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Successful Treatment Using Gentamicin Liposomes of *Salmonella dublin* Infections in Mice

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Gentamicin entrapped within stable multilamellar liposomes was used to treat mice after they were infected per os with *Salmonella dublin*. Of 10 mice, 8 survived after a single intravenous (i.v.) injection of 2 mg of gentamicin liposomes per kg compared with 0 of 10 treated with the same amount of free gentamicin. All mice survived after treatment with a single i.v. or intraperitoneal injection of 20 mg of gentamicin liposomes per kg, whereas that dose of free drug was completely ineffective and caused neuromuscular paralysis when injected rapidly i.v. In mice treated with gentamicin liposomes, there was a steady decrease in the number of salmonellae in spleens for 2 weeks after treatment. High concentrations of gentamicin were present in the spleen for at least 10 days after treatment. Although gentamicin was not detected in the mesenteric lymph nodes of mice treated with gentamicin liposomes, bacterial counts in the nodes also decreased over time. Small numbers of bacteria remained viable in the mesenteric lymph nodes and Peyer's patches but not in the spleens of mice treated with 20 to 80 mg/kg. Mice treated with doses of gentamicin liposomes as high as 80 mg/kg showed only a transient increase in blood urea nitrogen and no rise in serum creatinine. These results confirm that gentamicin in liposomes is less toxic in mice than is free gentamicin and is extremely effective therapy for disseminated *Salmonella* infections in mice.

It has been proposed that liposomes can be used as carriers of antibiotics in order to increase their therapeutic index (for a review, see reference 15). Since liposomes are cleared from the blood by and concentrate in fixed tissue macrophages (the reticuloendothelial system), one would predict that liposome-entrapped antibiotics would be particularly useful for the treatment of infections that localize in macrophages (22). The advantage of liposome-entrapped over free antibiotics should be particularly evident for antibiotics such as aminoglycosides, which are excluded from macrophages and are therefore ineffective therapy for typhoid fever despite excellent in vitro activity (7). Intracellular antimicrobial activity against *Mycobacterium avium* infection in human monocyte-derived macrophage cultures has been demonstrated (6). For nephrotoxic drugs such as gentamicin, another advantage of liposome entrapment is decreased renal excretion. Free gentamicin is usually rapidly cleared from the blood by glomerular filtration, exposing renal tubules to potentially toxic concentrations. When entrapped in liposomes, aminoglycosides are removed primarily by the liver and spleen with only a small percentage of drug appearing in the urine in the first 24 h (17). The ultimate fate of liposome-entrapped aminogylcosides has not been determined, but since only a small fraction is renally excreted acutely, the risk of renal tubular damage should be reduced substantially.

The theoretical therapeutic advantage of liposome-entrapped antibiotics has been confirmed by several investigators who have used animal models of infection with facultative intracellular pathogens. Tadakuma et al. showed that liposome-entrapped streptomycin was superior to the free drug for treating *Salmonella enteritidis* infections in mice (24). Fountain et al. used liposome-entrapped streptomycin to successfully treat *Brucella canis* and *Brucella abortus* infections in mice and guinea pigs (8, 12). Liposome-entrapped cephalothin also has been shown to be more active than free drug against *Salmonella typhimurium* in vitro and in mice (9, 10), and liposome-entrapped ampicillin is about 100 times more active than free ampicillin in murine listeriosis (5).

These results prompted us to study the therapeutic value of liposome-entrapped gentamicin (gentamicin liposomes) in a murine model of fatal *Salmonella dublin* infection induced by oral challenge. Using this model, we were able to determine that gentamicin liposomes had antimicrobial activity within Peyer's patches and the mesenteric lymph nodes as well as within the spleen.

MATERIALS AND METHODS

Mice. Female BALB/c mice were purchased from Simmonson Laboratories, Gilroy, Calif., and infected when they weighed 20 g. Animals were housed five per cage and allowed free access to food and water. BALB/c mice carry the *Ity*^{s} allele that makes them susceptible to *Salmonella* infections (19).

