Yonsei Medical Journal
Vol. 31, No. 4, 1990

Pharmacokinetics of Intravitreally Injected Liposome-Encapsulated Tobramycin in Normal Rabbits

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Bacterial endophthalmitis, which is a devastating complication of intraocular surgery or eye trauma, has a poor prognosis. Intravitreal injection of antimicrobial agents has become a part of the standard treatment of endophthalmitis. The authors investigated the pharmacokinetics of intravitreal liposome-encapsulated tobramycin as a possible method of prolonging the duration of therapeutic concentrations. Tobramycin was encapsulated into liposomes of phosphatidylcholine, phosphatidic acid, and alpha-tocopherol by the reverse phase evaporation method. The final liposomal suspension contained tobramycin, 7.0 mg/ml, 60.5% encapsulated. One eye received an intravitreal injection of either liposome-encapsulated tobramycin (LET), tobramycin phosphated-buffered saline (TS) or a mixture of tobramycin and liposome-encapsulated saline (TEL), and the results were as follows: 1. Concentrations of free tobramycin were significantly lower with LET than with TS or TEL at 1 hour after intravitreal injection. 2. Concentrations of free and total tobramycin were significantly higher with LET than with TS or TEL at 5 and 8 days after intravitreal injection. Concentrations of free tobramycin with TS were lower than the minimal inhibitory concentration(MIC) of tobramycin for Pseudomonas aeruginosa at 8 days after intravitreal injection, while those with LET were higher than the MIC of tobramycin for Pseudomonas aeruginosa 18 days after injection.

Key Words: Intravitreal injection, tobramycin concentration, liposome-encapsulated tobramycin

Endophthalmitis is a devastating complication of intraocular surgery or eye trauma and has a very poor prognosis (Peyman *et al.* 1974; Nelson *et al.* 1974). The generally poor visual results after true endophthalmitis may be caused by many factors: extreme sensitivity of the ocular structures to inflammation, poor host defense mechanisms, and poor penetration of antibiotics into the ocular tissues. It is believed that poor penetration of antibiotics is perhaps the most significant factor (Peyman and Schulman 1986). Poor penetration into the vitreous can be explained in part by the blood-retinal barrier which is composed of zonulae occludentes which interconnect cells of the retinal pigment epithelium and tight junctions which bind together the endothelial cells forming the walls of the retinal capillaries (Peyman and Bok 1972).

Conventional means of antibiotic therapy such as intramuscular or intravenous routes, topical application, and subconjunctival injection failed to attain the minimal inhibitory concentration (MIC) of antibiotics in the vitreous. (Leopold 1945; Leopold and Apt 1960; Furgiuele 1967; Litwack *et al.* 1969; Furgiuele 1970; Golden and Coppel 1970). The most logical means of bypassing physiologic and anatomic barriers that might prevent rapid attainment of high vitreous drug concentration appears to be through intravitreal injection. Intravitreal injection of penicillin was first studied by von Sallmann and associates (1944), and methicillin (Daily *et al.* 1973), gentamicine (Peyman *et al.* 1974; May *et al.* 1974; Cobo and Forster 1981), amikacin (Nelson *et al.* 1974), chloramphenicol (Koziol and Peyman 1974), kanamycin (Peyman *et al.* 1974), lincomycin (Schenk and Peyman 1974), tobramycin (Bennett and Peyman 1974), carbenicillin (Schenk *et al.* 1974), clindamycin (Paque and Peyman 1974), cephaloridine (Graham *et al.* 1975), netilmicin (Sloane *et al.* 1981) and moxalactam (Leeds *et al.* 1982) were administered intravitreally thereafter to treat experimental bacterial endophthalmitis in rabbits. However the duration of effective antimicrobial levels in the vitreous of the infected eye following a single injection of these antibiotics may not be adequate for

Received June 8, 1990
Accepted June 29, 1990
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optimal therapy. The administration of larger doses increases the duration of therapeutic concentrations, but may be toxic to the retina and lens due to higher initial concentrations. However, when a small dose is injected into the vitreous, the duration of an effective antimicrobial level above the MIC may be too short.

A dosage formulation allowing the slow release of an antimicrobial agent into the vitreous after injection would produce a lower initial concentration and increase the duration of therapeutic concentrations.

