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Antibacterial Efficacy of Gentamicin Encapsulated in pH-Sensitive Liposomes against an In Vivo *Salmonella enterica* Serovar Typhimurium Intracellular Infection Model

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Encapsulation of gentamicin in liposomes can be used to achieve intracellular delivery and broaden the clinical utility of this drug. We have previously described a novel, rationally designed, pH-sensitive liposomal carrier for gentamicin that has superior in vitro efficacy against intracellular infections compared to the efficacies of both free gentamicin and non-pH-sensitive liposomal controls. This liposomal carrier demonstrated pH-sensitive fusion that was dependent on the presence of unsaturated phosphatidylethanolamine (PE) and the pH-sensitive lipid *N*-succinyldioleoyl-PE. The pharmacokinetics and biodistribution of the free and liposomal gentamicin were examined in mice bearing a systemic *Salmonella enterica* serovar Typhimurium infection. Encapsulation of gentamicin in pH-sensitive liposomes significantly increased the concentrations of the drug in plasma compared to those of free gentamicin. Furthermore, the levels of accumulation of drug in the infected liver and spleen were increased by 153- and 437-fold, respectively, as a result of liposomal encapsulation. The increased accumulation of gentamicin in the liver and spleen effected by liposomal delivery was associated with 10^4-fold greater antibacterial activity than that associated with free gentamicin in a murine salmonellosis model. These pH-sensitive liposomal antibiotic carriers with enhanced in vitro activity could be used to improve both in vivo intracellular drug delivery and biological activity.

Gentamicin is an aminoglycoside antibiotic with broad-spectrum antibacterial activity against several serious bacterial infections, including typhoid fever. Infections caused by intracellular pathogens, such as *Brucella*, *Salmonella*, *Listeria*, *Legionella*, and *Mycobacteria* species, are difficult to treat by conventional antimicrobial therapies because of the requirement that antibiotics reach therapeutic levels at the intracellular site of infection. Since gentamicin is polycationic, it is freely soluble in water and has a low level of permeation through biological membranes. After intravenous (i.v.) or intramuscular administration the majority of the drug remains extracellular (7). Therefore, gentamicin and many other antibiotics that are active in vitro are often inactive against intracellular bacteria due to their poor penetration into cells. The development of new antibacterial formulations or carriers capable of intracellular delivery will improve therapy for infections that are presently difficult to treat.

The encapsulation of gentamicin in liposomal delivery systems has been used to alter the drug's biodistribution, increase its level of accumulation at the disease site, and reduce its nephrotoxicity that arises from multiple daily administrations (6). Gentamicin has been encapsulated into a variety of egg phosphatidylcholine (egg PC) and egg PC-cholesterol (chol) formulations and has been shown to have antibacterial activity in both in vitro and in vivo infection models (for a review, see reference 6). However, the formulations used by those workers were not specifically designed to facilitate the intracellular delivery of the antibiotic. Rather, the in vivo efficacies of these liposomal formulations resulted from the ability of liposomal carriers to increase the circulation lifetime of the encapsulated drug and achieve passive accumulation of drug at a site of infection. Similar effects have been demonstrated previously for other encapsulated antibacterial (15) and anticancer (16) agents. It is expected that a liposomal formulation that can achieve intracellular antibiotic delivery will demonstrate significantly enhanced antibacterial activity against intracellular pathogens beyond that conferred by either free drug or drug encapsulated in nonfusogenic carriers.

In a previous study, we described a novel pH-sensitive liposome composition that achieved very effective intracellular delivery of gentamicin into a mouse monocyte/macrophage cell line infected with *Salmonella enterica* serovar Typhimurium (8). This formulation comprises dioleoylphosphatidylethanolamine (DOPE), *N*-succinyl-DOPE, and polyethylene glycol (PEG)-ceramide and delivered drug to the cytoplasm of infected macrophages in a manner dependent on endosomal acidification (8). The antibacterial activity achieved by this liposomal carrier was significantly greater than that achieved by nonfusogenic control carriers (8).

The experiments reported on here were performed to determine if use of a pH-sensitive liposomal delivery system would also result in enhanced antibacterial activity in an in vivo infection model in which *S. enterica* serovar Typhimurium is an intracellular resident of macrophages.

