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Pharmacokinetics and In Vivo Activity of Liposome-Encapsulated Gentamicin

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Gentamicin sulfate was encapsulated in liposomes composed solely of egg phosphatidylcholine and administered via intravenous injection to rats and mice. The total gentamicin activity (regardless of whether it was free or liposome associated) in serum and selected tissues was determined for 24 h (serum) or up to 15 weeks (tissues) by using a microbiological assay. The mean half-lives in serum of a single 20-mg/kg dose of free (nonencapsulated) gentamicin in mice and rats were estimated to be 1.0 and 0.6 h, respectively, whereas a similar dose of encapsulated drug had apparent mean half-lives of 3.8 h in mice and 4.0 h in rats. In both species, the apparent half-life in serum of the liposomal formulation increased as the dose increased. Liposome encapsulation resulted in higher and more prolonged activity in organs rich in reticuloendothelial cells (especially spleen and liver). In acute septicemia infections in mice, the liposomal formulation showed enhanced prophylactic activity (as determined by calculation of the 50% protective dose). In a model of murine salmonellosis, liposomal gentamicin greatly enhanced survival when given as a single dose (10 mg/kg) at 1 or 2 days after infection as well as up to 7 days before infection.

Experiments in animals have shown that liposome encapsulation can dramatically alter the distribution of drugs in the body and their rate of clearance (9). These pharmacokinetic differences and other, less well understood effects can result in "targeting" a drug to particular organs or sites of disease, prolonged levels in serum or tissue, reduced toxicity, and/or enhanced efficacy of the encapsulated drug.

Liposome-encapsulated antibiotics have been studied by several groups (13). Most studies of antibacterial agents have focused on the efficacy of these preparations in the treatment of facultative, intracellular bacterial infections of the reticuloendothelial system such as salmonellosis (5, 19), brucellosis (6), listeriosis (1), and mycobacterial infections (4, 12, 21).

Workers in our laboratory recently described a liposomal formulation of gentamicin sulfate that showed enhanced therapeutic activity in two extracellular infections in mice—*Klebsiella* pneumonia and a thigh infection in neutropenic animals (7). One possible explanation for the enhanced efficacy in these models is the altered pharmacokinetic profile in the blood and lungs seen with liposomal gentamicin after a single bolus dose, resulting in greater peak levels and area under the concentration-time curves in these organs. Other workers have shown that liposome encapsulation can alter the half-life in serum and tissue distribution of gentamicin (11, 16).

The present studies were undertaken to further examine the concentration profile in serum and the distribution and retention in tissues of antibiotic activity after administration of gentamicin sulfate in a liposomal dosage form. In addition, we studied the prophylactic as well as therapeutic efficacy of liposomal gentamicin in a standard mouse protection test (3) and in murine salmonellosis.

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MATERIALS AND METHODS

Liposome preparation. Liposomes were prepared by a modification of the stable plurilamellar vesicle process (8). The lipid (95% pure egg phosphatidylcholine; Avanti Polar Lipids, Inc., Birmingham, Ala.) solution in methylene chloride and the drug (gentamicin sulfate, USP grade; Agvar Chemicals, Little Falls, N.J.) solution in normal saline were added to a large, round-bottom vessel. The solvent was evaporated under vacuum with agitation, and the lipid-drug mixture was hydrated with normal saline. The liposome suspension was subjected to tangential flow filtration to remove nonentrapped drug and liposomes outside of the desired size range. The concentration of gentamicin in the final liposome suspension was determined by a spectrophotometric assay (15) after disruption of the lipid membranes with 0.2% Triton X-100. Additionally, the antibacterial activity of the liposome suspension was determined (after solubilization and removal of the lipids by Bligh and Dyer extraction [10]) by using an agar well diffusion assay with *Bacillus subtilis* (ATCC 6633) as the indicator organism. The final liposome formulations used in these studies contained approximately 5 mg of active gentamicin per ml and about 55 mg of total phospholipid per ml (as determined by a Bartlett phosphorus assay [10]). The size of the liposomes was measured by laser diffraction (Particle Sizer 3600 E Type; Malvern Instruments, Malvern, England); more than 85% of the vesicles had diameters between 1.2 and 10.0 μm.

