isms requiring complement activation, but may also be advantageous in inhibiting destructive autoimmune processes requiring complement activation.

Key words: complement, complement inhibitors, cornea, aqueous humor

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References


**Pharmacokinetics of Subconjunctival Liposome-Encapsulated Gentamicin in Normal Rabbit Eyes**

Michael Darza,* Jules Baum,† and Francis Szoka, Jr.$

Subconjunctival injections of antibiotics produce very high corneal levels of drug that fall rapidly as the drug is dissipated. The authors studied the effects of liposome-encapsulation as a means of slowing release from the subconjunctival depot. Liposomes (0.1–1.0 μm) were made of phosphatidic acid, phosphatidylcholine, and α-tocopherol. The final suspension contained gentamicin 10 mg/ml with 60–70% encapsulated. Rabbits were given a single subconjunctival injection of liposome-encapsulated gentamicin, gentamicin with "empty" liposomes, or gentamicin alone. In each instance the dose of antibiotic was 5 mg. Gentamicin levels in the sclera and cornea, measured 3, 9, and 24 hr after injection, were generally markedly higher with the liposome-encapsulated drug than with the other two preparations. The differences were 5- to 20-fold in the cornea at 24 hr and were statistically significant for temporal cornea. Liposome-encapsulation may be a useful means of extending the effects of a subconjunctival injection of antibiotic. Invest Ophthalmol Vis Sci 25:486-490, 1984

Subconjunctival injections of antibiotics produce very high concentrations in the cornea. However, the levels fall off rapidly and are often low or undetectable 12–24 hr after injection.† Frequent topical applications of antibiotic, by producing modest but sustained concentrations in corneal tissue, may furnish as great an antibacterial effect as intermittent subconjunctival injections.‡ If, however, it were possible to insure a more gradual release of drug from the subconjunctival depot, persistently high corneal concentrations might be achieved that conceivably could be of benefit in the treatment of refractory infections.

Liposomes are small, biodegradable lipid vesicles with an aqueous core. Incorporation of drugs into liposomes provides a convenient way to retard their release from a relatively inert depot without changing the intrinsic characteristics of the agents.§ The rate of drug release will depend upon the size and composition of the liposomes. Recently, topically applied liposomes containing penicillin G were reported to enhance the transcorneal flux of the antibiotic, a phenomenon thought to have resulted from absorption of liposomes to the cornea with direct transfer of drug to the epithelial cell membrane.¶ In the present study, we examined the possibility that liposome-encapsulation might lead to more sustained concentrations of antibiotic in the cornea after subconjunctival injection.

**Materials and Methods.** Injectable gentamicin sulfate, 40 mg/ml, was obtained from Schering Corporation, Kenilworth, NJ, for these experiments. Phosphatidic acid and phosphatidylcholine were obtained from Avanti Polar Lipids (Birmingham, AL); α-tocopherol was from Sigma (St. Louis, MO).

Liposomes with a net negative charge were prepared of phosphatidic acid, phosphatidylcholine, and α-tocopherol in a molar ratio of 1/19/0.22 by the reverse-
phase evaporation method. The diameter of the vesicles was 0.1-1.0 μm. The initial preparation contained 50 μmoles of lipid per ml of the gentamicin sulfate solution (40 mg/ml). Nonencapsulated gentamicin was removed partially by dialysis against a 100-fold volume excess of phosphate-buffered saline (PBS). Typical preparations entrapped 25% of the gentamicin sulfate.

Samples of the suspension were diluted in PBS with or without Triton X-100 0.2% and were subjected to agar-diffusion bioassay using *Bacillus subtilis* ATCC 6633 as the test organism. Preliminary experiments showed that this concentration of Triton X-100 completely disrupted the liposomes, but produced only a faint zone of inhibition in the assay system. Standards of gentamicin in PBS were prepared with and without Triton X-100. The liposomal preparation used in the study was shown to contain gentamicin 14 mg/ml. Comparison of the results of assays with and without Triton X-100 revealed that 60-70% of the antibiotic was encapsulated, depending upon the preparation. The stock suspension was diluted in PBS to a gentamicin concentration of 10 mg/ml for the subsequent experiments.

As a control for the effect of liposomes upon the pharmacokinetics of extraliposomal gentamicin, empty liposomes were prepared in PBS as described above, but without antibiotic. Gentamicin was then added to yield a final concentration of 10 mg per ml of liposomal suspension.