MATERIALS AND METHODS

Materials. Commercially available lipids were obtained from Avanti Polar Lipids (Alabaster, Ala.) or Northern Lipids (Vancouver, British Columbia, Canada). PEG-C_{20}-ceramide (PEG-ceramide) was manufactured at Inex Pharmaceuticals by Zhao Wang as described previously (17). Gentamicin sulfate was

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obtained from Sigma Chemical Company (St. Louis, Mo.). ^{14}C-cholesterol hexadecyl ether (^{14}C-CHE) as well as ^{3}H-gentamicin were obtained from Amersham (Oakville, Ontario, Canada). The murine macrophage cell line J774A.1 was obtained from the American Type Culture Collection. Dulbecco's modified eagle medium, fetal bovine serum, and phosphate-buffered saline (PBS) plus Ca^{2+} and Mg^{2+} were obtained from Gibco (Burlington, Ontario, Canada). Octaethyleneglycol mono-*n*-dodecyl ether ($C_{12}E_8$) was obtained from Calbiochem (San Diego, Calif.). All other chemicals used were of reagent grade and were obtained from major suppliers.

Liposomal gentamicin preparation. Liposomal gentamicin formulations were prepared as described previously (8). Appropriate amounts of lipids were mixed in chloroform, and the solvent was evaporated by agitation under a nitrogen stream. Residual solvent was removed from the lipid mixture under high vacuum for at least 1 h. Dried lipid films (representing 25 mg of total lipid) were rehydrated by the addition of 0.5 ml of 100 mg of gentamicin (as the gentamicin base) per ml in 20 mM HEPES–150 mM NaCl (HBS; pH 7.4). Hydration of the lipid was facilitated by extensive vortexing and five freeze-thaw cycles between −196°C and room temperature. After hydration, the samples were diluted so that they contained lipid and drug concentrations of 25 and 50 mg/ml, respectively. This dispersion of multilamellar vesicles was converted to large unilamellar vesicles by 10 extrusions through two stacked 0.1-μm-pore-size filters (Poretics; AMD Manufacturing, Mississauga, Ontario, Canada) at 25 to 28°C by using a Thermobarrel Extruder (Lipex Biomembranes, Vancouver, British Columbia, Canada). Liposomes composed of dipalmitoylphosphatidylcholine (DPPC)-chol were extruded as described above but at 55°C. Removal of gentamicin from the outside surface of these anionic liposomes was achieved by adjusting the pH of the liposome suspension to pH 10 with 500 to 700 μl of glycine-buffered saline (2 M glycine, 150 mM NaCl [pH 10.0]) or 50 to 75 μl of 3 N NaOH with rapid vortexing. The liposomes were then passed through a 20- to 25-ml CM-Sepharose column equilibrated to pH 10.0 with 10 mM glycine–150 mM NaCl. The liposome fractions were collected and reequilibrated to pH 7.4 with 0.5 M HEPES–150 mM NaCl (pH 7.4).

Liposome sizes were routinely determined by quasielastic light scattering with a NICOMP 7600 submicron particle sizer (NICOMP Systems, Santa Barbara, Calif.). The data reported represent the mean ± standard deviation vesicle size fitted to a Gaussian particle size distribution. Lipid was quantified either by liquid scintillation counting (LSC) of samples containing a known quantity of ^{14}C-CHE or by phosphate assay (4). Gentamicin levels were determined by the addition of a known quantity of ^{3}H-gentamicin followed by LSC.

