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Therapy of *Mycobacterium avium* Complex Infections in Beige Mice with Streptomycin Encapsulated in Sterically Stabilized Liposomes

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Mycobacterium avium **complex (MAC) causes serious opportunistic infections in AIDS patients. Previous studies with MAC-infected beige mice have indicated that weekly administration of liposome-encapsulated streptomycin can reduce significantly the CFU in the liver and spleen. We examined whether streptomycin encapsulated in recently developed sterically stabilized liposomes with prolonged circulation times would have a therapeutic effect in this animal model. Two liposome types with prolonged circulation (polyethyleneglycol-distearoylphosphatidylethanolamine [PEG-DSPE]–distearoylphosphatidylcholine [DSPC]–cholesterol [chol] or phosphatidylinositol [PI]-DSPC-chol) and conventional liposomes (phosphatidylglycerol [PG]-phosphatidylcholine [PC]-chol) encapsulating streptomycin and administered twice weekly were bactericidal to MAC strain 101 in the spleen when the level of infection after treatment was compared with the level of infection before treatment. PI-DSPC-chol and PG-PC-chol liposomes encapsulating streptomycin were bactericidal in the liver. Although PG-PC-chol or PEG-DSPE–DSPE–chol liposomes encapsulating streptomycin were not bactericidal in the lungs, they reduced the level of MAC infection by more than 3 orders of magnitude compared with the level of MAC infection in untreated controls.**

Mycobacterium avium complex (MAC) causes serious disease, particularly in human immunodeficiency virus-infected individuals. The prognosis for MAC-infected AIDS patients is bleak, and most patients survive less than 1 year after diagnosis (26). MAC invades resident macrophages in the lungs, liver, spleen, lymph nodes, and intestines and is also found in blood during certain phases of the disease (19, 27, 30, 49, 50). To effectively combat this infection, it is essential to target antibiotics to macrophages in these tissues as well as to attain bactericidal levels in the bloodstream. We have demonstrated previously that liposome-encapsulated amikacin and streptomycin encapsulated in liposomes are more effective than the free antibiotics against MAC inside murine or human macrophages (5, 24, 32, 36). In the beige mouse model, intravenous administration of liposome-encapsulated aminoglycosides was effective against MAC infections in the liver and spleen even at weekly doses that were 1/10 to 1/5 of the daily dose of the free drug (17, 18). The superior efficacy of liposome-encapsulated antibiotics against MAC has also been demonstrated by other laboratories (11, 13, 33, 43).

Recently developed liposomes with prolonged circulation times in the bloodstream have been shown to extravasate into tissues and localize in tissue macrophages and have been used for cancer chemotherapy (2–4, 20, 29, 38, 39, 44, 47). Sterically stabilized liposomes with prolonged circulation times also appear to localize preferentially in *Klebsiella*-infected lung tissue rather than in normal tissue (8). To our knowledge, the antimicrobial effects of antibiotics encapsulated in such liposomes have not been investigated previously. In the study described here we examined the effect on MAC infection in beige mice of streptomycin encapsulated in liposomes of two different compositions that are known to have prolonged circulation times and compared the effect with the effect of antibiotic encapsulated in conventional liposomes.

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MATERIALS AND METHODS

Drugs and reagents. Streptomycin was obtained from Pfizer (New York, N.Y.), and *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) was purchased from Sigma Chemical Co. (St. Louis, Mo.). Egg yolk phosphatidylcholine (PC), phosphatidylglycerol (PG) prepared from PC, distearoylphosphatidylcholine (DSPC), and plant phosphatidylinositol (PI) were obtained from Avanti Polar Lipids (Alabaster, Ala.). Polyethylene glycol (PEG)-distearoylphosphatidylethanolamine (DSPE), synthesized with PEG with a molecular mass of 1,900 Da, was a gift from D. Papahadjopoulos (University of California, San Francisco). Cholesterol (chol) was purchased from Calbiochem (La Jolla, Calif.). NaCl and KCl were from Fisher (Pittsburgh, Pa.).

Organisms and infection of the animals. A predominantly transparent colony type of MAC (strain 101; serotype 1), maintained by repeated passages through beige mice to retain its virulence, was injected into 5- to 7-week-old male beige (C57B1/6/bg^j/bg^j) mice via the tail vein (approximately 7×10^6 viable units per animal). The animals were kept five to a cage in a biohazard room, with food and water being given ad libitum.