**Injections and Assay:** Pigmented rabbits weighing 1.5-2.5 kg, were used in these experiments. All studies were carried out in accordance with the ARVO Resolution on the Use of Animals in Research (as stated in Invest Ophthalmol Vis Sci 24:1156, 1983). The animals were given a single subconjunctival injection of one of three preparations in one eye: (1) liposome-encapsulated gentamicin; (2) empty liposomes plus gentamicin; or (3) gentamicin in PBS. In each instance the subconjunctival dose contained 5 mg of gentamicin in a volume of 0.5 ml and was delivered superiorly 3-4 mm from the limbus through a 27-gauge needle. Animals were tranquilized before injection with ketamine hydrochloride 50 mg/kg and the eye was anesthetized with proparacaine eyedrops (0.5%).

The rabbits were killed by an intravenous bolus of pentobarbital 3, 9, or 24 hr after antibiotics injection. Six animals were studied with each preparation at each time interval. A specimen of blood was obtained for antibiotic assay 0.5 hr after subconjunctival injection and at the time of death. The aqueous humor was aspirated into a syringe and set aside. The eyes were enucleated promptly and any residual bleb was removed carefully. The globe was rinsed briefly in PBS and was dissected as described previously. A 6-mm trephine was used to excise three discs from the superior, temporal, and nasal cornea. The vitreous humor was removed. The sclera was divided into four segments from which the attached choroid-retina was scraped. Discs of tissue were trephined from superior, inferior, temporal, and nasal sclera. The iris was likewise divided into four segments.

The tissues were assayed by the modified trephine-disc bioassay. The samples were weighed and placed on seeded agar plates. The zones of inhibition after overnight incubation were compared with those produced by standards absorbed by filter-paper discs. The inferred concentrations were corrected for differences in the amount of fluid contained in the tissue samples and in the filter-paper discs. We have shown previously that correction for differences in fluid content alone produced bioassay results that were within 13% of the radioactive assay value for gentamicin in nonpigmented ocular tissues. This technique measures the concentration of diffusible, bioactive drug and would fail to detect antibiotic that might be present within liposomes in the tissues. Triton X-100 was not added to the samples because of the uncertainty that it would penetrate into the tissue.

Aqueous and vitreous humor were assayed by agar-diffusion bioassay. Standards were prepared in PBS for the ocular humors and in pooled normal rabbit serum for the serum samples. The test organism was *B. subtilis* ATCC 6633 in all instances. The lower limit of sensitivity of the assay was approximately 0.1-0.2 μg/ml for fluids and 0.4 μg/g for tissues. To test for the presence of liposome-encapsulated drug in ocular fluids and serum, additional aliquots were assayed separately after addition of Triton X-100 in a final concentration of 0.2%, and compared with standards containing Triton X-100.

**Results.** Figure 1 shows the concentrations of gentamicin in superior and temporal sclera. The levels produced by injection of liposome-encapsulated gentamicin were clearly higher than those with the other preparations at all intervals. The mean and SE of concentrations in temporal and superior segments 24 hr after injection were 35 ± 15 μg/g and 26 ± 10 μg/g, respectively, with the liposome-encapsulated drug and less than 5 μg/g with the other preparations. The differences between the liposome-encapsulated drug and the other two preparations were significant (*P* < 0.05 by unpaired *t*-test) for temporal and superior sclera.

Figure 2 shows the concentrations of gentamicin in the temporal and superior cornea. The differences among the preparations were somewhat less marked than in the sclera. Nonetheless, corneal levels of gentamicin were higher after administration of liposome-encapsulated drug at all intervals except the 9-hr one. The effect is more striking when the data for the three segments of cornea at 24 hr are shown in detail (Figure 3). Levels with the liposome-encapsulated drug were about 5-fold higher than those with the other prepa-
Fig. 1. Antibiotic levels in temporal and superior sclera of pigmented rabbits with normal eyes after subconjunctival injection of 5 mg of gentamicin as liposomal gentamicin, gentamicin plus empty liposomes, or gentamicin in buffered saline (genta). Each point is the mean and SE of six values.

Fig. 2. Antibiotic levels in temporal and superior cornea of pigmented rabbits with normal eyes after subconjunctival injection of 5 mg of gentamicin as liposomal gentamicin, gentamicin plus empty liposomes, or gentamicin in buffered saline (genta). Each point is the mean and SE of six values.
Gentamicin concentrations in the aqueous humor 3 hr after injection were 2.7 ± 0.7 μg/ml with the liposome-encapsulated drug, 4.6 ± 0.7 μg/ml with gentamicin plus "empty" liposomes and 7.2 ± 1.6 μg/ml with gentamicin plus PBS. The differences were not statistically significant. No drug was detectable in the aqueous humor at the 9- and 24-hr intervals. There was no measurable gentamicin in the vitreous humor with any preparation at any time. Addition of Triton X-100 did not affect the assay results, suggesting that there was little or no liposome-encapsulated drug in these fluids.

