycin solution (14.5 mg/ml) for 24 hr (total dose of tobramycin, 17.4 mg). These animals served as the standard for comparison.

Group B received a single subconjunctival application of 0.4 ml of liposome-encapsulated tobramycin (dose, 14.0 mg). Group C received 20.0 mg of subconjunctival tobramycin (40 mg/ml) without liposomes. Group D received 0.4 ml of subconjunctival liposomes without tobramycin. Group E received hourly topical balanced salt solution only.

**Multivesicular Liposomes**

For Group B, 0.4 ml per treated eye of multivesicular liposomes (10–100 μm in diameter) were synthesized as previously described with the following modifications. Tobramycin solution (Alcon, Fort Worth, TX) for liposome encapsulation was prepared at 35...
mg/ml, pH 8.50. We added 1 ml dropwise to a vial containing the chloroform–lipid mixture. The chloroform was evaporated from the liposomal membranes by drying with nitrogen (81/min) in a flask containing 250 mM sucrose. We then added 25 ml of 290 mM sodium chloride, and this mixture was centrifuged at 600 × g for 10 min, producing a pure liposomal pellet at the bottom. The supernatant was withdrawn, and the liposomes were resuspended in the sodium chloride and recentrifuged. This procedure was repeated three times to remove unencapsulated drug. The liposomal tobramycin content for drug-capture efficacy was determined using an enzyme-multiplied immunoassay test (EMIT-SYVA, Palo Alto, CA).6 The samples were assayed using a Roche Cobos Fara centrifugal analyzer (Hoffman LaRoche, Montclair, NJ). We found the drug content capture and efficacy to be 35 mg/ml and 60%, respectively. The liposomes for Group D rabbits were synthesized as described except no tobramycin was added during their preparation.

Infection and Treatment

P. aeruginosa was grown overnight at 37°C in trypticase soy broth. The inoculum then was diluted with broth to yield 6.5 × 10^3 colony-forming units/ml.

Each animal received an intramuscular injection of xylazine 8.8 mg/kg and ketamine 50 mg/kg. We modified previous methods7,8 to produce pseudomonal keratitis in one eye of each rabbit. We injected 50 μl of the bacterial suspension containing 350 colony-forming units into the central corneal stroma of each animal with a 30-gauge needle on a microsyringe. The corneal epithelial surface then was scratched (six scratches) with the needle tip.

Twenty-four hours later, all eyes had a diffuse necrotizing sclerokeratitis with marked corneal infiltration and mucopurulent discharge. Drug therapy was initiated at this time. The animals were assigned randomly to five groups of ten rabbits each. After removal of the mucopurulent discharge from the corneal surface, Group A received hourly topical fortified tobramycin (14.5 mg/ml). Group B received 0.4 ml of liposomes (containing 14.0 mg tobramycin) administered subconjunctivally to the superotemporal quadrant. Groups C and D underwent procedures as described for Group B, except that Group C received free drug (20 mg) without liposomes and Group D received liposomes without tobramycin. In Group E, balanced salt solution was administered topically on an hourly basis.

Sampling

Five animals in each group were killed with pentobarbital at 12 and 24 hr after treatment began. Topical therapy was terminated 1 hr antemortem. The entire cornea was excised at the clear limbal margin, excluding any sclera or conjunctiva, rinsed with 5 ml of balanced salt solution, minced, placed in a test tube with 2 ml of trypticase soy broth, and homogenized. We removed a 0.1-ml sample of supernatant, and this was diluted serially from 10^{-1} to 10^{-6} with trypticase soy broth. We plated 100-μl samples of each dilution in duplicate onto 5% rabbit blood agar, and the number of colonies was counted after 24 hr. The final numbers were calculated, averaged for each duplicate pair, and expressed as relative viable bacteria per cornea.

The Mann-Whitney test was used to evaluate intergroup differences in the corneal colony counts. We considered a P value < 0.02 to be significant.

These studies were conducted in accordance with the ARVO Resolution on the Use of Animals in Research.

Results

At death, all eyes inoculated with Pseudomonas had marked corneal infiltrates and central stromal necrosis with no obvious clinical difference between the treated and untreated animals by gross inspection.

