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Liposome-Encapsulated-Amikacin Therapy of *Mycobacterium avium* Complex Infection in Beige Mice†

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Efficacy of liposome-encapsulated amikacin and free amikacin against *Mycobacterium avium* complex was evaluated in the beige mouse (C57BL/6J-bg^J/bg^J) acute infection model. Approximately 10^7 viable *M. avium* complex serotype 1 cells for which the MIC of amikacin was 8 μg/ml were given intravenously. Treatment was started with encapsulated or free amikacin at approximately 110 or 40 mg/kg of body weight 7 or 14 days later. In the former experiment, treatment was given two or three times per week. In the latter experiment, treatment was given daily for 5 days. The animals were sacrificed 5 days after the last dose. Liver, spleen, and lung were homogenized, and viable cell counts were determined on 7H10 agar. An analysis of variance and subsequent Tukey HSD (honestly significant difference) tests indicated that both encapsulated and free amikacin significantly reduced viable cell counts in each of the organs compared with counts in the control group. Compared with free amikacin, encapsulated amikacin significantly reduced viable cell counts in the liver and spleen. Liposome encapsulation of an active agent appears to be a promising therapeutic approach to *M. avium* complex infection.

Mycobacterium avium complex (MAC) is usually associated with chronic pulmonary disease in nonimmunocompromised patients (23) and disseminated infection in patients with acquired immunodeficiency syndrome (AIDS) (5, 24). In patients with AIDS, the blood, spleen, liver, lymph nodes, bone marrow, and lung have been involved (10, 12). In nonimmunocompromised patients, the intrinsic resistance of MAC to conventional chemotherapeutic agents is the primary problem with therapy (4). Patients with AIDS have the additional problem of a severely compromised immune system. There is currently no treatment regimen that is convincingly effective against MAC infection in patients with AIDS.

Liposome encapsulation of a variety of antimicrobial agents has improved therapeutic efficacy, particularly in the treatment of disseminated, facultative, intracellular bacterial infections (19). Liposomes given intravenously concentrate in organs with sinusoidal capillaries containing reticuloendothelial cells, such as the liver, spleen, and bone marrow (14–16), which are frequently the sites of localization of disseminated bacterial infections. Liposomes are taken up by macrophages in vitro and in vivo, and liposome-encapsulated antimicrobial agents show greater killing of intracellular bacteria, including MAC, in macrophage cultures in vitro than do nonencapsulated antimicrobial agents (3, 15, 19).

Various chemotherapeutic regimens for the treatment of MAC infection have been studied in normal (7, 13, 18) and beige (C57BL/6J-bg^J/bg^J) mice (5–8). Most drugs show little activity (7, 13, 18). Therapy with aminoglycosides may be effective but only at high doses for prolonged periods (8, 13, 18). Recently, Düzgünes et al. (5) showed that negatively charged large unilamellar and multilamellar vesicles containing amikacin at relatively low doses had enhanced efficacy in the treatment of early MAC infection in beige mice compared with similar doses of the free drug. The purpose of the present study was to evaluate the efficacy of liposome-encapsulated amikacin in MAC infections by using the beige mouse model. We studied more established infections and used neutral liposomes and higher drug doses.

MATERIALS AND METHODS

Drugs. Amikacin sulfate was prepared by titration of amikacin base with sulfuric acid and collection of the salt by lyophilization. The amikacin sulfate was dissolved in 0.9% saline to yield a final concentration of 5.5 mg of amikacin activity per ml.

Egg phosphatidylcholine (70 g) and amikacin sulfate (35 g) were used to prepare 1 liter of stable plurilamellar vesicles (9). The liposomes were washed by diafiltration. According to microbiological assays for drug content and phosphate assay for lipid content of the final suspension, 23% of the initial amikacin sulfate was entrapped, and the active drug-to-lipid ratio was 1:13.7 (by weight). The final composition was 5.5 mg of amikacin base as amikacin sulfate per ml and 74 mg of phospholipid per ml in 0.9% saline. The liposomes were stored at 4°C prior to use.

Drug distribution studies. Mice (outbred CD-1 males from Charles River Breeding Laboratories, Inc., Wilmington, Del.) were injected intravenously with free or encapsulated drug. At intervals, three to five mice from each treatment group were anesthetized with tribromoethanol and bled by cardiac puncture. Serum was collected and frozen for later bioassay. For determinations of drug levels in tissue, mice were killed by cervical dislocation, and selected organs were aseptically removed, weighed, diluted with distilled water, and homogenized. Samples of serum or tissue homogenate were assayed by an agar well diffusion bioassay using *Bacillus subtilis* ATCC 6633 as the indicator organism.