Liposome preparation. Streptomycin was encapsulated in liposomes composed of the following lipid mixtures: (i) PEG-DSPE–DSPC–chol (molar ratio, 1:9:6.7), (ii) PI-DSPC-chol (molar ratio, 1:9:6.7), and (iii) PG-PC-chol (molar ratio, 1:9:6.7). Lipids (200 μmol of phospholipid or 334 μmol of total lipid) were dried from a chloroform solution onto the sides of a round-bottom glass flask. The dried film was then hydrated by the addition of 3.5 ml of a sterile streptomycin solution (168 mg/ml in 10 mM HEPES [pH 7.4], adjusted to an osmolality of 300 mosM with water by using a Wescor [Logan, Utah] vapor pressure osmometer). In the case of negative-control liposomes, 100 μmol of phospholipid (or 167 μmol of total lipid) was dried and hydrated with 3.5 ml of HEPES buffer (10 mM HEPES, 140 mM NaCl, 10 mM KCl [pH 7.4]). Full hydration was achieved by vortexing. In the case of sterically stabilized liposomes that contained DSPC (whose gel-liquid crystalline phase transition temperature is 54°C in physiological saline), the flask was alternately placed in a 56°C water bath and vortexed. This was followed by four cycles of freezing (in a dry ice-ethanol bath) and thawing (in a 21°C water bath). The liposome preparations were extruded four times through dual polycarbonate membranes (a 0.4-μm-pore-diameter

membrane on top of a 0.1-μm-pore-diameter membrane; Nuclepore, Pleasanton, Calif.) under argon pressure in a high-pressure extrusion apparatus (Lipex Biomembranes, Vancouver, British Columbia, Canada). In the case of the liposome preparations containing DSPC, the extruder was preheated to 56°C by circulating water through the water jacket around the chamber. Unencapsulated streptomycin was removed by chromatographing the liposome suspension on sterile Sephadex G-75 equilibrated with argon-saturated HEPES buffer and eluting with the same buffer. The liposome suspensions were sterilized by passage through a 0.22-μm-pore-diameter filter in the case of the PG-PC-chol liposomes and a 0.45-μm-pore-diameter filter (Schleicher & Schuell, Keene, N.H.) in the case of the sterically stabilized liposomes. The mean diameter of identically prepared liposomes, estimated by dynamic light scattering with a Coulter (Hialeah, Fla.) NP-4 instrument, was 172 ± 40 nm.

The encapsulation of streptomycin was determined by a fluorescence assay involving the interaction of ninhydrin with guanidino groups as described previously (17). Phospholipid concentrations were determined by phosphate analysis (9). The amount of encapsulated streptomycin was in the range of 188 to 298 μg of drug per μmol of phospholipid for the different preparations. The liposomes containing PI or PEG-DSPE retained 100% of their contents upon storage for 2 weeks at 4°C and were used for all of the injections over the 2-week period of therapy. Liposomes containing PG leaked 38% of the encapsulated streptomycin upon storage at 4°C for 2 weeks. Therefore, in the case of the PG-containing liposomes, unencapsulated streptomycin was removed from half of the initial preparation and was used for the injections during the first week. Unencapsulated antibiotic was removed from the remaining half of the preparation 1 week later, to be used for the injections during the second week.

Treatment and its evaluation. Therapy was initiated 1 day after the infection of the animals. The infected animals were treated for 2 weeks by twice-weekly injections of the liposome-encapsulated antibiotics (at doses of 15 or 30 mg/kg of body weight for the PG-PC-chol or PI-DSPC-chol liposomes and 15 mg/kg for the PEG-DSPE–DSPC–chol liposomes) via the tail vein. Control animals received injections of HEPES buffer or buffer-loaded control liposomes. The amounts of lipid to be used for the latter controls were based on the amounts of lipid injected for each of the preparations (1.39 μmol for the PEG-DSPE–DSPC–chol liposomes, 2.01 μmol for the PI-DSPC-chol liposomes at the higher dose, and 3.19 μmol for the PG-PC-chol liposomes at the higher dose). Controls involving the intravenous injection of free streptomycin and free streptomycin plus buffer-loaded liposomes were omitted from this experiment, since they had been investigated in our earlier studies with large unilamellar PG-PC-chol liposomes (21). An additional positive control was the intramuscular injection of 150 mg of free streptomycin per kg 5 days per week for 4 weeks.