Pharmacokinetic studies. Mice (outbred CD-1 strain males, 20 to 25 g) and rats (Sprague-Dawley females, 150 to 250 g) were obtained from Charles River Breeding Laboratories, Inc., Wilmington, Mass. The animals were injected intravenously via the lateral tail vein with bolus doses of 20 mg of free gentamicin per kg or 5, 10, 20, or 40 mg of liposomal gentamicin per kg. A single lot of gentamicin liposomes was used and diluted with saline to administer the various doses. Therefore, animals receiving the 5-mg/kg dose received 8 times less lipid as well as drug when compared with animals receiving a 40-mg/kg dose. Blood was collected at 0.25, 0.5,

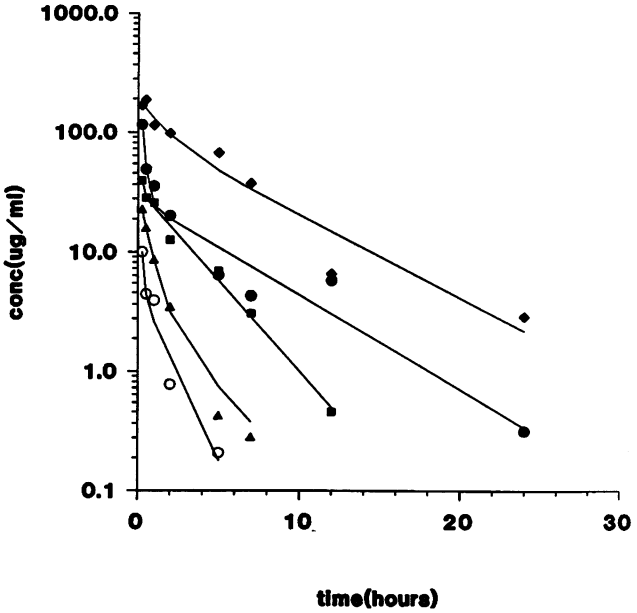

FIG. 1. Concentration of gentamicin activity in serum of mice after a single intravenous dose of gentamicin liposomes at 40 (◆), 20 (●), 10 (■), or 5 (▲) mg/kg or free gentamicin at 20 mg/kg (○). Each point represents the mean for five mice.

1.0, 2.0, 5.0, 7.0, 12.0, and 24.0 h postdose from the orbital sinus of rats or by cardiac puncture (after tribromoethanol anesthesia) in mice. Serum was separated and frozen for later bioassay. For tissue level determinations in mice, another group of animals were injected intravenously with a 20-mg/kg dose of free or liposomal gentamicin and killed (by cervical dislocation) at 1, 3, 7, 24, and 48 h after dosing. An additional group of rats received 20 mg of free or liposomal gentamicin per kg, and sets of three animals were killed (with CO_2) at 1 h, 3 days, 5, days, and 2, 3, 6, 7, 8, 9, 10, 11, 12, and 15 weeks after dosing. Selected organs (plasma, kidney, spleen, liver, lung, bone, adrenal gland, ovary, eyes, and thyroid) were aseptically removed, weighed, diluted with phosphate buffer (pH 8), and homogenized. Samples of serum or tissue homogenates were assayed by an agar well diffusion bioassay with *B. subtilis* (ATCC 6633) as the indicator organism. This assay system measures only the total activity and does not distinguish between free and liposome-associated drug. The sensitivities of the assay were 0.1 μg/ml in serum and 0.4 to 1.5 μg/g in tissues.