Strain of MAC. Strain Lpr (serotype 1) of MAC, obtained from a patient with AIDS, was used in these experiments. A cell suspension of a predominantly (>90%) transparent colony type was used.

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† This paper is dedicated to Emmet Lyons, whose lengthy illness was the inspiration for the efforts to develop improved therapy for MAC infections.

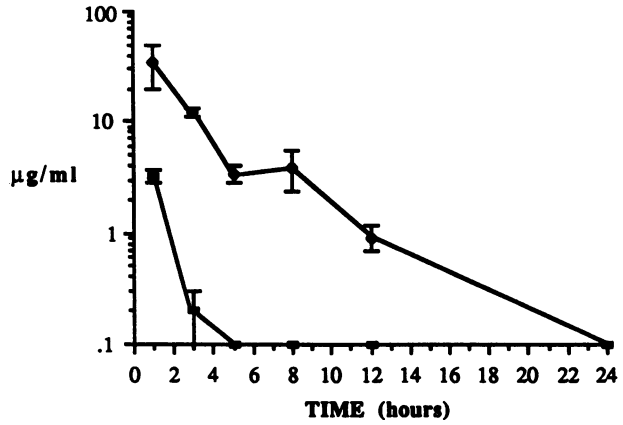

FIG. 1. Levels of amikacin in serum after an intravenous dose of 40 mg of amikacin or liposomal amikacin per kg. Symbols: ■, free amikacin; ◆, encapsulated amikacin.

Medium. The organism was grown in Middlebrook 7H10 broth (20) with Middlebrook OADC enrichment (Difco Laboratories, Detroit, Mich.) and 0.05% Tween 80 on a rotary shaker at 37°C for several days. The culture suspension was diluted in 7H10 broth to yield approximately 10^8 CFU/ml. The size of the inoculum was determined by titration and counting from duplicate 7H10 agar plates (BBL Microbiology Systems, Cockeysville, Md.) supplemented with Middlebrook OADC enrichment. The plates were incubated at 37°C for 3 to 4 weeks prior to counting.

Infection studies. Beige (C57BL/6J-bg^J/bg^J) mice (4 to 8 weeks old), bred in our own laboratories, were infected intravenously through one of the caudal veins. Each animal received approximately 10^7 viable organisms suspended in 0.2 ml of 7H10 broth. In each experiment, a control group of infected but untreated animals was compared with treated groups of animals. The activity of drug-free liposomes was compared with activity in an untreated control group to determine whether the liposomes themselves had an effect on the course of the mycobacterial infection. Animals were killed by cervical dislocation 5 days after the last dose of drug. The liver, spleen, and right lung were aseptically removed and ground in a tissue homogenizer; the number of viable organisms was determined by titration on 7H10 agar plates.

Statistics. The viable cell counts were converted to logarithms, which were then evaluated, when appropriate, with either two-tailed *t* tests or one or two variable analyses of variance. Statistically significant effects from the analyses of variance were further evaluated by the Tukey HSD (honestly significant difference) tests (11) to make pairwise comparisons among means. The results of the separate *t* tests and Tukey HSD tests for the liver, spleen, and lung are summarized below.

RESULTS

Amikacin levels in serum and tissue. Levels of amikacin activity in mouse serum after a single, intravenous bolus dose of 40 mg of free or liposomal amikacin sulfate per kg of body weight are shown in Fig. 1. The free drug showed a rapid decline in serum and was below the level of detection at 5 h after administration. The liposomal drug showed higher and more prolonged levels in serum. Levels of amikacin activity in tissue at 1, 3, and 24 h after drug administration in these mice are shown in Table 1. Comparable levels of activity were found in the kidneys of animals in both the free- and liposomal-drug groups. Activity was detected in the lungs of both treatment groups but was higher and more prolonged in the liposome-treated animals. Very high levels of activity were found in the spleens and livers of the liposomal-drug group, whereas activity was below the level of detection in these organs at all times in the free-drug group.