The baseline CFU 1 day following infection was determined in the liver, spleen, and lungs of three randomly selected mice following homogenization and plating of appropriate dilutions on 7H11 agar medium. The plates were incubated at 37°C, and the CFU was determined after 3 weeks of incubation. The treated animals were sacrificed at 2 and 4 weeks following infection to determine the CFU in the same organs. The results are expressed as the mean and standard deviation of the CFU from three mice per experimental point. The statistical significance of differences between experimental conditions was ascertained by Student's *t* test by using the Statview program (Brainpower, Inc., Calabasas, Calif.).

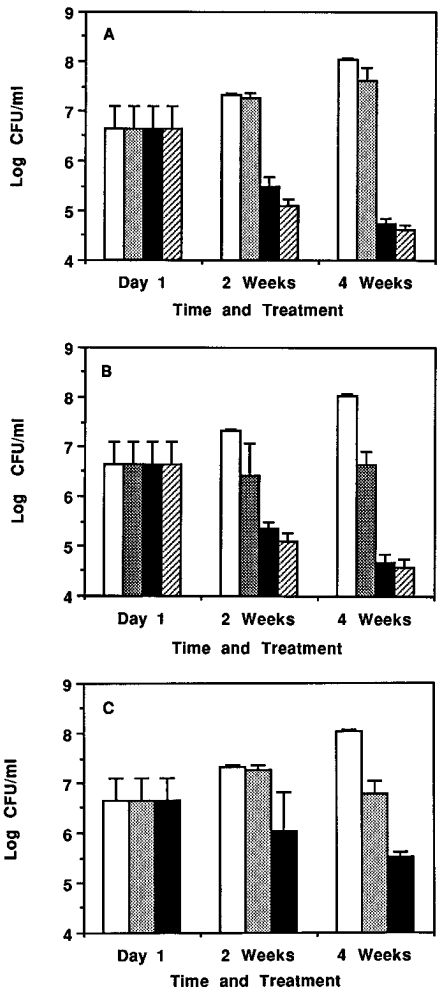

FIG. 1. CFU of MAC in the livers of mice treated with PG-PC-chol (A), PI-DSPC-chol (B), or PEG-PE–DSPC–chol (C) liposomes. Open bars, untreated controls; dotted bars, liposome controls; solid bars, 15 mg of streptomycin per kg in liposomes; striped bars, 30 mg of streptomycin per kg in liposomes. The error bars represent the standard deviations of the means obtained from three animals.

RESULTS

In the liver, streptomycin encapsulated in PG-PC-chol (Fig. 1A) or PI-DSPC-chol (Fig. 1B) liposomes was very effective in reducing the CFU counts. Compared with the initial (1 day) infection, 15 or 30 mg of streptomycin per kg in either liposome composition reduced the CFU by about 2 log units at the end of the 4-week experiment, indicating a bactericidal effect ($P \leq 0.005$ comparing CFU at day 1 and 4 weeks for both liposome types at 15 or 30 mg/kg). Compared with the untreated control at the end of the 4-week period, this corresponded to about a 3.4-log-unit reduction in CFU. Streptomycin encapsulated in PEG-DSPE–DSPC–chol liposomes (at a dose of 15 mg/kg) caused a 1.3-log-unit reduction in the CFU compared with the CFU of the initial infection ($P \leq 0.025$) and a 2.7-log-unit reduction in the CFU compared with CFU in the untreated controls at week 4. The difference between the CFU obtained with 15 mg of streptomycin per kg in PEG-DSPE–DSPC–chol and either PG-PC-chol or PI-DSPC-chol liposomes was significant ($P \leq 0.005$ and $P \leq 0.01$, respectively). Buffer-loaded control liposomes had no effect on the CFU in the case of PG-PC-chol liposomes, while liposomes of the two other compositions containing DSPC caused some reduction in the CFU compared with the CFU in untreated controls, particularly at 4 weeks (Fig. 1B and C). Intramuscular injections of free streptomycin (at a dose of 150 mg/kg at 5 days per week for 4 weeks) as a control caused a reduction in CFU of 3.6 log units at the 4-week time point compared with the CFU in untreated controls (data not shown). It should be noted that the total dose of antibiotic administered in the latter case was 25- to 50-fold higher than that given in liposomes.