Liposomes, which are small bilayer vesicles, have been used to entrap aqueous drug solutions for slow release. The drugs escape either by leakage through the membranes of intact liposomes or by diffusion from degraded or destabilized liposomes (Szoka and Papahadjopoulos 1980; Smolin *et al.* 1981; Barza *et al.* 1987). Because of the slow degradation of the lipid membrane within the tissues and the comparatively large size of the liposomes, the encapsulated drugs are retained in the circulation or at the site of the injection for a relatively long period (Gregoriadis 1973; Juliano and Tamp 1978; Morgan and Williams 1980; Assil and Weinreb 1987; Skuta *et al.* 1987). Slow release of the drug is important pharmacokinetically in the treatment of the bacterial endophthalmitis (Barza *et al.* 1984; Kim *et al.* 1988; Han *et al.* 1989), and liposome incorporation of the drug markedly attenuates the drug's toxicity in the rabbit eye (Barza *et al.* 1985; Trembley *et al.* 1985). Thus liposomes have been investigated as models of biologic membranes and as potential drug delivery systems in many different settings.

Nowadays the most causes of bacterial endophthalmitis are known to be gram-negative bacilli, the most common being *Pseudomonas aeruginosa* (Allen and Mangiaracine 1964; Allen and Mangiaracine, 1974). Aminoglycosides, which have a broad spectrum of action that includes both gram-positive and gram-negative organisms such as *Pseudomonas aeruginosa* have recently been administered in the treatment of bacterial endophthalmitis.

Fishman *et al.* (1986) investigated the effect of liposome encapsulation on the pharmacokinetics of gentamicin after intravitreal injection and reported that administration of liposome-encapsulated gentamicin improved the concentration time profile following intravitreal injection in normal rabbits. The activity of tobramycin against many species of bacteria susceptible to gentamicin makes tobramycin of further interest, as does its marked inhibitory action against many gentamicin-resistant strains of bacteria, for example, *Pseudomonas aeruginosa* (Meyer *et al.* 1971; Traub and Raymond 1972; Waterworth 1972).

We investigated the pharmacokinetics of intravitreal liposome-encapsulated tobramycin as a possible method of prolonging the duration of therapeutic concentration.

MATERIALS AND METHODS

Materials

Animals: Albino rabbits, each weighing 2 to 3 kg, were used.

Lipids and drugs: Phosphatidylcholine (PC), phosphatidic acid (PA), alpha-tocopherol (α-tocopherol) were obtained from Sigma (St. Louis, Mo.) Methanol, chloroform, diethyl ether were from Merck (Darmstadt, F.R. Germany) Tobramycin podwer was from Sigma Tobramycin injectable solution (Tenebra®, Dongwha Corp, Seoul, Korea)

Methods

Liposome preparation: Liposomes of phosphatidylcholine (PC), phosphatidic acid (PA) and α-tocopherol, with a molar ratio of 8:1:1 were prepared using the reverse-phase evaporation method (Szoka and Papahadjopoulos 1978). Liposomes were prepared by drying off the chloroform-methanol solvent containg 200 μmol of lipid in a 1 liter pear-shaped flask. The lipids were redissolved in diethyl ether, and 4ml of a solution of tobramycin sulfate, 40 mg/ml at pH 7.4 in PBS was pipetted into the flask. The resulting liposome suspension received ultrasonic irradiation for a 10 minute period with a bath-type sonicator (Branson B 52,240 watt) until the mixture became a homogenous opalescent dispersion. The mixture was placed on the rotary evaporator and diethyl ether was removed under reduced pressure at 20-25°C. Nonencapsulated tobramycin was partially removed by overnight dialysis at 4°C against a 400-fold volume of PBS. Following the dialysis, the liposomal suspension was centrifuged (100,000×g, 30 min) and resuspended in PBS to remove the remaining unentrapped tobramycin. 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 (Bennett *et al.* 1966;Barza *et al.* 1984).

The tobramycin concentration checked after treatment of Triton X-100 was considered to be that of the tobramycin both inside and outside of the liposome. The tobramycin concentration without treatment of Triton X-100 was considered to be that of the

tobramycin outside of the liposome only. The tobramycin concentration with Triton X-100 was revealed to be 7.0 mg/ml, with 60.5% of this encapsulated within the liposomes.