TABLE 1. Levels of amikacin activity in tissue after a single intravenous, 40-mg/kg dose of free or liposomal amikacin in mice

Tissue and type of amikacin	Amikacin activity (μg/g)[a] at:		
	1 h	3 h	24 h
Spleen			
Free	<0.5	<0.5	<0.5
Liposome	183.1 ± 82.4	212.5 ± 7.0	173.0 ± 13.0
Liver			
Free	<0.5	<0.5	<0.5
Liposome	22.5 ± 7.8	80.7 ± 1.5	47.6 ± 1.9
Lung			
Free	2.3 ± 0.9	0.7 ± 0.2	<0.5
Liposome	17.1 ± 8.3	9.6 ± 1.8	0.8 ± 0.2
Kidney			
Free	92.7 ± 14.2	62.8 ± 10.1	24.3 ± 3.5
Liposome	87.8 ± 13.5	80.2 ± 3.4	27.2 ± 4.7

[a] Mean for five mice ± standard error of the mean.

Effect of empty liposomes on MAC infection. Male mice were treated with empty liposomes (56.6 mg of lipid per ml; 0.3 ml) intravenously daily for 5 days beginning on day 7 after the mice were infected intravenously with 8.8×10^6 viable MAC cells. A control group consisted of untreated infected mice. The *t* tests indicated that therapy with empty liposomes resulted in significantly higher ($P < 0.01$) viable cell counts in the livers and spleens of treated mice compared with untreated mice. There was no significant difference between the two groups with regard to the viable cell counts in the lung (Fig. 2).

Effect of therapy with free or liposomal amikacin beginning on day 14 after infection with MAC. Amikacin or liposomal amikacin (approximately 40 mg/kg) was given intravenously daily for 5 days to male mice that had been infected with 2.8×10^7 viable MAC cells 14 days prior to the initiation of therapy. Analysis of variance and Tukey HSD tests indi-

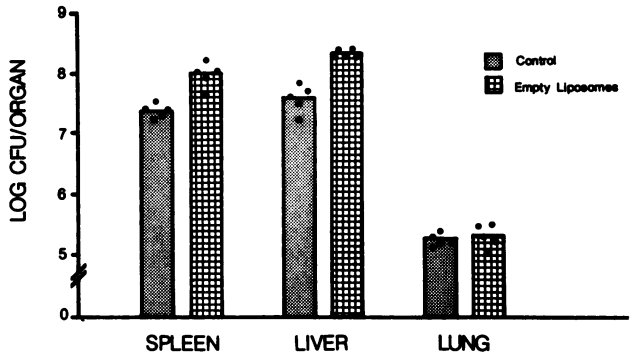

FIG. 2. Effect of empty liposomes on MAC infection in the beige mouse.

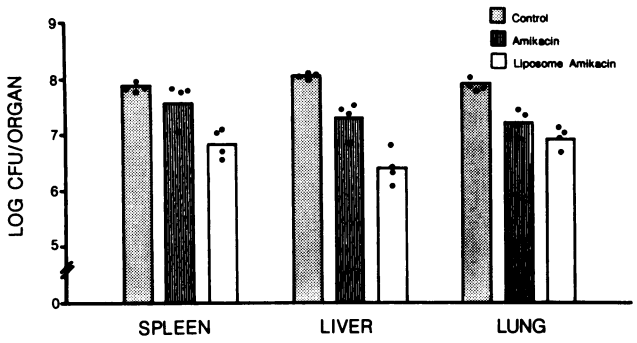

FIG. 3. Effect of therapy with free or liposomal amikacin beginning on day 14 after infection with MAC.

cated that liposomal amikacin reduced viable cell counts in the spleen, liver, and lung compared with counts in the control group ($P < 0.01$). Amikacin reduced the viable cell counts in the liver and lung compared with counts in the control group ($P < 0.01$). The viable cell counts in the spleen were not significantly different ($P < 0.05$) in these two groups. Liposomal amikacin was more active than amikacin. The former reduced the viable cell count in the liver ($P < 0.01$) and spleen ($P < 0.05$) compared with the latter. There was little difference in the activity of the two drugs on the viable cell count in the lung (Fig. 3).

