Reduced Toxicity of Liposome-Associated Amphotericin B Injected Intravitreally in Rabbits

Claude Tremblay,*† Michael Barza,*‡ Francis Szoka,§ Moshe Lahav,|| and Jules Baum‡

The ocular toxicity of liposome-intercalated amphotericin B and commercial amphotericin B were compared after intravitreal injection in healthy pigmented rabbits. Ophthalmoscopic observations over 5 weeks following a single intravitreal injection showed vitreal band formation and focal retinal damage after doses of commercial amphotericin B as low as 5 µg. Such lesions were not seen in animals given liposomal amphotericin B in doses up to 20 µg. Histopathologic examination showed areas of retinal atrophy or necrosis in five of 16 rabbits given commercial amphotericin B in doses of 5–20 µg but in none of 16 rabbits given the same doses of liposomal amphotericin B (P = 0.02). Small white vitreal bodies were seen clinically in virtually all animals given liposomal amphotericin B or “empty” (drug-free) liposomes but in only a few animals given commercial amphotericin B; these deposits may represent residual lipid. Concentrations of amphotericin B ranged from 0.4 to 1.0 µg per ml of vitreous humor 5 weeks after injection of 5–20 µg of either formulation. These studies indicate that liposome association markedly reduces the ocular toxicity of amphotericin B. Invest Ophthalmol Vis Sci 26:711–718, 1985

Amphotericin B is a drug of choice for a number of serious fungal infections of the eye including Candida endophthalmitis, postsurgical fungal endophthalmitis, and mucormycosis, and is an alternative to natamycin in the treatment of fungal keratitis.1–3 Unfortunately, intraocular penetration of amphotericin B is poor following systemic administration in rabbits,4 although modest levels were found in the aqueous and vitreous humor of two patients whose eyes were enucleated because of severe fungal infection.5 Subconjunctival injection of amphotericin B penetrates the ocular humors of rabbits very poorly6 and is prone to produce severe local tissue reactions.6

Direct intravitreal injection of amphotericin B is attractive because it produces high concentrations at the site of infection in fungal endophthalmitis. Axelrod et al7 found that normal rabbits tolerated intravitreal injections of 10 µg of amphotericin B without toxicity if the drug was administered slowly into the center of the vitreous humor. In contrast, Souri and Green8 reported marked retinal damage in this same species with intravitreal doses as low as 1 µg. In one patient with Candida albicans endophthalmitis, intravitreal injection of 5 µg of amphotericin B appeared to produce no residual ocular toxicity.8

Recent studies have indicated that incorporation of amphotericin B into liposomes markedly reduces the acute toxicity of the drug while retaining efficacy in animal models of fungal infection.9–11 We have developed a liposomal formulation of amphotericin B, which is approximately one-fifth as toxic as the commercial drug in terms of acute lethality after intravenous injection in mice but is fully active in vitro and in the treatment of systemic candidiasis in mice.11a In the present study we have compared the ocular toxicity of commercial amphotericin B with that of our liposomal formulation after intravitreal injection in rabbits.

Materials and Methods

Drug Preparations

Amphotericin B was obtained from the Squibb Institute (Princeton, NJ) as the commercial preparation for intravenous use. This formulation contains sodium desoxycholate as a solubilizing agent.

Small unilamellar vesicles were prepared by sonication in a bath type sonicator12 from a lipid composition of egg phosphatidylcholine (Avanti Polar Lipids; Birmingham, AL) cholesterol, and tocopherol succinate (Sigma Chemical; St. Louis, MO) in a

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From the Infectious Disease Division, Department of Medicine,* and the Department of Ophthalmology,¶ Tufts–New England Medical Center, Boston, Massachusetts, and the School of Pharmacy,§ University of California, California.

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Reprint requests: Michael Barza, MD, New England Medical Center, 171 Harrison Avenue, Boston, MA 02111.
molar ratio of 5:3:1. The lipids were deposited on the sides of a round bottom flask by evaporation from a chloroform solution. Amphotericin B in dimethylsulfoxide was added to the dried lipids at a 10 mole percent ratio. Phosphate-buffered saline (PBS; composition 137 mM NaCl, 2.6 mM KCl, 6.4 mM Na2HPO4, pH 7.4) was added to the flask to rehydrate the lipids and to give a final lipid concentration of 50 μmol/ml.