Gentamicin concentration in serum versus time data were fit to one- and two-compartment models with first-order elimination by using least-squares linear regression on an ESTRIP program (2). Elimination rate constants (k_{el}) were determined from the fit. The half-life was calculated as $0.693/k_{el}$. The area under the curve (AUC) was calculated using the trapezoidal rule with extrapolation to infinity. Total systemic clearance was determined by dose/AUC, and the volume of distribution was determined by dose/(AUC · elimination rate constant). A separate group of female Sprague-Dawley rats were given a single, intravenous 20-mg/kg bolus dose of free or liposomal gentamicin and housed individually in metabolic cages. Urine was collected daily for 10 days and assayed for gentamicin activity.

Mouse protection tests. Mice (male CD-1) were inoculated intraperitoneally with a rapidly lethal (death in untreated mice in 24 to 72 h) dose of bacteria suspended in 5% hog mucin (Sigma Chemical Co., St. Louis, Mo.). Treatment with free or liposomal gentamicin was given intravenously immediately postinfection or prophylactically. The 50% protective dose was calculated by the Reed and Muench method as described previously (3).

Mouse salmonellosis. *Salmonella typhimurium* (ATCC 14028) was grown in Trypticase soy broth (BBL Microbiology Systems, Cockeysville, Md.) at 35°C overnight. The MIC of gentamicin against this strain was 0.2 to 0.4 μg/ml when measured by a microdilution method. Samples of the suspension were frozen and maintained at −70°C. For use, cultures were thawed and diluted to contain 5×10^3 to 7×10^3 CFU/ml in 0.9% saline. Mice (male BALB/c) were inoculated intravenously (0.1 ml) via the lateral tail vein. Treatment with free and liposomal gentamicin was given intravenously at various times before or after infection. Mortality was monitored for up to 42 days after infection. There were 10 mice per treatment group.

RESULTS

Pharmacokinetics. Levels of gentamicin activity in serum of mice given a single dose of 5, 10, 20, or 40 mg of gentamicin liposomes per kg are compared with levels found in mice given a single 20-mg/kg injection of free gentamicin

TABLE 1. Mean pharmacokinetic parameters of free and liposome-encapsulated gentamicin in mice and rats[a]

Animals	Dosage form	Dose (mg/kg)	k_{el} (1/h)	Half-life (h)	AUC (μg · h/ml)	Clearance (ml/min per kg)	V (liters/kg)	C (0.25)[b] (μg/ml)
Mice	Free	20	0.67	1.03	8.08	41.25	3.68	10.05
	Liposomal	5	0.33	2.09	24.51	3.40	0.61	22.39
	Liposomal	10	0.35	1.97	91.16	1.83	0.31	39.57
	Liposomal	20	0.18	3.81	185.25	1.80	0.59	115.96
	Liposomal	40	0.16	4.33	764.23	0.87	0.33	167.32
Rats	Free	20	1.16	0.60	37.51	9.08	0.47	35.60
	Liposomal	5	0.51	1.37	58.00	1.50	0.18	21.28
	Liposomal	10	0.18	3.79	176.02	0.95	0.35	47.00
	Liposomal	20	0.17	4.02	426.55	0.79	0.28	72.10
	Liposomal	40	0.12	5.70	3,255.55	0.21	0.10	560.00

[a] Parameters were calculated by using a one-compartment model for rats given free gentamicin (20 mg/kg) or liposomal gentamicin (5 mg/kg). All other parameters were calculated by using two-compartment models. Abbreviations: k_{el}, elimination rate constant; AUC, area under the curve; C (0.25), concentration in serum at 0.25 h postdose.

[b] The concentration of gentamicin in the serum of animals given the liposomal drug represents the total activity (both free and liposome associated).