The choroid-retina and iris contained low levels of gentamicin (mean values up to 3 μg/g) at the 9- and 24-hr intervals when liposome-encapsulated drug was administered. The other preparations generally produced no detectable concentrations in these tissues.

Gentamicin levels in the serum 0.5 hr after injection were lower with liposome-encapsulated gentamicin (1.4 ± 0.1 μg/ml) than with gentamicin plus empty liposomes (4.3 ± 0.5 μg/ml) or gentamicin in PBS (6.5 ± 0.5 μg/ml). By 3 hr, mean levels were 0.8–1.1 μg/ml with the three preparations. Addition of Triton X-100 did not increase the values.

**Discussion.** Topical applications of antibiotics produce adequate tissue levels for the treatment of most corneal infections. However, subconjunctival injections clearly produce much higher corneal concentrations that could be of clinical value in certain relatively resistant infections. Unfortunately, antibiotics are quickly dissipated from the subconjunctival depot and corneal levels fall rapidly. Liposome-encapsulation, by retarding antibiotic release from the depot, offers the potential of producing more sustained concentrations in the cornea.

The results of the present study confirm this hypothesis. Levels in sclera and cornea following subconjunctival injection were markedly higher with the liposome-encapsulated drug than with gentamicin alone or gentamicin plus empty liposomes. Indeed, 24
hr after injection, corneal levels were 5- to 20-fold higher with the liposome-encapsulated drug than with the other two formulations. The differences were statistically significant for temporal cornea, but not for superior or nasal cornea.

The presumed mechanism of this effect is delayed release of gentamicin from the liposomes. Because of their size (0.1-1.0 μm), it seems doubtful that the liposomes themselves migrated into the cornea. In a separate set of experiments, we injected 125I-liposomes (label on p-hydroxybenzamidinophosphatidylethanolamine) of a similar composition to that used in these studies in a volume of 0.1 ml subconjunctivally in four normal rabbit eyes and dissected the eyes 3 hr later. We found a mean of 0.8% (range 0-2%) of the injected dose in the cornea, 3% (range 2-4%) in the sclera, none in choroid-retina, aqueous or vitreous, 38% (range 27-57%) in the bleb, and 7% (range 3-11%) in the serum (unpublished experiments). Thus, 43% of the injected dose could be accounted for and less than 1% was found in the cornea. Presumably some of the remainder leaked from the injection site into the tears. In the present experiments, the addition of Triton X-100 to samples of aqueous humor or serum failed to increase the measurable concentrations of gentamicin in these fluids. Taken together, these data suggest that virtually all of the gentamicin that reaches the cornea is in the form of free (nonliposomal drug). Indeed, small, sonicated, unilamellar liposomes of 0.03-0.08 μm in diameter are reported to be unable to traverse continuous capillaries into the extravascular space although they may be taken up by circulating or fixed phagocytic cells.9

The concentrations of gentamicin in the aqueous humor were low and in the vitreous humor were undetectable, with liposomal as well as nonliposomal preparations. This suggests that the liposomes did not facilitate drug entry across intact cellular membranes. In vitro experiments have suggested that liposomes may adhere to the corneal epithelium. This could conceivably facilitate the transcorneal flow of drugs.8,10 These effects appear to be more prominent with positively charged liposomes rather than the negatively charged ones used in our study. We are unable to estimate the proportion of gentamicin or liposomal gentamicin that might have entered by passage across the corneal epithelium rather than by subepithelial diffusion in this study.

If the results of liposomal-encapsulation of gentamicin are presumed to relate mainly to slow release of drug from the depot, it may seem surprising that gentamicin levels in cornea and sclera were higher with this preparation than with the others as early as 3 hr after administration. Possibly, a large proportion of gentamicin is dissipated from nonliposomal preparations even within this brief period. This is consistent with the finding that serum levels were fivefold higher with the control preparations than with liposome-encapsulated gentamicin 0.5 hr after injection, whereas there was no significant difference in serum levels by 3 hr. Evidently, enough gentamicin remained within liposomes in the subconjunctival bleb to continue to release drug into the cornea up to 24 hr.

Choroid-retina and iris showed low concentrations of gentamicin with liposome-encapsulated drug and no detectable antibiotic with the other formulations. This is probably related to the capacity of these pigmented tissues to bind gentamicin in a nondiffusible manner.8

Taken together, these data suggest that liposome-encapsulation may be a useful means of extending the effect of a subconjunctival injection of antibiotic so as to produce sustained, high corneal levels. Other liposomal formulations, eg, ones containing cholesterol, might be anticipated to produce even slower release and more prolonged levels that could be of clinical benefit.

Key words: pharmacokinetics, subconjunctival, liposome, gentamicin

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