In vitro lipid mixing assays. Liposome fusion was determined by measurement of lipid mixing as described previously (8). Briefly, donor vesicles containing encapsulated gentamicin were prepared as described above, but they also contained 0.5 mol% each *N*-4-nitrobenzo-2-oxa-1,3-diazole (NBD) phosphatidylethanolamine (NBD-PE) and *N*-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (Rh-PE). Acceptor vesicles were of the same composition as the donor vesicles but without the fluorescent molecules. Donor liposomes in 10 mM HBS (pH 7.4) were mixed in a quartz cuvette with acceptor liposomes (also in 10 mM HBS [pH 7.4]) and were brought to a final volume of 1,900 μl with 150 mM NaCl. The added volumes of donor and acceptor liposomes were adjusted to achieve final lipid concentrations of 0.1 and 1.0 mM, respectively. The cuvette was placed in an SLM Aminco-Bowman Series II luminescence spectroscope equipped with a stirring mechanism and maintained at 37°C. The fluorescence emission was measured at 535 nm (NBD moiety fluorescence) by using an excitation wavelength of 445 nm, with 4-nm slit widths and with the initial fluorescence set at between 50 and 70% of full scale. After a stable baseline was verified, 50 μl of an acid solution composed of 5% (vol/vol) acetic acid in saline or dilutions thereof was added. After a 2-min stabilization period to verify that no fusion had occurred, $CaCl_2$ was added directly to the cuvette to a final concentration of 20 mM. The fluorescence was measured continuously until a plateau was achieved, and then 100% dequenching of the NBD moiety was determined by the addition of 50 μl of 0.2 M $C_{12}E_8$ detergent. The final pHs of the solutions were measured with a pH meter. Data were processed with software provided by Aminco-Bowman. Percent NBD dequenching, equivalent to percent fusion, was determined as (final fluorescence − initial fluorescence)/($C_{12}E_8$ fluorescence − initial fluorescence) × 100.

Pharmacokinetics and biodistribution. Gentamicin (labeled with ^{3}H-gentamicin at approximately 2.0 μCi/mg of drug) was encapsulated in liposomes as described above. The liposomes were composed of DPPC-chol (55/45 mol%) or DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5 mol%) that were labeled at approximately 7 μCi/100 mg of lipid with ^{14}C-CHE, a nonexchangeable and nonmetabolizable radioactive tracer lipid (12). These specific activities provided levels in plasma greater than or equal to 880 and 620 dpm/ml for ^{3}H-gentamicin and ^{14}C-CHE, respectively, at their nadirs in the pharmacokinetic studies. For gentamicin, this represents a lowest measured concentration of 0.2 μg/ml of plasma in the pharmacokinetic studies. Pharmacokinetic studies were performed by injecting liposomal gentamicin formulations or free gentamicin i.v. via the lateral tail vein. In preliminary experiments, free and liposomal forms of the drug were administered to uninfected ICR mice at a dose of 10 mg of gentamicin/kg of body weight. In subsequent determinations, free and liposomal forms of the drug were administered to BALB/c mice at a dose of 4.5 mg of gentamicin/kg 24 h after i.v. infection with *S. enterica* serovar Typhimurium as described below. That is, the pharmacokinetics and biodistribution were determined under in vivo conditions identical to those used for the efficacy experiments described in the next section.

At various time points after drug administration (0.25, 0.5, 1, and 3 h for free gentamicin; 1, 3, 6, and 24 h for liposomal gentamicin), the mice were anesthetized and blood was recovered via cardiac puncture. Subsequently, the animals were killed with an overdose of CO_2; and the liver, spleen, lung, kidney, and thigh muscle were recovered. Plasma and tissue homogenates were assayed for gentamicin and lipid levels by LSC. The concentrations of lipid and drug in tissues were corrected for the concentration contribution from the blood as described previously (9). Pharmacokinetic parameters were calculated by using WinNonlin pharmacokinetic software (version 3.0; Pharsight, Mountain View, Calif.). Data were analyzed by using noncompartmental, one- and two-compartment models, and the best-fit results reported. The observed area-under-the-curve (AUC) values for lipid and drug accumulation in tissues were obtained from the linear-trapezoidal calculation in the noncompartmental model by using WinNonlin.

In vivo intracellular killing and antibacterial efficacy. *S. enterica* serovar Typhimurium SL1344 was grown overnight in Luria-Bertani broth in a 37°C shaking incubator. The culture was centrifuged and washed in PBS and was then diluted to achieve a final suspension containing 1,000 CFU/ml. Female BALB/c mice (age, 6 to 8 weeks) were injected i.v. in the tail vein with inocula containing 60 to 90 CFU per mouse.