There were significantly fewer colonies of bacteria present in the corneas of all drug-treated groups compared with the two untreated control groups at 12 and 24 hr (P < 0.02). The differences in colony counts among the three treated groups (A, B, and C) were not significant at 12 hr (P > 0.05). The mean log of the bacterial colony counts, along with the standard deviation for each group, at 24 hr, appears in Figure 1. Table 1 shows the mean log of bacterial colony counts in each group at 12 and 24 hr and compares the significance of colony counts present after 24 hr of therapy in Group A with each of the other groups. Of the 15 eyes in Groups A, B, and C, 10 corneas grew more than 2 × 10^3 colonies of bacteria after 12 hr of therapy, and at least one eye in each group had more than 3 × 10^4 colonies of Pseudomonas still present at that time.

At 24 hr of therapy, the untreated control animals in Groups D and E had approximately 6.65 and 5.67 mean log colony-forming units per cornea, respectively. By contrast, Group C eyes (which received a single subconjunctival unencapsulated tobramycin dose) had significantly fewer bacterial counts (mean log, 2.76 colony-forming units; P < 0.02). Treated eyes in Groups A and B (which received 24 doses of hourly topical fortified tobramycin and a single dose of liposome-encapsulated tobramycin, respectively) had even fewer colony counts at 24 hr (Table 1, Fig. 1). All corneas in these two groups had fewer than 300 colony-forming units of Pseudomonas present after 24 hr of therapy. The further decrease in colony counts observed in these two groups compared with
the eye. Small-diameter, negatively charged liposomes containing gentamicin were injected subconjunctivally in uninflamed rabbit eyes; this route was shown to provide sustained delivery of the drug to the cornea for 24 hr. The multivesicular liposomes we used were developed for sustained drug delivery to the eye because they: (1) can be prepared readily as a pure paste, (2) contain relatively large quantities of drug, (3) provide prolonged drug transit time, and (4) remain localized at the administration site. These liposomes can be synthesized using sterile technique and have a high drug-capture efficacy (greater than 60% for tobramycin) and a relatively stable shelf life.

We examined the efficacy of several delivery systems for tobramycin in treating a rabbit model of pseudomonal keratitis. There was no obvious clinical difference in corneal appearance at the conclusion of therapy (24 hr) between drug-treated and control eyes. Diffuse coagulative necrotizing sclerokeratitis from the heavy inflammatory process was present before therapy, and we would expect persistence of acute inflammation for several days, even in eyes sterilized by therapy. Culture results showed that a single subconjunctival injection of multivesicular megaloliposomes containing tobramycin and 24 doses of hourly topical fortified tobramycin were both effective in treating pseudomonal keratitis in this model.

In comparison with untreated controls (Groups D and E), a single dose of subconjunctival, unencapsulated tobramycin (20.0 mg in Group C) produced a near 1000-fold decrease in the number of colony-forming units of Pseudomonas in the cornea. Furthermore, when administered in the liposomes, a greater than 10,000-fold decrease in colony-forming units was achieved after a single administration (14.0 mg in Group B). These decreases in colony counts were both significant ($P < 0.02$). The further improvement observed in infection control with the administration of 24 hourly doses of topical fortified tobramycin (Group A) compared with a single subconjunctival administration of liposome-encapsulated tobramycin (Group B) was not significant ($P = 0.30$). This ob-

**Table 1. Intergroup comparison of colony-forming units (CFU)**

<table>
<thead>
<tr>
<th>Group X vs. Group A at 24 h (Mann-Whitney test)</th>
<th>Mean log CFU*</th>
<th>Group (n = 5)</th>
<th>12 hr</th>
<th>24 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>2.61</td>
<td>0.45</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>4.10</td>
<td>1.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>4.38</td>
<td>2.76</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7.29</td>
<td>6.65</td>
<td></td>
<td></td>
</tr>
<tr>
<td>E</td>
<td>7.10</td>
<td>5.67</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Mean log of CFU for each group, 24 hr postinoculation of treatment (48 hr postinoculation with *Pseudomonas*).
erved difference in the number of colonies recovered may be a result of the mechanical lavage, provided by hourly topical drops (Group A) compared with subconjunctival administration (Group B). In this regard, we found that, among untreated control rabbits (Groups D and E), animals receiving hourly topical balanced salt solution (Group E) had one order of magnitude fewer bacteria present, on average, than those receiving single subconjunctival therapy with empty liposomes (Group D; Table 1). This potential benefit of such frequent topical therapy should not be overlooked because it may wash away proteolytic enzymes and bacteria, helping to minimize tissue destruction.

