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

S. P. KLEMENS,[1] M. H. CYNAMON,[1]* C. E. SWENSON,[2] AND R. S. GINSBERG[2]

Veterans Administration Medical Center and State University of New York Health Science Center, Syracuse, New York 13210,[1] and The Liposome Company, Princeton, New Jersey 08540[2]

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The efficacy of liposome-encapsulated gentamicin and free gentamicin was evaluated with the beige (C57BL/6J-*bg*^{j}/*bg*^{j}) mouse model of disseminated *Mycobacterium avium* complex infection. Approximately 10^7 viable *M. avium* complex cells were given intravenously. Seven days later, treatment with either encapsulated or free gentamicin at 20 mg/kg of body weight was started. Treatment was given either daily for 5 consecutive days or twice weekly for 3 weeks. The mice were sacrificed 5 days after the last dose. Spleens, livers, and lungs were homogenized, and viable cell counts were determined. An analysis of variance and subsequent Tukey honestly significant difference tests indicated that both encapsulated and free gentamicin reduced viable cell counts in each of the organs compared with no treatment. Encapsulated gentamicin significantly reduced viable cell counts in the spleen and liver compared with the free gentamicin. A dose-response experiment was performed with a daily dose of 0.2, 2, or 20 mg/kg. Dose-related reductions in viable cell counts were observed for spleens and livers, although none of the regimens resulted in sterilization of these organs. Liposome-encapsulated gentamicin should be considered for further evaluation in the treatment of *M. avium* complex infection in humans.

Prior to the advent of the acquired immune deficiency syndrome (AIDS), diseases due to *Mycobacterium avium* complex (MAC) were usually pulmonary infections in individuals with chronic underlying lung disease (8, 17). Currently, disseminated infection with MAC is the most common systemic bacterial infection seen in patients with AIDS (24). The development of an effective regimen for treatment of MAC infection in patients with AIDS has been hindered both by the multiple-drug resistance exhibited by these organisms (3) and by the severely impaired immune system of the patients. Strategies for effective therapy of MAC infection include the development of new therapeutic classes of drugs, modification of existing antimycobacterial agents, the combined use of antimycobacterial drugs with immunostimulating agents, and the use of novel drug delivery systems such as liposome encapsulation.

Liposome encapsulation of aminoglycosides appears to be a promising approach to the treatment of mycobacterial infections. Liposome-encapsulated-streptomycin treatment of mice infected with *Mycobacterium tuberculosis* resulted in prolonged survival and a decrease in numbers of organisms recovered from the spleen compared with treatment with free streptomycin (22). The enhanced activity of liposomal amikacin against MAC in macrophage culture (1) and in the beige mouse model of disseminated infection (2, 7) has been reported.

The purpose of the present study was to evaluate the comparative activities of liposome-encapsulated gentamicin and free gentamicin against MAC infection with the beige mouse model. The liposomes used in these studies were composed solely of neutral phosphatidylcholine, which has been shown to have less potential for toxicity than liposomes containing charged lipids and/or cholesterol (23). We chose to use relatively large, plurilamellar liposomes, since these result in a higher entrapment efficiency during manufacture and are taken up by reticuloendothelial tissues in vivo more efficiently than small vesicles are (23). We assessed the activity of liposomal gentamicin against three isolates of MAC and evaluated activity of the liposomal preparation, which was given on a daily and an intermittent basis.

MATERIALS AND METHODS

Drugs. Gentamicin sulfate, USP grade, was obtained from Agvar Chemicals, Little Falls, N.J. The gentamicin sulfate was dissolved in 0.9% saline to yield a final concentration of 5.1 mg of gentamicin activity per ml.