Twenty-four hours after infection, the mice were treated by a single i.v. administration of free gentamicin (in saline) or liposomal gentamicin at doses between 0.2 and 20 mg of gentamicin/kg. The solutions of both free and liposomal gentamicin were sterilized by passage through a 0.2-μm-pore-size filter prior to administration. At 5 days postinfection, the mice were killed and the spleens and/or livers were removed and placed in sterile 6-ml WhirlPak bags on ice. Individual organs were aseptically homogenized in 3.0 ml of cold PBS, and then aliquots (100 μl) of the homogenates were serially diluted in PBS to a maximum of a 10^5-fold dilution and plated on duplicate MacConkey agar plates. The plates were incubated overnight at 37°C, and the resulting light red (*lac* mutant) colonies on plates containing between 30 and 300 colonies were counted. The results were analyzed by the nonparametric Kruskal-Wallis one-way analysis of variance on ranks method by Dunn's pairwise multiple comparison technique on SigmaStat statistical software (version 2.0; SPSS Science, Chicago, Ill.).

RESULTS

Pharmacokinetics and biodistribution studies with uninfected mice. In a previous publication (8) we demonstrated that a formulation that comprised DOPE–*N*-succinyl-DOPE–PEG-ceramide had significantly greater efficacy against intracellular *S. enterica* serovar Typhimurium infections than various nonfusogenic (i.e., DPPC-chol) liposomes by an in vitro killing assay. Therefore, preliminary in vivo screening experiments were performed with healthy (i.e., uninfected) mice to optimize the pharmacokinetics of liposome-encapsulated gentamicin for subsequent in vivo antibacterial efficacy studies. DPPC-chol (55/45), DOPE–*N*-succinyl-DOPE–PEG-ceramide (69.5/30/0.5 and 65/30/5; mol/mol/mol), and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5, 39.5/30/30/0.5, 49.5/30/20/0.5, and 45/30/20/5; mol/mol/mol/mol) preparations of liposomal gentamicin were compared for drug and lipid circulation lifetimes after i.v. administration (data not shown).

Free gentamicin was rapidly eliminated from the circulation (data not shown). In contrast, encapsulation of gentamicin in all of the DOPE–*N*-succinyl-DOPE- and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide-based carriers substantially increased the concentrations of gentamicin in the circulation after i.v. administration (data not shown). In some formulations, rapid in vivo leakage of drug from the liposomes was observed, but this could be ameliorated by the addition of chol to the formulation. Overall, the two formulations that provided maximal gentamicin concentrations in the plasma after i.v. administration for in vivo efficacy testing were DPPC-chol (55/45; mol/mol) and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5; mol/mol/mol/mol).

Pharmacokinetics and biodistributions of free and liposomal gentamicin in infected mice. In the preceding section, optimized liposomal formulations of gentamicin were identified on the basis of the pharmacokinetics of gentamicin and lipid in healthy mice. As a prelude to evaluating their antibacterial efficacies in a murine infection model, the pharmacoki-