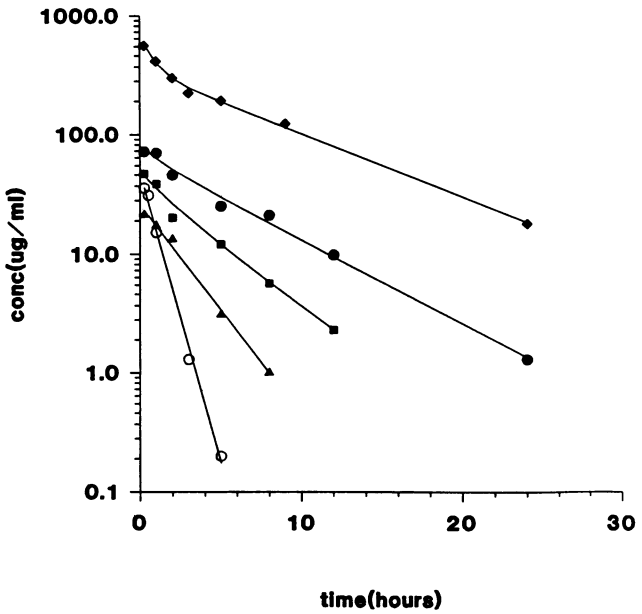

FIG. 2. Concentration of gentamicin activity in plasma of rats after a single intravenous dose of gentamicin liposomes at 40 (◆), 20 (●), 10 (■), or 5 (▲) mg/kg or free gentamicin at 20 mg/kg (○). Each point represents the mean for three rats.

sulfate in saline in Fig. 1. Pharmacokinetic parameters from these data are shown in Table 1. We calculated a half-life for free gentamicin in mice of 1.03 h, which is longer than that reported by others (22). This is probably due to the fact that we detected activity in the serum at 5 h after dosing and used a two-compartment model for analysis. If the 5-h point were disregarded and a one-compartment model was used, the half-life would be 0.51 h.

At all doses tested, the liposomal formulation resulted in higher concentrations of total gentamicin activity in serum at 15 or 30 min postinjection than did the free drug. The serum half-life and area under the concentration-time curve were higher at all doses tested for liposomal gentamicin than for the free drug. The systemic clearance decreased as the total dose of liposomal gentamicin increased. The apparent volume of distribution of free gentamicin was greater than the encapsulated form. Similar results were found in rats (Fig. 2 and Table 1).

Tissue distribution and excretion studies. The tissue distribution of gentamicin activity in mice given a single 20-mg/kg dose of free or liposomal gentamicin was determined at several intervals after administration (Table 2). The activity recovered in the spleens of animals given the liposomal formulation was 50 to 100 times greater than that found in animals given the free drug. Similarly, there was substantial activity in the livers of animals in the liposomal group but no detectable activity in the livers of animals in the free group. Peak levels in the kidneys occurred at 1 to 3 h after administration in the free group but was delayed to 7 to 24 h in the liposomal group.

TABLE 3. Tissue distribution of gentamicin activity at 1 h after a single 20-mg/kg intravenous dose of free or liposomal drug in rats[a]

Tissue	Free gentamicin		Liposomal gentamicin	
	μg/g	% of dose	μg/g	% of dose
Plasma[b]	17.6	2.63	73.4	10.99
Kidney	42.0	2.15	30.5	1.56
Spleen	1.5	0.002	490.6	7.90
Liver	1.6	0.35	61.6	13.38
Lung	2.6	0.13	15.6	0.77
Bone and marrow	1.9	~0.28	1.0	~0.15
Adrenal gland	0.5	<0.002	4.4	<0.002
Ovary	1.4	<0.002	1.9	<0.002

[a] Values shown are the means obtained from the tissues of three rats.
[b] Percentage of dose was calculated by assuming that plasma volume was 3 ml/100 g of body weight.

In rats given a single 20-mg/kg dose, substantially higher levels of gentamicin activity were found in the livers and spleens of animals given the liposomal drug compared with those in animals given the free drug at 1 h (Table 3). Gentamicin activity was not detected after the 1-h time point in any organ other than the kidney (where up to 100 μg/g was found for 3 weeks) in animals injected with the free drug. In animals that received the liposomal drug, gentamicin activity was detectable in the kidney and spleen for 15 weeks, in the liver and adrenal glands for 2 weeks, and in the lung and bone for 3 days after a single dose.