Liposomes were prepared by a modification of the stable plurilamellar vesicle process (13). The lipid (95% pure egg phosphatidylcholine) and methylene chloride solution and the gentamicin sulfate and normal saline solution were added to a large round-bottom vessel. The solvent was evaporated under vacuum with agitation, and the lipid-drug mixture was rehydrated with normal saline. The liposome suspension was subjected to tangential flow filtration to remove nonentrapped drug and liposomes outside the desired size range. The concentration of gentamicin in the final liposome suspension was determined by a spectrophotometric assay (19) after disruption of the lipid membranes with 0.2% Triton X-100. The antimicrobial activity of the liposome suspension was determined, after solubilization and removal of the lipids by Bligh and Dyer extraction (15), by using an agar well diffusion assay with *Bacillus subtilis* ATCC 6633 or *Staphylococcus epidermidis* ATCC 12228 as the indicator organism. The final liposome formulations used in these studies contained approximately 5 mg of active gentamicin and 55 mg of total phospholipid per ml, as determined by a Bartlett phosphorus assay (15). The size of the liposomes was measured by laser diffraction (Particle Sizer 3600 E Type; Malvern Instruments, Malvern, England), and more than 85% of the vesicles had diameters between 1.2 and 9.6 μm. The pharmacokinetics and tissue distribution of this

* Corresponding author.

liposomal preparation in mice have been previously reported (20).

MAC isolates. Isolates A, B, and C were obtained as clinical isolates from patients with AIDS at State University of New York Health Science Center, Syracuse. The gentamicin MIC for isolate A (serotype 1) was 4 μg/ml by agar dilution. Gentamicin MICs for isolates B and C were 8 μg/ml. A cell suspension of a predominantly (>95%) transparent colony was used for infection. The organisms were passaged through beige mice every 3 months to maintain virulence.

Media. The organisms were grown in Middlebrook 7H10 broth (21) with Middlebrook OADC (oleate-albumin-dextrose-catalase) enrichment (Difco Laboratories, Detroit, Mich.) and 0.05% Tween 80 on a rotary shaker at 37°C for 3 days. The culture suspension was diluted with 7H10 broth to yield approximately 5×10^7 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 weeks prior to being counted.

Infection studies. Four- to eight-week-old beige (C57BL/6J-*bg*^{j}/*bg*^{j}) mice, bred at our own facility, 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 mice was compared with groups of treated mice. In all experiments except the prophylaxis study, either free or liposome-encapsulated gentamicin was given intravenously starting 7 days after infection. Animals were sacrificed by cervical dislocation 5 days after the last dose of drug. The spleens, livers, and right lungs were aseptically removed and ground in a tissue homogenizer, and the number of viable organisms was determined by titration on 7H10 agar plates.

Statistical evaluation. The viable cell counts for each organ 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 using Tukey honestly significant difference (HSD) tests (16) to make pairwise comparisons among means. The results of the separate *t* tests and Tukey HSD tests for the spleens, livers, and lungs are summarized in the following section.