The dispersion was hydrated under nitrogen for 10 min and then sonicated under nitrogen for 60 min at room temperature. The slightly opalescent yellow preparation was dialyzed at 4°C against 200 volumes of PBS, changed two times over a 24-hr period to remove nonintercalated amphotericin B and dimethylsulfoxide. A typical preparation retained 70% of the initial amphotericin B (3525 μg amphotericin B per 50 μmoles total lipid per ml). The liposomes were sterilized by filtration through a sterile 0.22 μm filter (Millipore; Bedford, MA) into a sterile container.

Drug concentration in the liposome dispersion was determined by diluting an aliquot of the final preparation 1/1000 in methanol, measuring the absorbance at 388 nm and 406 nm, and comparing it with a standard curve prepared from solid amphotericin B or liposomal amphotericin B in a dosage of 1, 5, 10, or 20 μg, or a control preparation consisting of PBS or drug-free (“empty”) liposomes. The latter were prepared in the same way as drug-containing liposomes. The controls given empty liposomes received the same amount of lipid as was present in the 20 μg injection of liposomal amphotericin B.

Each of the forty eyes was randomly assigned to receive one of 10 intravitreal regimens: amphotericin B or liposomal amphotericin B in a dosage of 1, 5, 10, or 20 μg, or a control preparation consisting of PBS or drug-free (“empty”) liposomes. The latter were prepared in the same way as drug-containing liposomes. The controls given empty liposomes received the same amount of lipid as was present in the 20 μg injection of liposomal amphotericin B. Each preparation was administered to four eyes in four animals. The volume injected was 0.1 ml in each instance.

In preparation for the intravitreal injections, the rabbits were tranquilized with an intramuscular injection of ketamine 90 mg and acepromazine maleate 1 mg. Proparacaine hydrochloride 0.5% was then applied topically. The intraocular volume was decreased by aspiration of 0.1–0.2 ml of aqueous humor through a 25-gauge needle. The superior rectus muscle was gently grasped with a forceps to stabilize and proptose the globe. A 27-gauge needle attached to a tuberculin syringe was introduced about 5 mm posterior to the limbus and was inserted to the approximate center of the vitreous humor. The solution (0.1 ml) was injected slowly.

Ophthalmoscopic and Histologic Studies

The rabbits' eyes were examined once a week with a direct ophthalmoscope and a slit lamp after dilatation with atropine sulfate 1%. All examinations were made by one observer who did not know which preparation had been administered.

After 5 weeks, the animals were killed with an intravenous injection of pentobarbital, and the eyes were promptly enucleated. The vitreal lesions appeared stable by this time. About 0.4 ml of vitreous humor was aspirated through a 25-gauge needle for assay of the amount of amphotericin B remaining (see below); this volume was promptly replaced by an equal volume of phosphate-buffered saline to maintain the volume of the globe.

The eyes were placed in 10% phosphate-buffered formalin. After fixation, they were properly oriented and a pupil–optic nerve section was cut along the vertical axis. A central segment was processed for paraffin embedding, sectioned at 6 μm, and stained with hematoxylin and eosin. Selected sections were stained with periodic acid–Schiff reagent. Sequential sections were observed for inflammatory changes in the vitreous humor and for retinal abnormalities. All interpretations were made by one observer who was not aware of which preparation had been administered. The changes were graded as follows:

*Vitreal inflammation*: Vitreal inflammation was graded as follows: ± = few monocular cells along vitreous base per high power field (40X on AO Ultrastar microscope, American Optical USA; Buffalo, NY); 1+ = 10–20 cells along the vitreous base per high power field; 2+ = 20–40 cells per high power field at the vitreous base and elsewhere; 3+ = >40 cells per high power field at the vitreous base and elsewhere.

*Retinal atrophy or necrosis*: These changes were designated as focal or diffuse as estimated by the
anteroposterior involvement in sections from the central segment of the globe.

Assay of Amphotericin B in Vitreous Humor

Residual amphotericin B was assayed by an HPLC method followed methanol extraction, with o-nitrophenol serving as internal standard. Activity was read spectrophotometrically at 388 nm.