Table 4 shows the cumulative gentamicin activity excreted in the urine of rats over a 10-day period after the administration of a single 20-mg/kg dose of free or liposomal drug. Virtually 100% of the administered dose was recovered in the urine within 24 h in the animals that received the free drug. After 24 h, only 52% of the activity was recovered in the urine of animals given the liposomal preparation. Gentamicin activity was detected in the urine for up to 10 days after dosing in these animals, at which time the mean cumulative percentage of dose recovered was 82%.

Mouse protection tests. When treatment was given imme-

TABLE 2. Tissue levels of gentamicin activity in mice after a single 20-mg/kg dose of free or liposome-encapsulated drug[a]

Tissue	Formulation	Mean μg/g ± SEM at time (h) after administration:				
		1	3	7	24	48
Spleen	Free	2.5 ± 1.9	2.3 ± 1.3	1.8 ± 1.4	<1.0	<1.0
	Liposome	106.2 ± 38.0	220.5 ± 67.0	238.0 ± 111.0	86.7 ± 45.0	57.5 ± 8.2
Liver	Free	<1.0	<1.0	<1.0	<1.0	<1.0
	Liposome	8.2 ± 4.2	15.7 ± 3.3	20.7 ± 1.6	39.7 ± 6.3	25.0 ± 2.1
Kidney	Free	34.0 ± 3.0	37.9 ± 4.7	26.2 ± 2.3	18.2 ± 1.4	10.1 ± 0.7
	Liposome	17.4 ± 3.3	25.8 ± 3.2	26.8 ± 3.1	38.4 ± 2.4	7.4 ± 0.8

[a] There were five mice per group per time point.

TABLE 4. Urinary excretion of gentamicin activity in rats after a single, intravenous 20-mg/kg dose of free or liposomal gentamicin[a]

Days postdose	Mean cumulative activity (% of dose administered ± SEM)	
	Free gentamicin	Liposomal gentamicin
1	112.0 ± 3.1	52.4 ± 10.6
2	114.0 ± 2.9	63.1 ± 10.9
3	114.6 ± 2.9	68.5 ± 10.8
4	115.3 ± 2.8	72.0 ± 10.5
5	115.5 ± 2.9	74.7 ± 10.3
6	115.9 ± 2.9	76.6 ± 10.1
7	116.1 ± 3.0	78.3 ± 10.1
8	116.3 ± 3.0	80.1 ± 10.2
9	116.5 ± 3.0	81.0 ± 10.1
10	116.6 ± 3.0	82.2 ± 10.2

[a] Values shown are the means from five rats in each treatment group.

diately after infection (0 h), the 50% protective dose for liposomal gentamicin against *S. typhimurium* and *Klebsiella pneumoniae* was slightly higher than that for the free drug (Table 5). With *Escherichia coli* and *Staphylococcus aureus*, the 50% protective dose for the liposomal preparation was slightly lower than that of the free drug. In general, the differences were not great. When treatment was given 4 h to 7 days before infection, however, the liposomal drug appeared to provide much greater protection than the free drug (Table 5).

Mouse salmonellosis. The mean median survival time for untreated mice infected intravenously with 700 CFU of *S. typhimurium* was 6.5 days. When mice were treated immediately postinfection (0 h) with free drug, there was a slight increase in survival time (Table 6). No other dosage regimen of free drug tested had any effect on the survival of infected mice. The majority of mice treated with a single dose of liposomal gentamicin as early as 7 days before infection or up to 2 days after infection survived until the termination of the study. Empty liposomes (at a lipid dose equivalent to that administered to gentamicin liposome groups) given 1 day after infection did not prolong survival above that seen in the untreated animals.