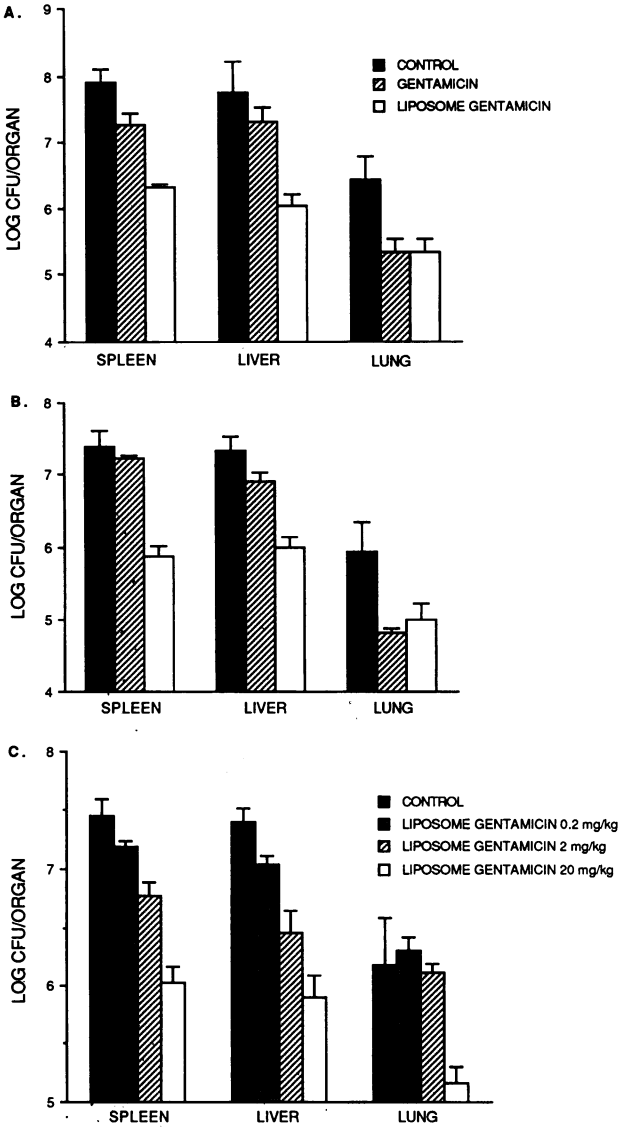

FIG. 1. Comparative activities of free and liposome-encapsulated gentamicin against MAC. (A) Isolate B; (B) isolate C; (C) dose-response study of daily liposome-encapsulated gentamicin on isolate A. Results are means of five mice per group. Error bars, Standard deviation.

RESULTS

Comparison of free and liposome-encapsulated gentamicin. (i) Isolate B. Gentamicin or liposomal gentamicin at 20 mg/kg of body weight was given intravenously daily for 5 days to male mice which had been infected with 8.0×10^6 viable MAC, isolate B, cells. Analysis of variance and Tukey HSD tests indicated that gentamicin reduced viable cell counts in the spleens and lungs compared with no treatment ($P < 0.01$). Liposomal gentamicin was more active than the free drug was with regard to reducing the number of organisms in spleens and livers ($P < 0.01$) but not lungs (Fig. 1A).

(ii) Isolate C. Gentamicin or liposomal gentamicin at 20 mg/kg was given intravenously daily for 5 days to female mice which had been infected with 5.6×10^6 viable MAC, isolate C. Analysis of variance and Tukey HSD tests indicated that the unencapsulated drug reduced viable cell counts to a significant degree in the liver and lung ($P < 0.01$) compared with the no treatment. The liposomal gentamicin further reduced cell counts in the spleen and liver ($P < 0.01$), but there was little difference in the activities of the two drugs on the viable cell counts in the lung (Fig. 1B).

Dose-response study of daily liposome-encapsulated gentamicin against isolate A. Liposomal gentamicin at either 0.2, 2, or 20 mg/kg was given intravenously daily for 5 days to male mice which had been infected with 1.3×10^7 viable MAC, isolate A, cells. Treatment with 0.2 mg/kg reduced viable cell counts in the spleens and livers ($P < 0.05$) compared with no treatment. Similarly, treatment with 2 mg/kg reduced cell counts in these same organs compared with no treatment ($P < 0.01$) and treatment with 0.2 mg/kg ($P < 0.01$). Treatment with 0.2 and 2 mg/kg had no effect on organisms in the lung compared with no treatment. The cell counts in the spleens, livers, and lungs of the 20 mg/kg-treated group were significantly lower than those of the 2 mg/kg-treated group ($P < 0.01$) (Fig. 1C).

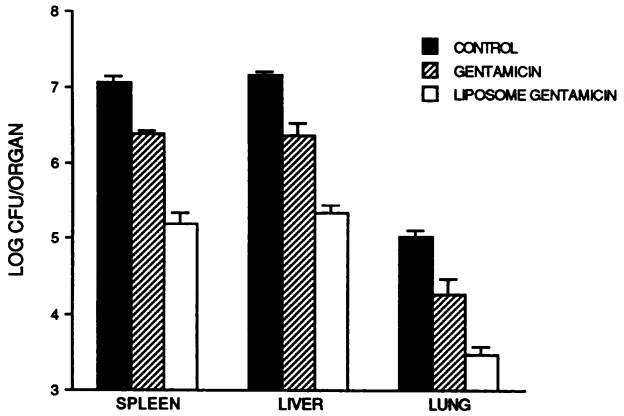

FIG. 2. Comparison of prophylaxis with free and liposome-encapsulated gentamicin against MAC infection.