Comparison of twice-weekly amikacin with liposomal amikacin on MAC. Amikacin or liposomal amikacin (approximately 110 mg/kg) was given intravenously twice a week for 3 weeks to female mice that had been infected intravenously with 1.8×10^7 viable MAC cells 7 days prior to the initiation of therapy. Analysis of variance and Tukey HSD tests indicated that liposomal amikacin and amikacin reduced the viable cell counts in the liver, spleen, and lung compared with counts in the control group ($P < 0.01$). The encapsulated amikacin was more active than the free drug for organisms in the liver ($P < 0.01$) and spleen ($P < 0.01$) but not for organisms in the lung (Fig. 4).

Liposomal amikacin three times weekly for 3 weeks in MAC infection. Liposomal amikacin (approximately 110 mg/kg) was given intravenously three times a week for 3 weeks to male mice that had been infected intravenously with 1.6×10^7 viable MAC cells 7 days prior to the initiation of therapy. Initial analyses indicated that the viable cell counts in each organ were significantly lower after 1 week of therapy (day 14) than the corresponding cell counts in mice sacrificed at day 7 prior to the initiation of therapy. In contrast, the viable cell counts for spleens and lungs from untreated mice were

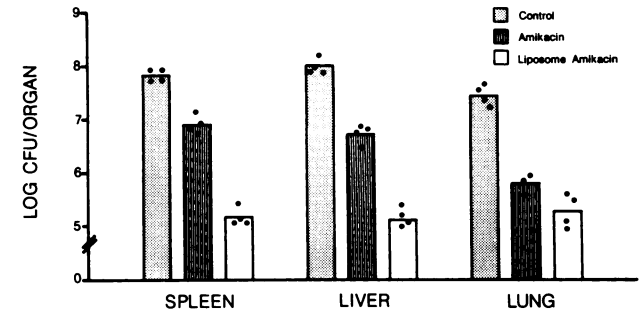

FIG. 4. Comparison of effects of twice-weekly amikacin with liposomal amikacin on MAC infection.

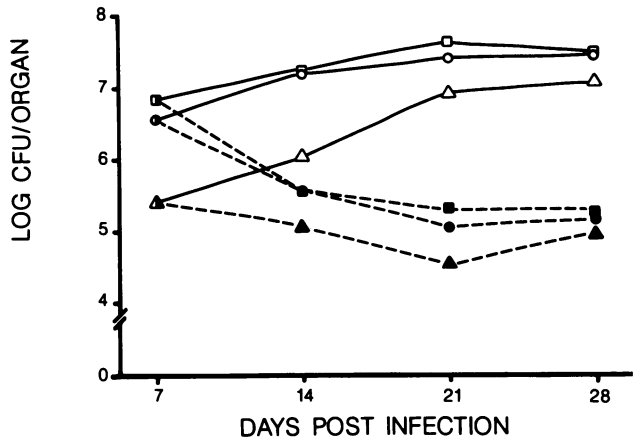

FIG. 5. Effect of liposomal amikacin three times a week for three weeks on MAC infection. Results are shown for control experiments (○, spleen; □, liver; △, lung) and for experiments with liposomal amikacin (●, spleen; ■, liver; ▲, lung).

significantly higher at day 14 compared with day 7. Analyses of variance and Tukey HSD tests further indicated that liposomal-amikacin treatment led to significantly lower viable cell counts in each organ compared with counts in the untreated control after 1 (day 14), 2 (day 21), and 3 (day 28) weeks of therapy. More important, significant ($P < 0.05$) interactions between group and time of measurement in each organ indicated that the differences between treated and untreated animals continued to increase following day 14. Maximum differences occurred at day 21, when amikacin-treated animals showed significant reductions in cell count relative to counts on day 14 in all three organs ($P < 0.05$), while untreated animals exhibited significant cell count increases in the liver and lung. Some reversals and no further significant reductions for treated animals or increases for untreated animals were observed in comparisons between days 21 and 28 (Fig. 5).

Comparison of prophylaxis with amikacin or liposomal amikacin on MAC infection. Male and female mice (two of each per group) were given amikacin or liposomal amikacin (approximately 50 mg/kg) intravenously on day 1. The control group did not receive therapy. During the morning of day 2, all mice were infected with 3.6×10^6 viable MAC cells intravenously. Amikacin and liposomal-amikacin groups received their respective drugs that afternoon and on days 3 and 4. Analysis of variance and Tukey HSD tests indicated that both liposomal amikacin and amikacin reduced the viable cell counts in each organ compared with counts in the control group ($P < 0.01$). Liposomal amikacin was more active than amikacin in the liver and spleen ($P < 0.01$) but had similar activity in the lung ($P > 0.05$) (Fig. 6).