Infection. *S. dublin* Lane was suspended in 0.1 M $NaHCO_3$ to 1×10^8 CFU/ml, and 0.1 ml of the suspension was delivered into the stomach by gavage. Untreated, the infection spreads from the gut to the mesenteric lymph nodes, liver, and spleen, and the mice die within 10 days (13).

Liposome formulation. Oligolamellar gentamicin liposomes composed of partially hydrogenated egg phosphatidylcholine, egg phosphatidylglycerol, cholesterol, and alpha-tocopherol were prepared aseptically by a solvent injection method developed by Liposome Technology, Inc. (G. West and F. Martin, U.S. patent 4,781,871, 1988). The liposomes were extruded through a 1-μm-pore-size polycarbonate filter. Unencapsulated gentamicin was removed by washing the gentamicin liposomes in isotonic buffer and

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centrifugation. Gentamicin concentration in the final liposome formulation was determined by EMIT assay (Syva, Palo Alto, Calif.) after mild detergent treatment. Greater than 97% of the total drug was liposome associated. The mean (average) diameter of the particles in the liposome formulation was determined by using the multichannel counter (model TAII; Coulter Electronics, Inc., Hialeah, Fla.) to be 1.1 μm. The gentamicin liposomes and the control samples were shown to be negative in the *Limulus* amebocyte lysate assay (Associates of Cape Cod, Inc., Woods Hole, Mass.) for endotoxin. The gentamicin liposomes were stable throughout the entire study period of 9 months (25).

Organ and tissue cultures. Organs and tissues were removed aseptically and homogenized in 2 ml of 0.9% saline as previously described (13). The homogenates were cultured on Trypticase (BBL Microbiology Systems, Cockeysville, Md.) soy agar adjusted with 0.1 M HCl to pH 5.7 to reduce the activity of any residual gentamicin that might be in the tissue. At pH 5.7, the MIC of gentamicin for *S. dublin* Lane was >20 μg/ml, whereas the MIC at neutral pH was 1.25 μg/ml. Peyer's patches were cultured on eosin-methylene blue agar (EMB; Difco Laboratories, Detroit, Mich.). Representative non-lactose-fermenting colonies were subcultured and confirmed to be *S. dublin* by standard methods.

Gentamicin assay. Tissues were homogenized in saline. The homogenate was centrifuged at 10,000 × *g* for 10 min to sediment debris and residual bacteria. Supernatants were frozen at −70°C until they were thawed for assay. Gentamicin was measured by using the EMIT system with a sensitivity of 1 μg/ml.

RESULTS

Mice were treated with 2, 10, or 20 mg of gentamicin liposomes per kg given either intravenously (i.v.) or intraperitoneally (i.p.) on day 4 of infection. Control mice received either an equal dose of aqueous gentamicin, placebo liposomes, or buffer (the 20-mg/kg i.v. dose of aqueous gentamicin had to be administered slowly to avoid neuromuscular blockade and death). There were 10 mice in each group. Control mice began to die 2 days after treatment, and all were dead by 5 days after treatment (day 9 after infection) (Fig. 1). Aqueous gentamicin had no significant effect on mortality. In contrast, 8 of 10 mice survived for 30 days after receiving one i.v. injection of 2 mg of gentamicin liposomes per kg, even though they did appear ill (decreased activity, ruffled coat, and weight loss). Mice treated with 10 or 20 mg of gentamicin liposomes per kg gained weight and appeared healthy with 3 days of treatment, and only one mouse in these groups died. When gentamicin liposomes were given i.p., they were slightly less effective relative to i.v. dosing but more effective than the free drug. Of 10 mice, 8 treated with 2 mg/kg i.p. died compared with only 2 of 10 treated with that dose given i.v. (χ^2, $P < 0.01$). The 10- and 20-mg/kg doses were equally effective when given i.p. or i.v. (these results were reported previously [25]). Because the i.p. route of administration of liposomes is less effective (18) and would not be used clinically, in all subsequent experiments the drug was given exclusively by injection into tail veins.