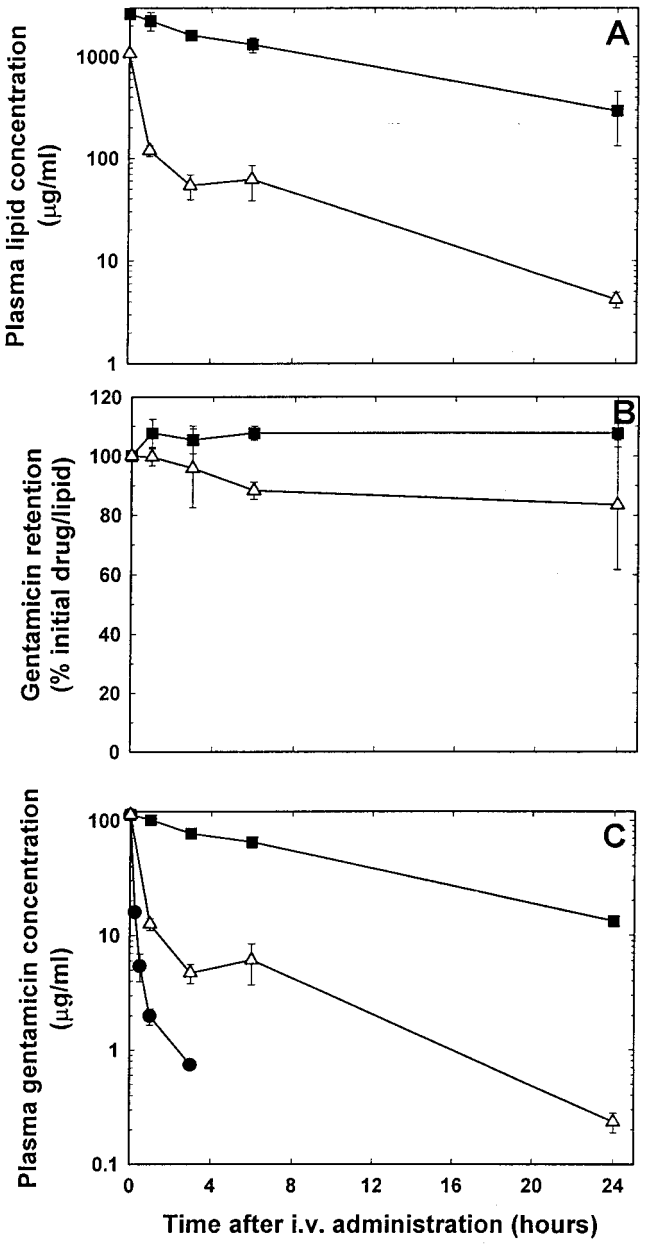

FIG. 1. Pharmacokinetics of free and liposomal gentamicin after i.v. administration of free and liposomal gentamicin in mice bearing an *S. enterica* serovar Typhimurium infection. Plasma lipid concentrations (A), plasma gentamicin/lipid ratios (B), and plasma gentamicin concentrations (C) are shown for free gentamicin (●) and for gentamicin encapsulated in liposomes composed of DPPC-chol (55/45; ■) or DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5; △). Data are means ± standard deviations for three animals. Error bars are plotted for all datum points; where error bars are not visible, they are smaller than the size of the symbol.

netics and biodistributions of these liposomal gentamicin formulations in animals bearing an *S. enterica* serovar Typhimurium infection were characterized (Fig. 1A to C).

As observed in healthy mice (data not shown), the clearance of DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) formulations from the plasma was more rapid than the clearance of DPPC-chol formulations (Fig. 1A). The pharmacokinetics of the DPPC-chol form of liposomal gentamicin was best described by a noncompartmental model ($r = 0.9997$) with an estimated terminal elimination half-life ($t_{1/2\lambda}$) of 8.49 h. In contrast, the elimination of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) formulation best fit a two-compartment model ($r = 0.9998$), with calculated values for the distribution phase half-life ($t_{1/2\alpha}$) of 0.22 h and for the elimination phase half-life ($t_{1/2\beta}$) of 8.69 h. For both liposomal formulations of gentamicin, there was negligible leakage of drug, as indicated by the drug/lipid ratio (Fig. 1B), after administration to infected mice. Consequently, the pharmacokinetics of drug elimination (Fig. 1C) from the plasma of mice bearing an *S. enterica* serovar Typhimurium infection were very similar to those described above for liposome elimination rates (contrast Fig. 1A with C). That is, the elimination of gentamicin removal from the circulation of infected mice treated with the DPPC-chol form of liposomal gentamicin was best described by a noncompartmental model ($r = 0.9991$) with a calculated $t_{1/2\lambda}$ of 8.13 h. The elimination of drug administered as the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) form of liposomal gentamicin best fit a two-compartment model ($r = 0.9998$) with calculated values of $t_{1/2\alpha}$ of 0.24 h and $t_{1/2\beta}$ of 8.88 h. While the elimination of free gentamicin was best described by a two-compartment model ($r = 1.000$), the calculated $t_{1/2}$s were significantly shorter, with a $t_{1/2\alpha}$ of 0.07 h and a $t_{1/2\beta}$ of 0.49 h. These elimination rates were associated with observed AUC values of 17.23, 129.3, and 1,195.4 μg · h/ml for free gentamicin and for the DPPC-chol and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) liposomal gentamicin formulations, respectively. These represent 7.5- and 69.4-fold increases in the plasma AUCs for the DPPC-chol and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposomal formulations of gentamicin, respectively, compared to that for the free drug.