DISCUSSION

Liposome encapsulation increased the delivery of amikacin to the liver and spleen, and in each of our experiments, liposomal amikacin was more active than free amikacin in reducing the number of hepatic and splenic organisms. Whether targeting a particular organ is sufficient to account for the improved efficacy or whether specific cellular and subcellular targeting (which has been shown in the case of liposomal meglumine antimoniate in the treatment of *Leishmania donovani* infection [22]) is necessary is not clear. Since one mechanism by which some organisms (e.g., *My-*

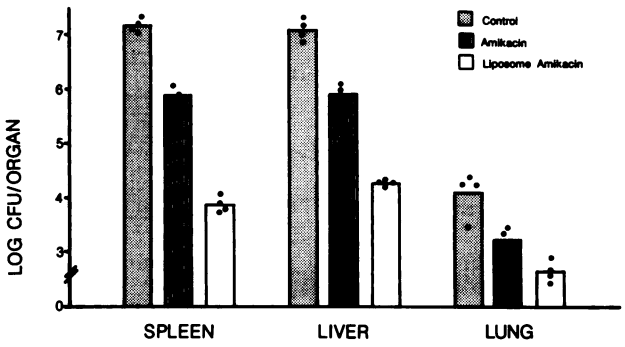

FIG. 6. Comparison of prophylaxis with amikacin or liposomal amikacin on MAC infection.

cobacterium tuberculosis) escape destruction within phagocytic cells is by preventing phagolysosomal fusion, it is unlikely that liposomes are delivered directly to the pathogen in these cases. Nonetheless, two groups have shown that liposome encapsulation can enhance the activity of antibiotics against disseminated *M. tuberculosis* in mice (15, 21). The actual intracellular location of MAC is unknown; however, it has been shown that liposomes enhance the activity of antibiotics against MAC in macrophage cultures in vitro (3), suggesting that intracellular delivery plays a role in this disease.

One of the major rationales for the use of liposomes to deliver antimicrobial agents involves targeting the drug to intracellular infections of the reticuloendothelial system. Such targeting is possible because of the natural uptake of liposomes and other particulates by these tissues. The actual amount taken up by these organs is somewhat dependent on the dose of lipid administered. With small doses, most intact liposomes are retained by the liver. With larger doses, the ability of the liver to clear the particles from the circulation may be overwhelmed, and the amount remaining in the circulation that can be taken up by the spleen and bone marrow thus increases (17). Still larger doses can produce a temporary reticuloendothelial system blockade which results in a longer circulation time but eventually a similar tissue distribution (1). In our studies, we found very high levels of amikacin activity in the spleen and liver as well as sustained levels in the serum, suggesting that we were saturating the reticuloendothelial system to some extent with the lipid doses used. We found that treatment with empty liposomes resulted in increased numbers of organisms in the spleens and livers of mice. Orozco et al. (15) also found that empty liposomes increased the number of bacteria in organs of mice infected with *M. tuberculosis*. Bakker-Woudenberg et al. (2) found that *Listeria* infections in mice were worse after treatment with placebo liposomes at high lipid doses but not at low lipid doses. Since distribution to nonhepatic reticuloendothelial system tissues (such as the spleen, bone marrow, and lung) and sustained levels in serum would appear to be desirable in the treatment of MAC, it may be necessary to carefully balance the advantages and potential disadvantages of high lipid doses to achieve the optimum therapy of this disease.

The ability of both free and liposomal amikacin to reduce the viable count of organisms per organ was directly related to the interval between infection and the initiation of therapy. The earlier therapy was started, the greater the reduction in bacterial load. This has also been demonstrated in MAC-infected beige mice treated with rifabutin and clofazamine (7).

It is difficult to compare the results of our studies with those of Düzgünes et al. (5) because the time of initiation of therapy, the lipid composition, and the total lipid doses used in the two studies were different. Although in neither study was the infection completely eliminated, it is apparent that in both cases, liposome encapsulation provided a therapeutic benefit unavailable with the free drug, at least in the spleen and liver. Simply reducing the bacterial load in patients infected with MAC may reduce morbidity and is therefore a worthwhile goal. It is likely that further work to optimize liposome formulation as well as treatment regimens (i.e., dosage, dosage interval, length of therapy, and combination with other agents) will be necessary to provide further therapeutic advantage.

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