At the end of the experiment, 30 days after infection, we autopsied three surviving mice from each of the three i.v. treatment groups and enumerated bacteria in their organs. The most striking difference between the groups was in the mesenteric lymph nodes. Of three mice that received 2 mg/kg, two had frank abscesses in their lymph nodes, and each of the abscesses contained >10^6 CFU. In contrast, all

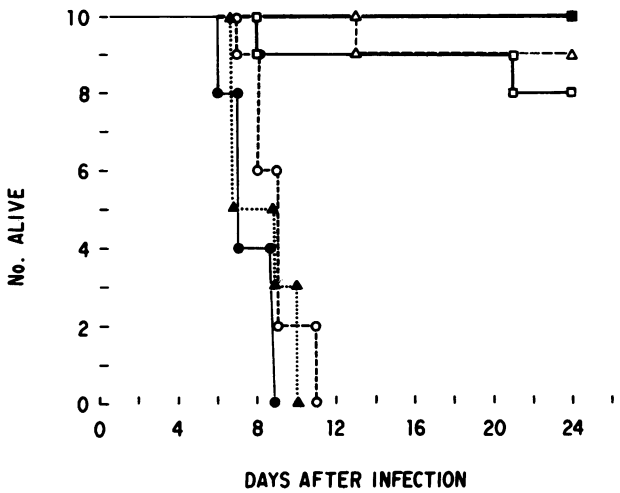

FIG. 1. Effect of a single i.v. dose of gentamicin on mortality after oral infection with *S. dublin*. Treatment was given on day 4 after infection. For simplicity, the only controls that are shown are citrate buffer (●), placebo liposomes only (equivalent to the amount in the 20-mg/kg dose) (▲), and 20 mg of free gentamicin per kg (○). Treatment was with 2 (□), 10 (△), or 20 (■) mg of gentamicin liposomes per kg. Mice were sacrificed when they appeared moribund to reduce their suffering.

of the nodes from mice that had received the higher doses contained <10^3 CFU. Although all three doses suppressed bacterial growth in the spleen, only mice treated with 20 mg/kg had sterile spleens. There were relatively few viable salmonellae remaining in Peyer's patches, and there was no significant difference between the counts from the 2- and 20-mg/kg groups (data not shown).

We then determined the kinetics of the in vivo antibacterial activity of gentamicin liposomes. As described above, BALB/c mice were infected orally with *S. dublin* and 3 days later were treated with either free gentamicin or gentamicin liposomes at 10 mg/kg. At various intervals after treatment, we sacrificed mice and enumerated surviving bacteria in the spleen, mesenteric lymph nodes, and Peyer's patches. At the start of treatments, there were 10^2 CFU per spleen and approximately 10^3 CFU in both the mesenteric lymph nodes and Peyer's patches (Fig. 2). Three days later, mice that had received free gentamicin had over 10^6 CFU per spleen, and counts of *S. dublin* had increased nearly 2 logs in the nodes and 1 log in Peyer's patches. None of the gentamicin-treated mice survived until the next time point. Three days after injection of gentamicin liposomes, there was only a fivefold increase in CFU in the spleen; thereafter, viable counts gradually decreased. Gentamicin liposomes also inhibited bacterial growth in the nodes and Peyer's patches by approximately 1 log compared with controls. On day 3 after treatment, there were small increases in the mean of bacterial counts at these sites, but after that, viable bacteria decreased in the mesenteric lymph nodes but stabilized in Peyer's patches.

To determine whether there would be added benefit from multiple injections of gentamicin liposomes, we treated 10 mice with one or two doses of 10 mg/kg; half of the mice received a second dose of 10 mg/kg 7 days after the first dose, while the remaining mice were given a second injection of placebo liposomes. For comparison, five mice received a single dose of 20 mg/kg. Fourteen days after the first dose, we determined the number of surviving bacteria in the spleens and mesenteric lymph nodes of the mice. Two doses