The liposomal suspension was diluted in PBS to a tobramycin concentration of 4.5 mg/ml (LET). A second preparation contained only the tobramycin sulfate injectable solution with the tobramycin concentration of 4.5 mg/ml (TS). As a control for the effect of liposomes upon the pharmakokinetics of extraliposomal tobramycin, empty liposomes were prepared in PBS as described above, but without tobramycin. Tobramycin was then added to the formed liposome and diluted with PBS to 4.5 mg/ml (TEL).

Intravitreal injection: Rabbits were sedated with an intravenous injection of pentobarbital sodium (35 mg/kg) and the eyes were anesthetized with proparacaine eyedrops, 0.5%. Immediately before intravitreal injection, 0.1ml of aqueous was aspirated by limbal paracentesis with a 28-gauge needle on a tuberculin syringe to prevent an increase in intraocular pressure (Peyman *et al.* 1974; Kane *et al.* 1981; Barza *et al.* 1985).

Twenty-seven of the 57 rabbits (54 eyes) received a 0.1ml injection of 450 μg/0.1ml of liposome-encapsulated tobramycin with a 28-gauge needle through the pars plana 2mm from the limbus into the center of the vitreous under direct visualization (LET group). Three rabbits were sacrificed with an intravenous injection of sodium pentobarbital at each of the following time intervals: 1, 24, 72, 120, and 192 hours, 11, 15, 18, and 23 days. Fifteen of the remaining 30 rabbits (30 eyes) intravitreally received 0.1 ml of 450 μg/0.1ml of free tobramycin saline solution (TS group) and the remaining 15 rabbits (30 eyes) received 0.1ml of 450 μg/0.1ml of tobramycin with empty liposome (TEL group). Three rabbits were sacrificed in each group at 1, 24, 72, 120, and 192 hours after injection. Eyes were enucleated, washed in PBS, and dried. The aqueous humor was aspirated as previously described, removing as much aqueous as possible. The sclera was dissected at the posterior pole area and the vitreous humor was aspirated into a 5-ml syringe. The vitreous was repeatedly forced through a 24-gauge needle to be homogenized.

Assay of tobramycin: The concentration of tobramycin was assayed by agar-diffusion bioassay using *Bacillus subtilis* (ATCC 6633) as the test organism. To determine the total and free (released from liposomes) tobramycin concentration within the vitreous, 0.8ml of vitreous was dialysed against 0.8ml of PBS at 37°C for two hours, using a multichamber dialysis machine [Q factor (surface of dialysis membrane/half cell working volume):8.8]. After equilibrium dialysis, vitreous was recovered from the vitreous chamber and PBS from the PBS chamber. The concen-

Table 1. Total and free tobramycin concentrations[a] of vitreous following intravitreal injection of liposome-encapsulated tobramycin (LET), tobramycin in PBS (TS), and tobramycin with empty liposome (TEL) in rabbits

Time after injection	Tobramycin concentration		
	LET	TS	TEL
1 hour	total: 270.3±35.3	265.0±25.1	269.6±27.1
	free: 198.2±21.0	251.8±26.0	235.0±33.4
	%free[b]: 73.6± 4.5	95.0± 3.4	86.8± 6.5
24 hours	total: 185.0±27.8	172.3±35.5	180.1±32.6
(1 day)	free: 149.0± 5.6	148.4±26.8	160.4±34.8
	%free: 81.8±10.2	86.6± 5.1	88.9± 6.1
72 hours	total: 77.3±20.7	69.2±12.0	64.0±15.9
(3 days)	free: 54.6±14.8	61.4±14.4	53.2±16.4
	%free: 70.5± 4.8	87.9± 5.6	82.1± 7.1
120 hours	total: 57.1±13.3	16.1± 4.5	28.9± 9.9
(5 days)	free: 41.8± 9.2	15.0± 4.4	21.9± 7.7
	%free: 74.6±16.3	92.8± 6.5	76.4± 8.7
192 hours	total: 33.0± 9.4	4.1± 1.9	11.4± 3.8
(8 days)	free: 16.8± 5.0	3.4± 1.2	8.6± 3.6
	%free: 53.3±19.7	83.3±11.7	73.8± 5.9