Results

Ophthalmoscopic Examination

During the first week after injection, all but four eyes showed a moderate diffuse inflammatory cellular reaction in the vitreous humor, particularly in the anterior part, and all but six eyes showed scattered small white vitreal bodies. By the second and third weeks, the diffuse inflammatory reaction decreased or disappeared although the vitreal bodies remained. These opacities were sparkling white, were less than 1 mm in diameter, were mostly located in the lower part of the eye, and did not move when the animal’s head was moved. In some eyes, bands of “veil-like” vitreal condensations began to appear, sometimes seeming to exert traction on the retina.

During the fourth and fifth weeks, the vitreal bodies and vitreal bands remained unchanged. Retinal damage was noted in a few eyes, being manifested by a silvery sheen of the retina, generally in areas adjacent to the fibrotic vitreal bands. In a few animals, slit-lamp examination revealed cataract formation.

A grading system, summarized in Table 1, was used to quantify the extent of these gross changes 5 weeks after injection when vitreal lesions appeared to be stable. Intermediate values were scored with half-increments (eg, between 1+ and 2+ = 1.5). Figure 1 shows the density of vitreal bodies for each of the four eyes in the eight treatment groups and two control groups (PBS and drug-free or “empty” liposomes). As can be seen, vitreal bodies were evident in most animals given liposomal amphotericin B or empty liposomes. It is not clear that these opacities signify a toxic reaction; they may, instead, represent residual liposomes. However, some were seen in eyes that received no liposomes.

Figure 2 shows the extent of vitreal band formation and of retinal damage seen clinically in these same animals. These reactions, which were clearly due to adverse drug effects, were evident in most eyes given amphotericin B in doses of 5 μg or more, but were absent from all but one of the eyes treated with liposomal amphotericin B.

Cataracts were observed in four eyes, one each treated with amphotericin B, 5 μg, 10 μg, or 20 μg and one given liposomal amphotericin B, 5 μg. A faint focal cortical opacity was seen in one eye treated

Table 1. Scoring system for vitreal and retinal abnormalities observed on direct ophthalmoscopy

<table>
<thead>
<tr>
<th>Grade</th>
<th>Vitreal opacities</th>
<th>Vitreal bands</th>
<th>Retinal damage</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>None</td>
<td>None</td>
<td>None</td>
</tr>
<tr>
<td>1+</td>
<td>5–10 per eye</td>
<td>Minimal</td>
<td>Silvery sheen or atrophy of retina occupying 0.5–1 whole field visible at one time</td>
</tr>
<tr>
<td>2+</td>
<td>10–25 per eye</td>
<td>Moderate</td>
<td>Retinal sheen or atrophy occupying &gt;1 field visible at one time</td>
</tr>
<tr>
<td>3+</td>
<td>&gt;25 per eye</td>
<td>Severe</td>
<td>More extensive than above (no such lesions actually found)</td>
</tr>
</tbody>
</table>

Fig. 1. Gross toxicity of intravitreal amphotericin B. Extent of vitreal opacities observed ophthalmoscopically 5 weeks after intravitreal injection of commercial amphotericin B (AMB) or liposomal amphotericin B (lip-AMB) in the indicated dosages in pigmented rabbits. Each bar is the result in one eye using the scoring system shown in Table 1. Control eyes received phosphate-buffered saline (PBS) or empty (drug-free) liposomes.
with liposomal amphotericin B, 10 µg. The lenticular opacities were located in the posterior cortex.

Histologic Studies

The results of histologic examination are summarized in Table 2. Vitreal inflammation, manifested primarily by round cells along the vitreous base, was generally slight. Although some inflammation was noted in all groups, there was a suggestion of a somewhat more marked reaction in animals receiving higher doses of liposomal amphotericin B. We did not analyze vitreal band formation histologically because of the possibility that some of the effects observed may have been caused by aspiration of the vitreous humor for assay of amphotericin B.