TABLE 5. Efficacy of intravenous treatment with free and liposome-encapsulated gentamicin in mice infected systemically by the intraperitoneal route

Organism	Strain	Log CFU per mouse	Time of treatment	PD_{50}[a] (mg/kg) for gentamicin	
				Free	Liposome
Salmonella typhimurium	ATCC 14028	4.69	0 h	15	20
Staphylococcus aureus	ATCC 29740	8.30	0 h	4	1
Escherichia coli	ATCC 25922	6.69	0 h	1.55	1.09
E. coli	ATCC 25922	6.69	−4 h	>50	21.75
E. coli	ATCC 25922	6.69	−1 day	>50	30.17
E. coli	ATCC 25922	6.69	−2 day	>50	>50
Klebsiella pneumoniae	DTS	6.00	0 h	0.12	0.25
K. pneumoniae	DTS	6.00	−1 day	>16	2
K. pneumoniae	DTS	6.00	−2 day	>16	2.40
K. pneumoniae	DTS	6.00	−3 day	>16	5.30
K. pneumoniae	DTS	6.00	−7 day	>16	8.80

[a] PD_{50}, 50% protective dose.

TABLE 6. Comparison of free and liposomal gentamicin in the treatment of murine salmonellosis[a]

Time of treatment	Gentamicin dose (mg/kg)	Median days of survival	
		Free gentamicin	Liposomal gentamicin
−7 days	10	7	>42[b]
−2 days	10	6	>42
−1 day	10	6	>42
0 h	10	10	>42
+1 day	20	6	>33[c]
+1 day	10	6	>33
+1 day	5	5.5	>33
+1 day	2.5	5.5	22
+1 day	1.25	5.5	27
+ 1 day and + 3 days	20	6.5	>14[d]
+ 1 day and + 3 days	10	6.5	>14
+ 1 day and + 3 days	5	6.5	>14
+ 2 days	5	5.5	>14

[a] There were 10 mice per treatment group.
[b] The experiment was terminated at 42 days postinfection.
[c] The experiment was terminated at 33 days postinfection.
[d] The experiment was terminated at 14 days postinfection.

DISCUSSION

The pharmacokinetics and tissue distribution of gentamicin were substantially altered when gentamicin was administered in a liposomal form to rodents as compared with a conventional, aqueous dosage form. The liposomal delivery system, composed solely of egg phosphatidylcholine, provided prolonged levels of gentamicin activity in blood and increased and prolonged concentrations of drug in certain tissues.

Studies of aqueous-phase and membrane liposome markers delivered intravenously to animals have demonstrated the importance of liposome size, charge, dose, and stability in vivo on the pharmacokinetic behavior of these markers (9). It has been shown that the blood clearance kinetics of large liposomes (>0.5-μm diameter) and high lipid doses (>1 mg of phospholipid per 25 g of mouse body weight), such as that used in these studies, is compatible with a saturable pathway of elimination (17). We found that the half-life of activity of gentamicin liposomes in serum was substantially prolonged and that the apparent rate of elimination and clearance of active drug from the blood was dose dependent. As the total dose increased, the rate of elimination and systemic clearance decreased. This is consistent with a saturable process (such as phagocytosis by fixed or circulating cells) as a primary mechanism in the removal of drug from the blood.

We did not distinguish free and liposome-encapsulated drug in the circulation of animals given the liposomal formulation. All samples were frozen and thawed before the assay, a procedure that is known to disrupt liposomal membranes and allow leakage of entrapped drug. The activity in serum that was treated with 0.2% Triton X-100 (which visibly disrupts gentamicin liposomes in saline) was not significantly different from untreated, frozen-thawed serum using our biological assay (data not shown). It is likely that freeze-thawing (in addition to overnight incubation in complex agar medium at 35°C) disrupts the liposomes sufficiently such that we were able to measure the total drug (both free and encapsulated) in the serum.

That a significant proportion of the drug was lipid or liposome associated in the circulation is clear from the fact that the distribution was markedly different from that of the

free drug. It might be expected that drug circulating in intact liposomes would not be as biologically active or available as drug circulating in the free form. The classic mouse protection test is a rapid and simple method for estimating the bioavailability of an antimicrobial agent. In these acute infections with rapidly growing organisms primarily localized in nonvascular, non-reticuloendothelial system sites (the peritoneal cavity), liposomal gentamicin was roughly as effective as or somewhat less effective than the free drug when administered intravenously immediately postinfection. This may be due to a slightly slower penetration of antibacterial activity when gentamicin is administered in a liposomal form to the site of this infection. Nonetheless, the differences were not great, suggesting that even though a substantial portion of the drug in animals given the liposomal formulation may be circulating in intact liposomes, it is still available and antimicrobially active. When given prophylactically, liposomal gentamicin showed substantially greater efficacy in the mouse protection test. This may be due to prolonged levels in serum and tissue.