In the spleen, all three liposome compositions caused a 1.8- to 2.4-log-unit reduction in CFU at the 4-week point compared with the CFU at the initial infection (Fig. 2). The difference between the CFU on day 1 and that obtained with any of the three liposome types (15 mg of streptomycin per kg) at 4 weeks was highly significant ($P \leq 0.0005$). At a dose of 15 mg of streptomycin per kg, the reductions in CFU compared with the CFU in the untreated controls at 4 weeks were 4.3, 4.0, and 4.5 log units for PG-PC-chol, PI-DSPC-chol, and PEG-DSPE–DSPC–chol liposomes, respectively. The differences in CFU between the liposomes of the different formulations were not significant. Control liposomes in all three cases did not have a significant effect on the MAC CFU in the spleen. The intramuscular injection of free streptomycin as a control produced

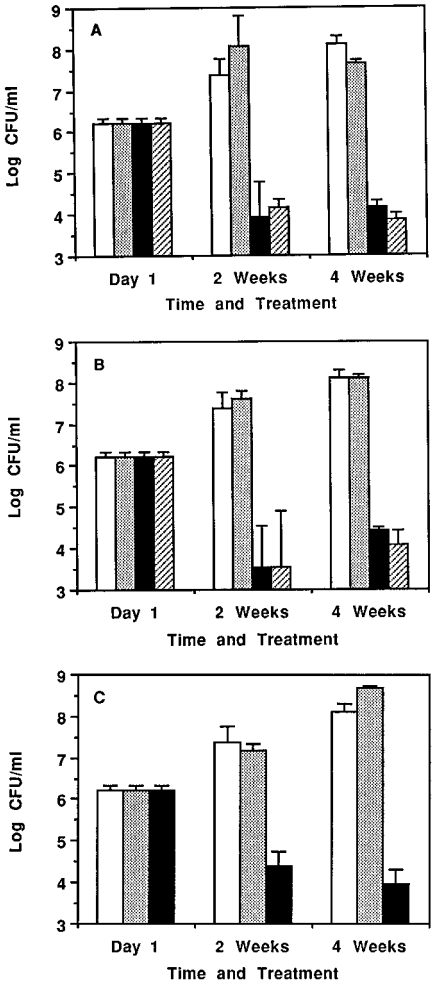

FIG. 2. CFU of MAC in the spleens of mice treated with PG-PC-chol (A), PI-DSPC-chol (B), or PEG-PE–DSPC–chol (C) liposomes. Open bars, untreated controls; dotted bars, liposome controls; solid bars, 15 mg of streptomycin per kg in liposomes; striped bars, 30 mg of streptomycin per kg in liposomes. The error bars represent the standard deviations of the means obtained from three animals.

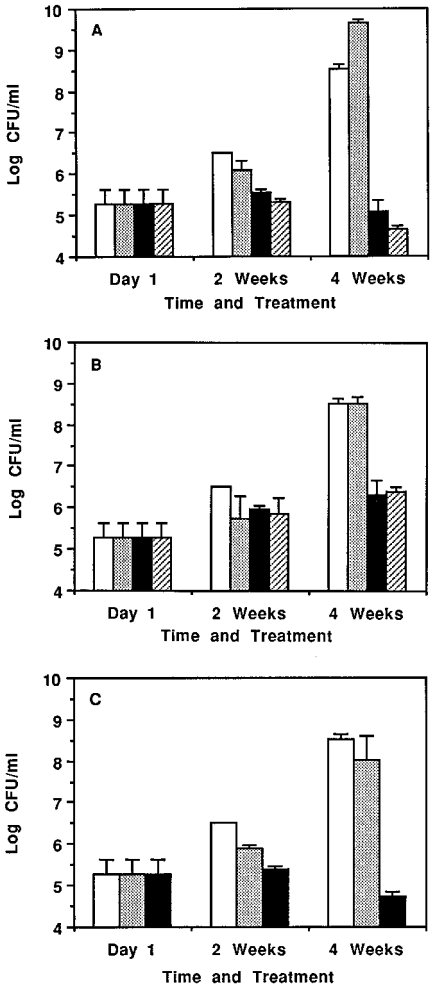

FIG. 3. CFU of MAC in the lungs of mice treated with PG-PC-chol (A), PI-DSPC-chol (B), or PEG-PE–DSPC–chol (C) liposomes. Open bars, untreated controls; dotted bars, liposome controls; solid bars, 15 mg of streptomycin per kg in liposomes; striped bars, 30 mg of streptomycin per kg in liposomes. The error bars represent the standard deviations of the means obtained from three animals.

a CFU reduction of 4.8 log units at 4 weeks compared with the CFU in the untreated controls (data not shown).