When administered in liposomes, a smaller amount of drug (14 mg in Group B) achieved better results than did a higher dose given without liposomes (20 mg in Group C). This observation may be related, in part, to the slow, steady release of the drug by liposomes over the course of the study. Alternatively, the liposomes may block local lymphatic and vascular drainage pathways, resulting in elevated drug levels at the injection site.46-47 Another possibility is that a significantly greater amount of drug is lost quickly through the needle tract when administered without liposomes. Finally, it is possible that the liposomes may obstruct the nasolacrimal drainage system,30 thus decreasing the clearance rate by that route.

Twenty-four hours after treatment began (48 hr after inoculation with Pseudomonas), all eyes treated with either hourly fortified topical tobramycin (Group A) or a single subconjunctival liposome-encapsulated tobramycin (Group B) were nearly sterilized (<300 colony-forming units in each). Although this study suggests that, with appropriate therapy, corneas may become sterilized within 24 hr, 12 hr of therapy is not adequate. Ten of 15 eyes (Groups A, B, and C) grew more than $2 \times 10^5$ colonies of bacteria after 12 hr of therapy (36 hr after inoculation with Pseudomonas). At least one eye in each group had greater than $3 \times 10^5$ colonies of Pseudomonas still present at that time. Furthermore, among the three drug-treated groups, no single form of therapy was significantly superior to any other at 12 hr ($P > 0.05$). Finally, there was a significant further decrease ($P < 0.02$) in colony counts observed in each of these three groups after 24 hr (versus 12 hr) of treatment.

Although these data suggest that, even without liposomes, a single subconjunctival injection of free drug (Group C) may provide persistence of bactericidal activity beyond 12 hr, such persistence may not be the only factor responsible for the decline in Pseudomonas colonies between 12 and 24 hr of therapy. Even among the untreated eyes in Group E, there were higher numbers of bacterial colonies present at 12 hr (mean log, 7.10) than at 24 hr (mean log, 5.67) after initiation of "therapy." This apparent spontane-ous decline by 48 hr after inoculation with bacteria (24 hr after intervention) may reflect the natural delay in mounting a maximal immune response. Alternatively, the number of Pseudomonas colonies present 36 hr after initiation of infection may represent the maximum number of bacteria that can be supported by the substrate (cornea). When suspended in nutrient broth, for example, Pseudomonas colony-forming units will increase in number over several days to approximately $1 \times 10^6$; after this time, they spontaneously diminish in number.

We believe that subconjunctival administration is the simplest route for prolonged drug delivery to the ocular surface from liposomes. In a separate study (submitted for publication) evaluating topically administered liposomes, we found that single topical administration of liposome-encapsulated tobramycin was not as effective as frequent therapy with fortified drops. Although single topical therapy with liposomes became more effective when the liposomes were emmeshed in a fibrin sealant, this form of application was technically more demanding than subconjunctival injection.

This study indicates that a single subconjunctival administration of tobramycin produces a substantial decrease in corneal bacterial counts in a rabbit model of pseudomonal keratitis. Furthermore, when delivered within multivesicular megaliposomes, tobramycin is significantly more effective than subconjunctival drug administered alone; it is nearly as effective as 24 doses of hourly topical fortified drug in this model. By diminishing the need for frequent administration, treatment compliance may be improved. This modality also may be beneficial in treating pediatric, disabled, and otherwise poorly compliant patients, or it may serve a useful adjunctive role to frequent topical therapy in treatment of keratitis caused by *P. aeruginosa*. Further studies evaluating the toxicity and pharmacokinetics of this system must be done.

Key words: antibiotic, keratitis, multivesicular liposome, *Pseudomonas aeruginosa*, tobramycin

Acknowledgments

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References