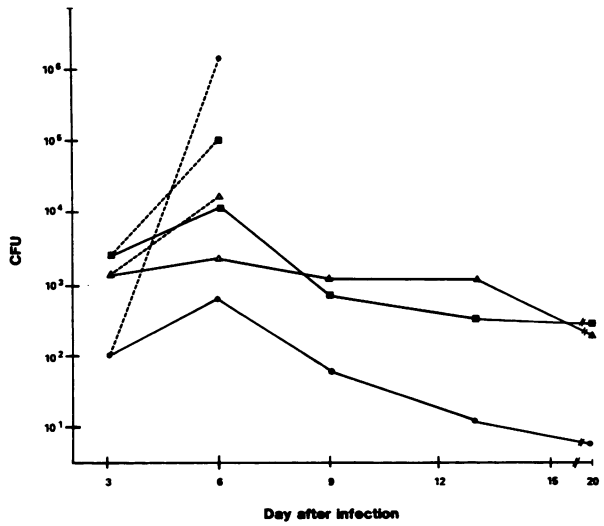

FIG. 2. Viable *S. dublin* recovered from organs after treatment with 10 mg of gentamicin liposomes per mg administered via a tail vein. Gentamicin liposomes were given on day 3. Symbols: ----, treatment with free gentamicin; ——, treatment with gentamicin liposomes; ●, spleen; ■, mesenteric lymph nodes; △, Peyer's patches. Each datum point is the mean of three mice.

of 10 mg of gentamicin liposomes per kg were more effective than one dose (Table 1). All the mice treated with two doses had sterile spleens. In contrast, 4 of 5 spleens were infected in the group which had received one dose of gentamicin liposomes, although they contained only a mean of 10^2 CFU per spleen. One dose of 20 mg/kg was about as effective as two doses of 10 mg/kg; only 1 of 5 spleens yielded viable bacteria, and that spleen contained only 80 CFU. There was no significant difference between the antibacterial effects of the three treatments on bacteria in mesenteric nodes.

Because we did not sterilize lymph nodes by treating mice with 20 mg/kg in either one or two divided doses, we attempted to eradicate *S. dublin* by using even higher doses of gentamicin liposomes. Mice were infected as described above and 3 days later were given either 40 or 80 mg of

TABLE 1. Effects of one versus two doses of gentamicin liposomes on antimicrobial activity[a]

Gentamicin		CFU ($\bar{x}$ ± SD)	
Amt (mg/kg)	No. of doses	Spleen	Mesenteric lymph nodes
10	1	104 ± 100 (1)	1,520 ± 600 (0)
10	2	0 (5)	1,204 ± 1,100 (1)
20	1	16 (4)	596 ± 360 (0)

[a] All mice were treated i.v. with one dose of gentamicin liposomes on day 3 after oral infection. Some mice were retreated on day 10. Autopsies were done on day 17 after infection. There were five mice per group. The numbers of sterile organs are in parentheses.

gentamicin liposomes per kg. (It was not possible to administer free drug at these high doses, because the infected mice tend to die immediately because of acute neuromuscular toxicity.) Mice were sacrificed 3 and 14 days after treatment. Again, both doses of gentamicin liposomes effectively inhibited bacterial growth in the spleen (Fig. 3). While neither dose was able to sterilize mesenteric lymph nodes or Peyer's patches, there were relatively few viable bacteria in either of these organs in both treatment groups. Of note, the spleens from mice given 80 mg/kg were sterile by day 3 after infection.

The prolonged bactericidal effect of gentamicin liposomes reflected persistently high concentrations of drug in the spleen. In Fig. 4, we show the absolute amount of gentamicin extracted from the spleens of infected mice treated with three different doses of gentamicin liposomes. There was a roughly proportionate relationship between the administered dose and the amount of gentamicin retained in the spleen at both 3 and 12 to 14 days after treatment. There was a tendency for the rate of disappearance from the spleen to be higher in mice treated with higher doses of drug. The data are expressed as the amount of drug per spleen rather than per gram of tissue, because spleen weights decreased drastically as the infection was cured. For instance, in the 10-mg/kg group, the absolute amount of gentamicin decreased 35% from day 3 to day 10 whereas the concentration (micrograms per gram) fell only 21%.