[a]: mean±SD μg/ml, n=6

[b]: free tobramycin concentration/total tobramycin concentration

tration of tobramycin in the recovered vitreous or PBS from each chamber was checked after addition of Triton X-100 at a final concentration of 0.2% and compared with tobramycin standards containing Triton X-100. Two times the concentration of tobramycin of the PBS was considered as the free tobramycin concentration in the vitreous. The sum of the tobramycin concentration of the liposome and that of the PBS was considered as the total tobramycin concentration. Wilcoxon rank sum test was applied for the analysis of data.

RESULTS

Total and free vitreous tobramycin concentration (μg/ml; mean±SD) following liposome-encapsulated tobramycin (LET), tobramycin in PBS (TS) and tobramycin in empty liposome (TEL) intravitreal injections are given in Table 1, 2, and Fig. 1, 2.

At 1 hour after injection, the total tobramycin injections were similar for LET, TS, and TEL group ($p>0.05$), but the free vitreous tobramycin concentration was significantly less in the LET group than TS and TEL groups ($p<0.001$). At 1 and 3 days after injection, the total and free tobramycin concentrations were similar for LET, TS, and TEL groups ($p>0.05$). The total and free tobramycin concentrations were significantly greater ($p<0.01$) at 5 and 8 days following injection with LET than with TS and TEL. The total tobramycin concentration 5 day following injection, and total and free tobramycin concentrations 5 and 8 days following injection with TEL were greater than with TS ($p<0.01$). The free tobramycin concentrations were still high at 23 days in cases where LET was injected.

Table 2. Total and free tobramycin concentrations[a] of vitreous from 11 to 23 days after intravitreal injection of liposomeencapsulated tobramycin (LET) in rabbits

Time after injection	Tobramycin concentration LET
11 days	total: 32.5± 7.5
	free: 16.5± 2.8
	%free[b]: 51.8± 8.1
15 days	total: 29.1± 8.5
	free: 13.0± 2.7
	%free: 40.8±16.6
18 days	total: 23.5± 7.9
	free: 15.8± 8.7
	%free: 65.0±17.2
23 days	total: 24.7± 8.5
	free: 16.3± 8.8
	%free: 59.9±26.1

[a]: mean±S.D. μg/ml, n=6

[b]: free tobramycin concentration/total tobramycin concentration

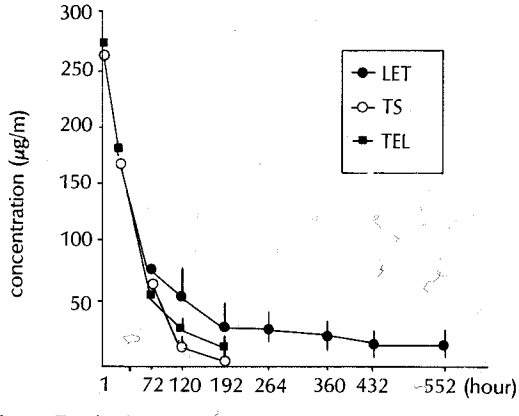

Fig. 1. Total tobramycin concentrations of vitreous following intravitreal injection of liposome-encapsulated tobramycin (LET), tobramycin in PBS (TS), and tobramycin with empty liposome (TEL) in rabbits. Each dot represents mean±SD μg/ml, n=6.

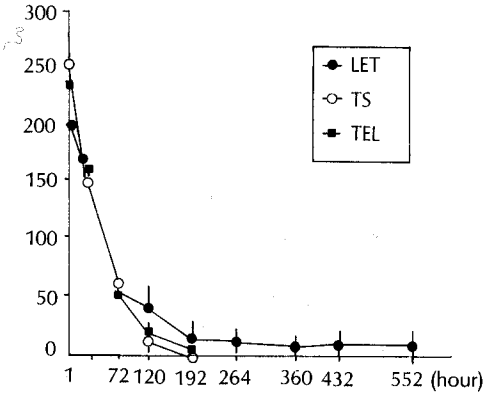

Fig. 2. Free tobramycin concentrations of vitreous following intravitreal injection of liposome-encapsulated tobramycin (LET), tobramycin in PBS (TS), and tobramycin with empty liposome (TEL) in rabbits. Each dot represents mean±SD μg/ml, n=6.