Comparison of prophylaxis with gentamicin and liposomal gentamicin on MAC infection. Male mice were given gentamicin or liposomal gentamicin at 20 mg/kg intravenously on day 1. On the morning of day 2, all mice were infected with 1.0×10^7 viable MAC, isolate A, cells. Gentamicin and liposomal gentamicin groups received their respective drugs in the afternoon of day 2 and on days 3 and 4. Analysis of variance and Tukey HSD tests indicated that both gentamicin and liposomal gentamicin reduced viable cell counts in each organ compared with no treatment ($P < 0.01$), but liposomal gentamicin was more active than the free drug in each organ ($P < 0.01$) (Fig. 2).

Comparison of twice-weekly free gentamicin with twice-weekly liposomal gentamicin. Free or liposomal gentamicin at 20 mg/kg was given intravenously twice weekly for 3 weeks to female mice which had been infected with 1.4×10^7 viable MAC, isolate A, cells. Analysis of variance and Tukey HSD tests indicated that free gentamicin reduced viable cell counts in the spleens, livers, and lungs compared with no treatment ($P < 0.01$). Liposomal gentamicin was more active than the free drug against organisms in the spleens and livers ($P < 0.01$) (Fig. 3A).

Dose-response study of twice-weekly liposomal gentamicin. Liposomal gentamicin at either 20, 40, or 60 mg/kg was given intravenously twice weekly for 3 weeks to female mice which had been infected with 1.0×10^7 MAC, isolate A, cells. Treatment with all doses on an intermittent basis reduced viable cell counts in spleens, livers, and lungs compared with no treatment ($P < 0.01$). The reduction in viable cell counts observed with treatment at 40 mg/kg compared with the reduction in the 20 mg/kg-treated group was not significantly different for any organ ($P > 0.05$). Treatment with 60 mg/kg reduced viable cell counts in the liver compared with treatment with lower doses ($P < 0.01$), but the reduction in counts observed in the spleens was not significantly different. The reduction in cell counts observed in the lung of the 60 mg/kg-treated group was not significantly different from that seen with treatment at 40 mg/kg but was significant compared with the reduction in the 20 mg/kg-treated group ($P < 0.01$) (Fig. 3B).

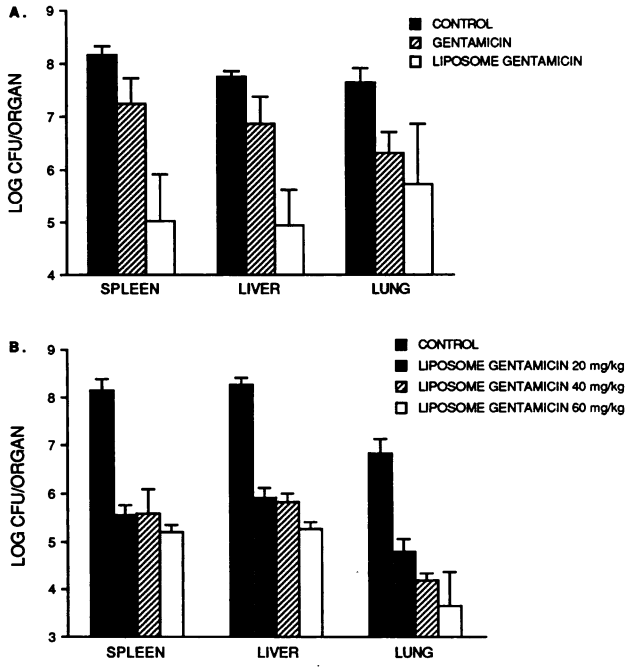

FIG. 3. Activity of intermittent liposome-encapsulated gentamicin. (A) Comparison of twice-weekly free gentamicin with twice-weekly liposomal gentamicin; (B) dose-response study of twice-weekly liposomal gentamicin.