Mice tolerated large doses of gentamicin liposomes with

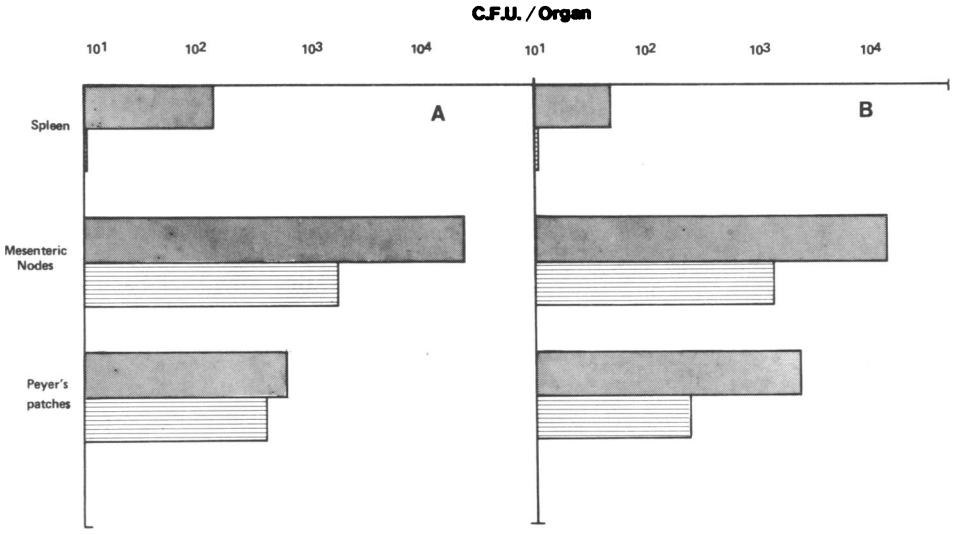

FIG. 3. Comparative antimicrobial activity (CFU) from mice treated with 40 (■) and 80 (▤) of gentamicin liposomes per kg. There were five mice in each group. (A) Day 3 after treatment; (B) day 10 after treatment.

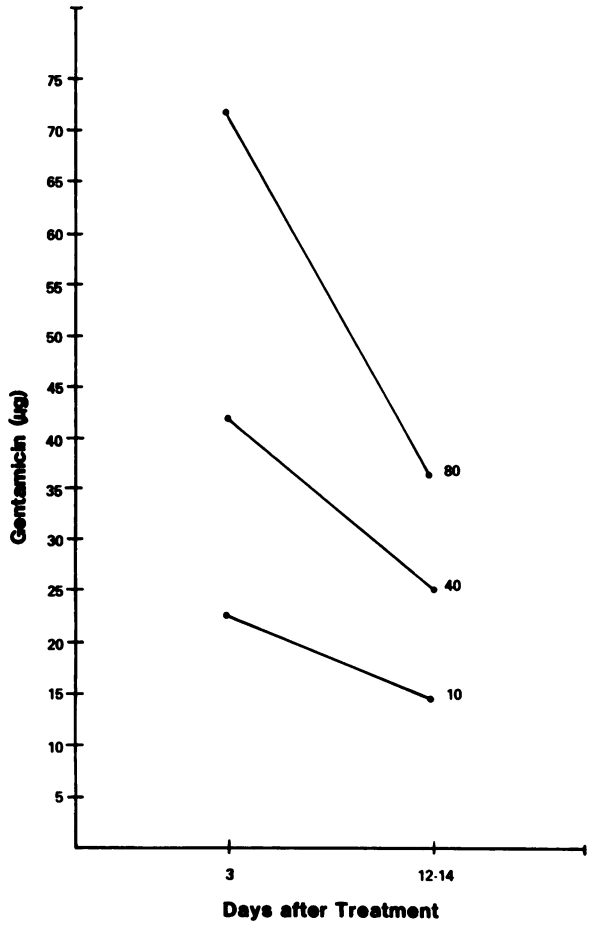

FIG. 4. Gentamicin extracted from spleens 3 and 12 or 14 days after treatment. The doses (milligrams per kilogram) of gentamicin liposomes are indicated. Each datum point is the mean of five mice.