DISCUSSION

In bacterial endophthalmitis, *Pseudomonas aeruginosa* is known to be the most frequent organism cultured, and the MIC of gentamicin for ≥90% of *Pseudomonas aeruginosa* strains is 8.0 μg/ml and that of tobramycin is 6.3 μg/ml. It is known that there is no difference between the toxicity of gentamicin and that of tobramycin (Ristuccia and Cunha 1982), and it may be more effective to inject liposome-encapsulated tobramycin than liposome-encapsulated gentamicin in the treatment of bacterial endophthalmitis. Four and fifty μg of liposome-encapsulated tobramycin was injected because the maximum amount of tobramycin to be injected into rabbit eyes without retinal degeneration is known to be 500μg (Bennett and Peyman 1974).

Liposomes can be classified as positively charged, neutrally charged, and negatively charged liposome (Schaeffer and Krohn 1982), and the negatively charged liposomes are relatively larger in size (Szoka and Papahadjopoulos 1980). In this study, phosphatidic acid was added to the lipid mixture so that negatively charged liposomes were formed (Szoka and Papahadjopoulos 1980; Morgan and Williams 1980). α-Tocopherol was added to decrease the permeability of the liposome (Barza *et al.* 1987).

Various methods are available for the preparation of liposomes and the reverse phase evaporation method (Szoka and Papahadjopoulos 1978) was adopted in this study to create a high concentration of tobramycin encapsulated in the liposome. Many procedures, such as dialysis, column chromatography, and centrifugation were introduced to separate the liposome-encapsulated drug from the free drug (Watt *et al.* 1978; Szoke and Papahadjopoulos 1980). Dialysis was performed first and ultracentrifugation was done thereafter, because previous results (unpublished data) showed low harvest rates of liposome-encapsulated tobramycin with simple repeated centrifuging.

The vitreous can be removed intact from the eyeball and frozen in liquid nitrogen after enucleation (Bennett and Peyman 1974; Boyle 1976; Cobo and Forster 1981), but the simple aspiration method was applied instead of the freezing method in this study because liposome stability, such as leaking of encapsulated tobramycin after repeated freezing and thawing, was not confirmed (Fishman *et al.* 1986).

In the equilibrium dialysis procedure, although preliminary data using tobramycin in PBS showed that equilibrium was achieved after 2 hours at 37°C, liposome breakdown at 37°C (Szoka and Papahadjopoulos 1980; Barza *et al.* 1987) would increase dialysate tobramycin concentration, and might show higher free tobramycin concentrations in the LET group than in vivo.

Barza *et al.* (1987) demonstrated that encapsulation of ^{51}Cr-EDTA (chromium-51-ethylenediaminetetraacetic acid complex), whose half-life in the vitreous was found to be similar to that of gentamicin, prolonged its half-life by sevenfold. In this study, the concentration of free tobramycin 1 hour after injection in the LET group was statistically lower than that of the TS and TEL groups. The relatively low peak value 1 hour after injection may decrease the toxic effects on the intraocular pressure. Intravitreal concentration 1 hour after injuection in LET may reflect the concentration of free tobramycin, which had already persisted as the free form before injection, and the moderate intravitreal concentration 1 day after injection may come from the sum of concentrations of tobramycin leaked outside from within the liposome and that already existing as the free form. The relatively higher concentration of LET than TS or TEL 5 days after injection indicates slow release of tobramycin from the liposome and the tobramycin from multilamellar liposomes might play some role in the concentration of tobramycin at a later period.

The tobramycin concentrations of TEL were higher than those of TS from 5 days after injection, though the injection solution used in TEL was a simple mixture of tobramycin and empty liposome. It probably came from the charge interaction between the positively-charged tobramycin and negatively-charged liposome in the neutral pH (Kubo *et al.* 1986), because the amount of precipitates observed decreased as the pH of the solution became alkaline.

If similar slow release of tobramycin can occur in human eyes without damage to the intraocular structure, liposome-encapsulated tobramycin may be a good tool for the eradication of intraocular infection.

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