DISCUSSION

Since the discovery of streptomycin in 1944, the aminoglycosides have had a major role in the therapy of mycobacterial diseases. Although streptomycin continues to be an important drug in treatment regimens for tuberculosis, its use in the treatment of infection due to nontuberculous mycobacteria is not well defined (4, 6, 14). Other aminoglycosides, such as kanamycin and the cyclic peptide capreomycin, appear to have limited value in the treatment of nontuberculous mycobacterial disease (9).

Recent work has focused on the activity of amikacin against the nontuberculous species, in particular MAC. Its activity against MAC under simulated in vivo conditions and in the beige mouse model has been reported (11, 12). Although controlled studies of efficacy in human MAC infection have not been reported, amikacin is in use as part of multidrug regimens for treatment of disseminated infection in patients with AIDS (24).

Antimycobacterial activity of gentamicin has not been well studied. Although it has been found to have good in vitro antituberculous activity by the broth dilution method, gentamicin showed poor activity compared with streptomycin in a murine tuberculosis model (18). Variable in vitro activity of gentamicin against MAC has been reported. Sanders et al. reported a gentamicin MIC range from 1.6 to 6.2 μg/ml for 10 isolates designated *Mycobacterium intracellulare* (18), Davis et al. reported MICs of 4 μg/ml or less for 13 of 20 isolates tested (5), and Gangadharam and Candler reported MICs greater than 64 μg/ml for all 20 *M. intracellulare* isolates tested (10). In vitro MICs for the small sample of isolates used in our studies were 4 to 8 μg/ml. Comparative in vitro studies of amikacin and gentamicin would be useful to confirm gentamicin activity against MAC and to determine the degree of cross-resistance between these aminoglycosides.

In each of our experiments, unencapsulated gentamicin showed modest activity in the beige mouse model of MAC infection. Gentamicin activity against organisms in the spleen and liver was markedly enhanced by liposomal encapsulation. Treatment with the liposomal preparation at 20

mg/kg produced a 1.5-log reduction in viable cell counts after only 5 days of therapy. The activities of the liposomal preparation given on a daily basis were comparable for each of the three isolates tested.

Intermittent liposomal gentamicin at 20 mg/kg for 3 weeks produced a 2.5-log reduction in cell counts in the spleens and livers. Effective therapy for disseminated MAC infection may require an initial intensive-treatment phase followed by an intermittent phase which utilizes an injectable agent as part of a multidrug regimen. A liposomal preparation may provide advantages of convenient intermittent administration in addition to enhanced activity in comparison with the free aminoglycoside.

In each experiment except the prophylaxis study, liposomal encapsulation failed to enhance the activity of gentamicin against organisms in the lung. This finding is similar to those in our study of amikacin and liposome-encapsulated amikacin, in which a stable plurilamellar liposome preparation was used (2). It is difficult to directly compare results of these studies, since the lipid compositions and total lipid doses used in the two studies were different. Although this finding may have implications for the use of liposome-encapsulated aminoglycosides in the treatment of pulmonary MAC infection, it is possible that changes in the liposome formulation may optimize activity of the preparation in the lung.

It is unclear why the liposome-encapsulated gentamicin did not have stronger sterilizing activity for the spleen and liver. It is unusual for antimycobacterial agents (encapsulated or free) to sterilize the spleen or liver in murine models of mycobacterial infection. Düzgüneş et al. observed a 2- to 3-log reduction of viable organisms in the spleen and liver of *M. avium*-infected mice treated with liposome-encapsulated amikacin for 8 weeks (7). Vladimirsky and Ladigina observed less than a 1-log reduction in viable organisms in the spleen of *M. tuberculosis*-infected mice treated with liposome-encapsulated streptomycin (22). The goal of therapy for human immunodeficiency virus-infected patients with disseminated MAC may be to reduce the viable mycobacterial population in order to provide symptomatic improvement. This approach is similar to the suppressive therapy of other opportunistic infections in patients with AIDS.

Studies that will assess activity of the liposomal gentamicin in combination with other antimycobacterial agents to see if activity can be further enhanced are in progress. Comparison of regrowth of MAC following cessation of therapy with either free or encapsulated drug would also be of interest. Liposomal encapsulation of an aminoglycoside may improve therapy for MAC infection in patients with AIDS.

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