minimal toxicity. There was no evidence of neuromuscular paralysis even after an i.v. dose of 80 mg/kg. Three days after treatment with 40 or 80 mg/kg, the blood urea nitrogen (BUN) was significantly elevated but the serum creatinine was normal. By 2 weeks, the BUN returned to normal (Table 2). Three normal mice treated with 80 mg of gentamicin liposomes per kg also had transient increases in BUN (30- to 37-mg/dl range) but not serum creatinine, making it likely that treatment resulted in mild renal damage. This probably reflects the slow release of gentamicin from the liver and spleen (Fig. 4) and the subsequent efficient renal excretion of free gentamicin. In support of this, we found gentamicin in the kidneys of all three mice tested 3 days after a 10-mg/kg dose of gentamicin liposomes ($\bar{x}$ = 20 μg/g of tissue), whereas none of the kidneys from mice given free gentamicin had measurable levels. The transient rise in BUN may have been caused by the gentamicin, but we cannot exclude a prerenal cause for the elevated BUN, such as dehydration or increased catabolism due to the infection.

TABLE 2. Effects of gentamicin liposomes on renal function

Gentamicin (mg/kg)	Concn (mg/dl) in serum at[a]:			
	Day 3		Day 14	
	BUN	Creatinine	BUN	Creatinine
10	ND	ND	21.7 ± 6.3	0.4 ± 0
20	ND	ND	24.3 ± 4.5	0.5 ± 0.05
40	39.5 ± 5.5	0.5 ± 0.14	25.6 ± 3.65	0.4 ± 0.07
80	39.3 ± 2.5	0.45 ± 0.06	23.4 ± 2.88	0.4 ± 0.05

[a] Values are expressed as means ± standard deviations. Normal levels for BUN and creatinine are 22 to 26 (range) and 0.4 ± 0.05 mg/dl, respectively. ND, Not determined.

DISCUSSION

The animal model we used to test the efficacy of gentamicin liposomes was chosen to provide a stiff challenge for the drug. *S. dublin* is a highly virulent strain in BALB/c mice, which are genetically susceptible to salmonellae (19). By withholding therapy for 3 or 4 days, we allowed the infection to spread from the intestine to the mesenteric lymph nodes, liver, and spleen (13). This allowed us to determine whether the antibiotic was active in various organs that are affected in the course of a naturally acquired invasive *Salmonella* infection, not just the major reticuloendothelial system organs, i.e., the liver and spleen, which were expected to accumulate most of the antibiotic. Using this experimental model, we demonstrated that gentamicin liposomes provide an extraordinarily effective therapy for *S. dublin* infections in mice. As little as a single injection of 2 mg of gentamicin liposomes per kg lowered the mortality from 100 to 20% when given i.v. Higher doses of gentamicin liposomes (10 or 20 mg/kg) were effective by either the i.p. or i.v. route. The 2-mg/kg dose was much less effective when given i.p., probably because liposomes are not completely absorbed into the bloodstream from the peritoneum (18).

Since streptomycin liposomes are effective treatment for murine salmonellosis (24), we anticipated that gentamicin liposomes also would be an effective treatment for *S. dublin* in mice. However, we were concerned that insufficient amounts of antibiotic might reach some peripheral tissues that are infected after ingestion of salmonellae, such as mesenteric lymph nodes and Peyer's patches, because they are less well endowed with macrophages and are less well perfused than the liver and spleen (16). This concern proved to be founded. Although 80% of mice treated with 2 mg of gentamicin liposomes per kg survived infection, 2 of 3 developed suppurative mesenteric lymphadenitis. This complication did not occur in mice treated with 10 or 20 mg of gentamicin liposomes per kg. In fact, 27 days after treatment with either 10 or 20 mg/kg, mice had very few viable bacteria in their lymph nodes, even though gentamicin was not detected in their nodes (data not shown). Because there are relatively few macrophages in the lymph nodes and the gentamicin was concentrated inside macrophages and not distributed uniformly throughout the node, we suspect that by assaying a homogenate of whole nodes we were unable to detect the small but functional concentration of gentamicin in the nodes. The ability of gentamicin liposomes to limit and then reduce the growth of bacteria in the nodes (Fig. 2) suggests that gentamicin does reach the nodes. Nevertheless, it was not possible to sterilize the lymph nodes or Peyer's patches with doses of gentamicin liposomes as high as 80 mg/kg, though the numbers of surviving bacteria were quite low. We anticipate that eventually the residual salmonellae would have been eliminated by the immune response of the host.