Microscopic examination of the retina revealed damage in five eyes, all of which had been treated with amphotericin B. In one eye injected with amphotericin B, 5 µg, there was atrophy and necrosis over 80% of the inferior retina. Another eye treated in the same manner exhibited focal (2 × 1 mm) retinal atrophy (Figs. 3A and B). One eye injected with amphotericin B, 10 µg, displayed retinal necrosis in one area inferiorly (4 × 4 mm) and hypertrophy of the retinal pigment epithelium. A fourth eye, which had been treated with amphotericin B, 20 µg, showed retinal atrophy and focal atrophy of the retinal pigment epithelium (3 × 1 mm). In the fifth eye, injected with amphotericin B, 20 µg, diffuse necrosis was seen over the lower half of the retina (Figs. 4A and B). The first three eyes described above had been noted to have retinal damage on ophthalmoscopic examination. Overall, five of 16 eyes treated with amphotericin B, but none of 16 treated with liposomal amphotericin B, exhibited histologic retinal abnormalities (P = 0.0217 by Fisher exact test, two-tailed). All of the microscopic abnormalities were observed with doses of amphotericin B of 5 µg or more. None of the eyes injected with “empty” liposomes showed retinal damage histologically.

Vitreal Concentrations of Amphotericin B

Figure 5 shows the concentrations of amphotericin B measured in the vitreous humor 5 weeks after injection. Several specimens were lost for technical reasons. No drug was detectable in eyes given 1 µg of amphotericin B and an unexpectedly high concent-

Table 2. Histologic abnormalities in rabbits given intravitreal amphotericin B (AMB) or liposomal amphotericin B (lip-AMB) (each group included four eyes)

<table>
<thead>
<tr>
<th>Dose of AMB</th>
<th>AMB</th>
<th>Lip-AMB</th>
<th>Retinal abnormalities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>±</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>0 (empty liposomes)</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>2</td>
<td>1</td>
</tr>
</tbody>
</table>
| Total       | 8   | 4       | 0                    | 5   | 7       | 1

VITREAL BAND FORMATION

RETNAL DAMAGE

Fig. 2. Gross toxicity of intravitreal amphotericin B. Extent of vitreal band formation and retinal damage observed ophthalmoscopically 5 weeks after intravitreal injection of commercial amphotericin B (AMB) or liposomal amphotericin B (lip-AMB) in the indicated dosage in pigmented rabbits. Each bar is the result in one eye using the scoring system shown in Table 1. Control eyes received phosphate-buffered saline (PBS) or empty (drug-free) liposomes.
Fig. 3. A, top. Equatorial retinal lesion in a rabbit injected intravitreally with 5 µg of amphotericin B. Note atrophy of all layers, more marked in the inner layers of the retina. The outer nuclear layer is thinned (hematoxylin and eosin, ×120). B, bottom. Equatorial retina in a rabbit injected with 5 µg of liposomal amphotericin B. The retinal architecture is normal (hematoxylin and eosin, ×120).

A concentration of 1.4 µg/ml was detected in the only eye studied after 1 µg of liposomal amphotericin B. Concentrations were in the range of 0.4–1.0 µg/ml in eyes injected with 5–20 µg of amphotericin B or liposomal amphotericin B. There was no obvious difference in concentration between eyes treated with liposomal or nonliposomal preparations. If one considers that, in these small rabbits, the initial dose of
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four half-lives, suggesting a vitreal half-life of amphotericin B of approximately 9–12 days.

Discussion

Amphotericin B has been a mainstay in the treatment of fungal endophthalmitis. Jones has documented that within the past two decades, candidal endophthalmitis has emerged as the most common ocular fungal infection. Several case reports support the effectiveness of amphotericin B, given by subconjunctival or intravitreal injection in the treatment of fungal endophthalmitis. However, the toxicity of amphotericin B may limit its effectiveness.

Recent experiments in our laboratory have shown that liposome association of amphotericin B strikingly reduces the toxicity of the drug after systemic administration in mice. The acute LD$_{50}$ of commercial amphotericin B in this animal is approximately 2.27 mg/kg, whereas the acute LD$_{50}$ of the liposomal preparation is 11.75 mg/kg. Similar reductions in toxicity with liposomal formulations of amphotericin B have been reported by others. We have also shown that liposomal amphotericin B retains full antifungal activity in vitro. Finally, we have demonstrated that liposomal amphotericin B can be given safely in higher doses than commercial amphotericin B to animals with systemic fungal infections thereby achieving higher survival rates. The small unilamellar vesicles that we used have a diameter of approximately 0.1 μm so that they can be sterilized by passage through a 0.22 μm filter. The reduced systemic toxicity of liposomal amphotericin B led us to test the preparation for ocular toxicity after intravitreal injection in rabbits.