The results obtained in the lungs were of particular interest because of the inability of previous treatment regimens with liposome-encapsulated antibiotics used in our laboratories to affect the CFU significantly. Twice-weekly administration of streptomycin for 2 weeks in either PG-PC-chol (30 mg/kg) or PEG-DSPE–DSPC–chol (15 mg/kg) liposomes effected a 3.9- or 3.8-log-unit reduction in CFU, respectively, at the 4-week time point compared with the CFU in untreated controls (Fig. 3A and C). Compared with the initial level of infection on day 1, these CFU represented about a 0.5-log-unit reduction, which was statistically significant ($P \leq 0.025$ for PG-PC-chol and $P \leq 0.05$ for PEG-DSPE–DSPC–chol liposomes). PI-DSPC-chol liposomes, on the other hand, caused only about a 2-log-unit reduction in the CFU compared with the CFU in the untreated controls at 4 weeks (Fig. 3B). Control liposomes did not show a significant effect on the CFU except for the enhancement noted at 4 weeks for the buffer-loaded PG-PC-chol liposomes. The intramuscular injections of free antibiotic as a positive control resulted in a 4.4-log-unit reduction in CFU compared with the CFU in untreated controls at 4 weeks.

DISCUSSION

Currently available treatments for MAC infections in humans require chemotherapy with multiple drug combinations, which may clear bacteremia, but they have not been effective in treating MAC infections in tissues and in prolonging patient survival (1, 28, 37). In some studies, although a reduction in the level of bacteremia and improvements in clinical symptoms were observed, a large percentage of patients developed adverse reactions (12, 31). While monotherapy with clarithromycin appears to be effective (10, 15, 16), the relatively high MBC of the drug and the potential development of drug resistance (25) may eventually limit the efficacy of this agent. Therefore, the development of novel drugs and treatment modalities is essential for a complete cure of the disease. Our previous studies with an animal model have shown that aminoglycoside antibiotics, particularly amikacin and streptomycin, could be useful either by themselves or in combination with other drugs like clofazimine, ethambutol, and rifabutin (22, 23). However, these treatments require prolonged administration of high doses of aminoglycosides (50 mg/kg for amikacin and 150 mg/kg for streptomycin) that could be toxic.

One strategy for reducing the dose and frequency of administration of antibiotics is to target them to infected tissues and cells. We and others have shown that liposome-encapsulated aminoglycosides can reduce the levels of MAC in the livers and spleens of infected animals (11, 13, 17, 18, 33, 43). The once-weekly dosage used in our studies, however, was not effective in the lungs, despite the localization of the antibiotic in this tissue. This result was ascribed to the trapping of liposomes in lung capillaries and their inability to localize in lung macrophages (17). Other studies have used daily, twice-weekly, or three-times-weekly doses of liposome-encapsulated amikacin or gentamicin and observed some efficacy in reducing the CFU counts in the lungs compared with the counts in untreated controls (13, 35). Therefore, in the present study we administered liposomes twice weekly.

In the current study we explored the use of liposomes of three different compositions that have been shown to have different half-lives in the bloodstream when they are prepared and administered under similar experimental conditions. Liposomes with prolonged circulation times, termed "stealth" liposomes because they avoid immediate recognition and removal by the mononuclear phagocyte system (the reticuloendothelial system), are generally composed of saturated phospholipids, cholesterol, and a glycolipid or synthetic lipid component that confers stability in serum as well as prolonged circulation times. Thus, these liposomes have also been designated as being sterically stabilized or surface modified (45, 46). Sterically stabilized liposomes have been shown to localize in implanted tumors, most likely because of their prolonged circulation times in blood and increased microvascular permeation (20, 39, 48). Such liposomes also have a propensity to localize in infected lung tissue (8). The percentage of the intravenous dose remaining in the blood 24 h after injection is 23.6 ± 3.1 for PEG-DSPE–DSPC–chol liposomes, while the percentages are 0.3 ± 1 for PG-PC-chol liposomes, 4.1 ± 1.3 for PI-PC-chol liposomes, and 12.2 ± 3.4 for hydrogenated PI-DSPC-chol liposomes (45). Clearly, there are considerable differences in the circulation times of PG-PC-chol and PEG-DSPE–DSPC–chol liposomes. Our liposomes had compositions similar to those of the liposomes listed above, but they were not tested for their circulation times. The PI-containing liposomes that we used contained nonhydrogenated PI in combination with DSPC. Thus, the levels of these liposomes in blood at 24 h may be expected to be intermediate between 4.1% (PI-PC-chol liposomes) and 12.2% (hydrogenated PI-DSPC-chol liposomes). Despite these potential differences in circulation times between the different liposome types, the reductions in CFU in the tissues examined were not drastically different.