Assuming that gentamicin inhibited bacterial growth in the lymph nodes and Peyer's patches, it is uncertain how the gentamicin entered these tissues. In normal mice, nearly all the injected liposomes are taken out of the bloodstream by

the liver and spleen, because these organs have fenestrated capillaries and are well perfused (20, 21). Liposomes do not pass through the intact endothelium. However, circulating monocytes and polymorphonuclear leukocytes also ingest liposomes (20), and these cells would be attracted to inflammatory foci. Thus, monocytes and polymorphonuclear leukocytes that are recruited to the infected tissues could come "armed" with gentamicin. Another possibility is that capillary integrity may be compromised sufficiently at sites of inflammation to allow egress of liposomes from the bloodstream. Once liposomes enter the exudate, they would be taken up by local phagocytes or degraded by phospholipases releasing free gentamicin. We cannot exclude the possibility that released free gentamicin killed the salmonellae in the lymph nodes, although this seems very unlikely since we could not detect gentamicin in the nodes. Others also have observed unexpected antimicrobial activity of liposome-entrapped drug in organs or tissues (such as skin) that are poorly endowed with macrophages (3). The most likely explanation for these results is that macrophages outside the reticuloendothelial system do take up liposomes, but more sensitive methods for tracing the fate of injected liposomes are needed to test that hypothesis.

By encapsulating gentamicin in liposomes, we eliminated the curarelike effect of free gentamicin. Rapid i.v. administration of 20 mg of free gentamicin per kg caused paralysis and death, and we could not safely administer higher doses. In contrast, mice remained fully active even after receiving 80 mg of gentamicin liposomes per kg. We also found little evidence of renal toxicity even after large doses of gentamicin liposomes. Mice treated with 40 or 80 mg of gentamicin liposomes per kg had only a transient rise in BUN and no rise in serum creatinine 3 days later. By day 10, the BUN had returned to normal. We do know that gentamicin is slowly released from the spleen, and we could detect gentamicin in kidneys from mice that received a dose of 10 mg/kg 3 days earlier (mean, 20 μg/g). It is possible, therefore, that prolonged release of gentamicin from the reticuloendothelial system and subsequent renal excretion of gentamicin might cause renal damage. However, this is minimal compared with what occurs after treatment with much smaller doses of free gentamicin. More than 70% of free gentamicin is excreted in the urine in 24 h, primarily by glomerular filtration. This results in high urine concentrations and renal tubular damage. Less than 10% of gentamicin in liposomes is excreted in the same time period (17). Exactly how much gentamicin can be given safely within liposomes remains to be established, but our results suggest it will be possible to safely administer much more gentamicin in liposomes than free gentamicin.

Liposomes themselves are remarkably nontoxic and did not appear to affect the course of the *S. dublin* infection in these experiments (1, 2). The ultimate fate of gentamicin liposomes is not known. Liposomes are degraded by phospholipases in lysosomes after they fuse with the phagocytic vesicles (21, 23). The phospholipids in liposomes are hydrolyzed, and components are utilized by host cells. In the process, the contents of the liposome are released (11). Gentamicin is not metabolized in the body, and this highly charged antibiotic is a potent inhibitor of mammalian phospholipases (4, 14). In itself, this is unlikely to adversely affect the liver or spleen, as tissue macrophages are renewed from the bone marrow at a high rate.

From the results of these experiments and previously published experiments with animals infected with salmonellae (2, 25), brucellae (12), listeriae (5), or leishmaniae (3), it is demonstrated that liposome-entrapped antimicrobial agents are an improved dosage form for the treatment of infections with obligate and facultative intracellular pathogens that localize to the reticuloendothelial system. Liposomes target the antimicrobial agents to the infected cells, thereby increasing activity and decreasing toxicity. If inflammation allows liposomes to reach other sites of infection, this method of drug delivery may have even wider applications.

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