As with other liposomal delivery systems (9), a large portion of the injected dose localized in organs rich in reticuloendothelial cells, particularly the liver and spleen. At 1 h after injection in rats, over 20% of the dose was found in these two organs. Similarly, in mice there was about 50 times more activity in the spleens and at least 8 times more activity in the livers at 1 h after injection in liposome-treated animals than in those given free drug. The bone marrow and blood sinuses of the lungs and adrenal capillaries are also considered part of the reticuloendothelial system (14), and we found higher and more prolonged levels of gentamicin activity in these organs also. This tissue distribution may be particularly advantageous in the treatment of certain disseminated, intracellular bacterial infections such as salmonellosis, brucellosis, listeriosis, and mycobacterial infections (18). In experimental murine salmonellosis, where infection is by the intravenous route, the initial septicemia is followed by localization of the organism within the major reticuloendothelial system organs. The bacteria can survive and multiply within macrophages and will cause a chronic infection unless death intervenes. The mechanism of the enhanced therapeutic effect of gentamicin liposomes in this model is probably related to targeting of the antibiotic to the organ, cellular, and possibly subcellular site of bacterial residence.

Gentamicin activity was detected for 2 weeks in the liver and up to 15 weeks in the spleen after a single dose of the liposome formulation. The persistence is most probably due to the nature of the aminoglycosides. These drugs are highly stable and not metabolized in vivo. Because of their highly polar nature, they penetrate cells very poorly, but, once inside, their intracellular retention is very high (20). Therefore, once the drug is delivered intracellularly by the liposome, a large proportion of it will probably remain there, regardless of the fate of the liposomal lipids, for the lifetime of the cell.

This persistence may be beneficial in the prophylaxis of certain bacterial infections. We found that a single dose of liposomal gentamicin could protect mice from the lethal effects of *Klebsiella* and *Salmonella* infections, even when given 7 days before infection. Since there would be no detectable activity in the serum of animals at the time of infection, the protective effect must have been due to the persistence of activity in the tissues.

These results suggest that liposomal gentamicin may be a convenient and effective agent for the prophylactic and therapeutic treatment of patients susceptible to disseminated bacterial infections. Recently, it has been shown that liposomal gentamicin is more effective than free gentamicin in the treatment of *Mycobacterium avium* complex infection in beige mice (S. P. Klemens, M. H. Cynamon, C. E. Swenson, G. S. Palmer, and R. S. Ginsberg, Abstr. Annu. Meet. Am. Soc. Microbiol. 1989, U55, p. 164). In addition, the prolonged action of liposomal gentamicin may allow less frequent dosing (and perhaps the administration of less total drug) in the treatment of serious gram-negative infections. This has recently been demonstrated in models of thigh and pulmonary infection with *K. pneumoniae* in neutropenic mice (J. Leggett, W. A. Craig, B. Vogelman, S. Ebert, R. Ginsberg, and C. Swenson, Program Abstr. 29th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 163, 1989).

The benefits of liposome encapsulation of gentamicin (or any drug) must be weighed against the potential risks. It is not yet known what effects high and prolonged serum and tissue levels may have on the development of antibiotic resistance and/or toxicity. Preliminary work suggests that encapsulation changes the natural history of gentamicin nephrotoxicity (A. Whelton, R. L. Stout, J. London, C. Swenson, and R. Ginsberg, J. Clin. Pharmacol. **29**:856, 1989). This and other aspects of the pharmacodynamics and pharmacotoxicology of liposomal gentamicin require further study.

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