The results presented in this report show that liposomes of all three compositions used to encapsulate streptomycin are effective in reducing by several orders of magnitude the level of MAC infection in the liver and spleen. Comparing these results with those obtained after intramuscular injections of free streptomycin, it is clear that similar reductions in MAC CFU can be obtained with 25- to 50-fold lower total doses of liposome-encapsulated antibiotics, in agreement with earlier observations (17, 18, 21). Significantly, twice-weekly administration of streptomycin encapsulated in PG-PC-chol or PI-DSPC-chol liposomes was bactericidal to MAC in the liver, while the antibiotic encapsulated in liposomes of all three compositions was bactericidal in the spleen. PEG-DSPE-containing liposomes may have been less effective than the other liposomes in the liver because of increased levels of circulation in blood and uptake in other tissues (3, 46). While PI-containing liposomes were effective in the liver and spleen, they were the least effective liposome composition in terms of the CFU reduction in the lungs. The reasons for this observation are not obvious at present. Previous studies with *Klebsiella pneumoniae*-infected rats have indicated that the percentage of hydrogenated PI-containing liposomes that localize in the infected lungs is much lower than that of PEG-DSPE-containing liposomes (7, 8). However, the degree of localization of the former liposomes in infected lungs was significantly higher than that of PG-containing liposomes (8). Thus, it is difficult to correlate localization in the lung with enhanced efficacy, particularly since the two animal models are different. Furthermore, our previous studies have indicated that the enhanced localization of streptomycin in multilamellar liposomes in the lung compared with that of unilamellar liposomes does not result in enhanced efficacy against MAC (17).

Our previous studies have shown that liposome-encapsulated streptomycin is more effective than the free antibiotic against MAC even in cultured murine peritoneal (5) or human peripheral blood monocyte (36) macrophages, suggesting that liposomes can mediate the intracellular transport of streptomycin and its localization at high concentrations in the same compartments as MAC. A similar mechanism is likely to operate in vivo. In addition, liposomes facilitate the localization of the antibiotic in tissue macrophages since they are avidly phagocytosed by these cells (14, 34, 40–42).

Liposomes containing solid-phase lipids, such as DSPC, and cholesterol are known to be more stable in plasma than those composed of fluid-phase lipids or those not containing cholesterol (42). It is possible that the improved liposome stability in plasma may prevent the release of encapsulated antibiotics once the liposomes are endocytosed by macrophages. For example, the intracellular release of ampicillin encapsulated in solid liposomes composed of PG-DSPC-chol (molar ratio, 1:10:10) was delayed compared with that of ampicillin encapsulated in fluid liposomes (phosphatidylserine-PC-chol [molar ratio, 1:4:5]), as evidenced by the killing of intracellular *Listeria monocytogenes* (6). Our results with DSPC-cholesterol-containing liposomes demonstrate, however, that encapsulated streptomycin is available in MAC-infected tissues and cells and can kill or inhibit the growth of MAC. Thus, sterically stabilized liposomes with prolonged circulation times, such as those composed of PEG-DSPE–DSPC–chol, can be used effectively to deliver antibiotics to tissues and infected macrophages. These liposomes did not exhibit a particular advantage over conventional liposomes in reducing the CFU in the tissues examined in the present study. However, since sterically stabilized liposomes have been shown to extravasate into deep tissues and localize in tissue macrophages, it is likely that they may be able to transport antibiotics to MAC-infected macrophages in the intestines, lymph nodes, and other tissues. Liposomes with prolonged circulation times may also be effective in reducing the level of bacteremia. These aspects will be examined in future studies.

Our observations also demonstrate that twice-weekly administration of streptomycin encapsulated in PG-PC-chol or PEG-DSPE–DSPE–chol liposomes can reduce the level of MAC infection in the lungs by several orders of magnitude. This result demonstrates that liposome-encapsulated aminoglycosides can reduce the MAC CFU counts in the lungs at doses much lower than the free intramuscular doses required for achieving the same effect (17, 21). The ability to reduce levels of mycobacterial infection in the lungs with liposomes may also provide the opportunity to treat *Mycobacterium tuberculosis* infections by the same technique.

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