During the first 1 to 2 weeks after intravitreal injection of amphotericin B or liposomal amphotericin B, a vitreal haze consistent with leukocytic infiltration was observed in most eyes. Thereafter, the major clinical changes consisted of vitreal “bodies”, ie, small white sparkling opacities mainly located in the lower part of the eye, vitreal condensations, and retinal abnormalities. Vitreal bodies were noted predominantly in animals given liposomal preparations. They may represent residual lipid. However, they were also noted in some eyes treated with nonliposomal formulations. Their histologic counterparts may have been eluted during tissue processing. Further studies will be needed to elucidate the nature of these particles and to determine how long they remain in the eye.

Vitreal band formation and retinal damage of grade 1+ or greater were clinically evident only in eyes treated with amphotericin B alone. This strongly suggests that liposome association reduces the acute inflammatory effect of the drug. Cataract formation was observed in one of 20 eyes given amphotericin B and in three of 20 eyes given liposomal amphotericin B. Although we cannot exclude trauma as a cause of the cataracts, we were careful to direct the needle posteriorly. Neither the distribution of cataracts among the treatment groups nor their morphologic appearance permits us to interpret their cause or significance.

On histologic examination, vitreal inflammation, chiefly in the form of a round-cell infiltrate along the vitreous base, was noted in over half of the eyes, including two eyes treated with PBS. Inflammation of grade 1+ or 2+ was noted in four of 16 eyes injected with amphotericin B but eight of 16 injected with liposomal amphotericin B, suggesting a somewhat more irritating effect of the liposomal drug. However, the relation between vitreal inflammation and substance or dose of substance administered was inconclusive.

If we consider retinal necrosis or atrophy as the most significant histologic lesions, there was a marked benefit of liposomal amphotericin B. Retinal damage was detected histopathologically in five of 16 eyes treated with amphotericin B but none of 16 given liposomal amphotericin B ($P = 0.0217$ by Fisher exact test). This corresponds to the clinical finding that significant retinal damage was confined to the animals given amphotericin B alone. Although it is conceivable that needle trauma caused the atrophic changes noted in two of the eyes, it is unlikely to have produced the more extensive changes observed in the other three eyes. Moreover, only animals given nonliposomal drug manifested these abnormalities, which should not be the case if trauma due to the injection were responsible for the changes. We did not control for the direction of the needle bevel in making the injections, but we presume that this variable was randomly distributed among the eyes. We cannot rule out the possibility that the desoxycholate solu-
bibilizer present in commercial amphotericin B played some role; however, Axelrod et al.\(^7\) found that deoxycholate was not toxic in the doses used in this study.

The mechanism by which liposome-association reduces the toxicity of amphotericin B without reducing its antifungal activity is not clear. In contrast to water-soluble molecules, which are trapped in the interior (aqueous phase) of liposomes, amphotericin B is presumably intercalated in the wall of the liposome.\(^19\) The antibiotic has an affinity for sterols present in the liposomes and in human and fungal cellular membranes. Its greatest affinity is for ergosterol, which occurs naturally in fungal cells but not in human cell membranes. It may be that the interaction between amphotericin B and human cell membranes is reduced by the competitive effect of the liposomal cholesterol but that the drug is readily captured by fungal ergosterol.

Concentrations of amphotericin B of 0.4–1.0 \(\mu\)g/ml were found in the vitreous humor 5 weeks after a single intravitreal injection of 5–20 \(\mu\)g. The apparent clearance rate of amphotericin B from the vitreous humor was similar for liposomal and nonliposomal preparations. However, our data do not permit us to determine whether amphotericin B administered in liposomes was cleared as the drug–liposome complex or after the drug had become separated from the liposomes. More detailed pharmacokinetic studies underway in our laboratory suggest that amphotericin B has a long half-life in the vitreous humor, choroid, and retina following intravitreal injection. Accordingly, intravitreal injections may not have to be repeated often to maintain relatively high levels of drug in pertinent sites.

The results of this study offer some hope that liposome-intercalation may reduce the toxicity of intravitreal amphotericin B, thereby permitting this valuable drug to be used more safely in the treatment of fungal endophthalmitis. Our data suggest that further study of the safety and efficacy of intraocular liposomal amphotericin B is warranted.

**Key words:** amphotericin B, liposomes, intravitreal, toxicity, rabbit

**References**