The effects of liposomal encapsulation of gentamicin on drug biodistribution are shown in Fig. 2A to C and are summarized in Table 1. Encapsulation of gentamicin in either liposome formulation redirected gentamicin to both the liver and the spleen (Fig. 2A and B; Table 1) and substantially reduced the level of drug accumulation in the kidneys (Fig. 2C and Table 1). The increase in drug accumulation conferred by liposomal encapsulation was most pronounced in spleens, in which DPPC-chol liposomes increased drug quantities by 497-fold and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide formulations increased drug quantities by 437-fold (Table 1). The two formulations increased the quantities delivered to the liver by factors of 80- and 153-fold, respectively (Table 1), whereas delivery of increased quantities to the lungs was observed only for the DPPC-chol liposomal formulation of gentamicin (5.9- versus 1.05-fold for DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide; data not shown). The gentamicin/lipid ratios in the livers and spleens of mice receiving liposomal gentamicin were very similar to the gentamicin/lipid ratios of the injected formulations (Table 1). This result suggests that the liposomes with the encapsulated gentamicin were accumulating intact in the liver and spleen. In contrast, the gentamicin/lipid ratios were significantly higher in the kidneys of these animals, by 2.4- and 13.3-fold for the DPPC-chol and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide formulations, respectively (Table 1). This result suggests that gentamicin leaking from these liposomal delivery formulations rapidly accumulated in the kidneys in a fashion identical to that observed after the administration of the free drug (Table 1).

Taken together, these data demonstrate that the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) formulations were rapidly removed from the circulation (Fig. 1) and accumulated primarily in the liver and spleen (Fig. 2). It is likely

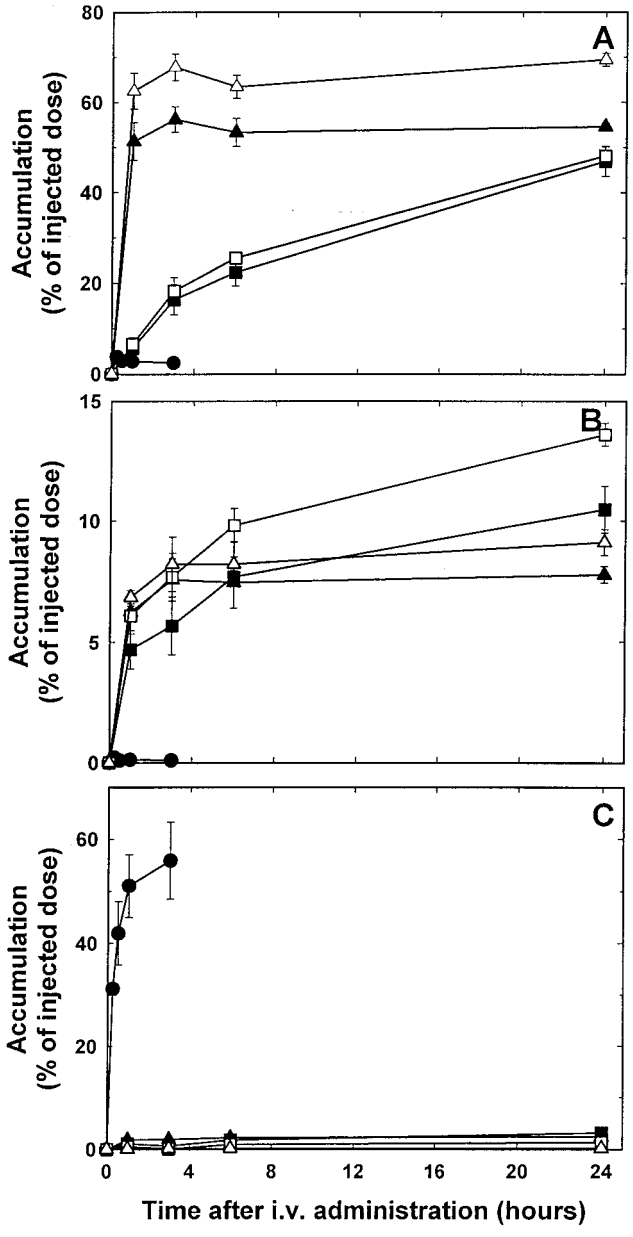

FIG. 2. Accumulation of gentamicin and lipid in tissues after i.v. administration of free and liposomal gentamicin in mice bearing an *S. enterica* serovar Typhimurium infection. The proportion of the injected dose of gentamicin (●, ■, ▲) and lipid (□, △) in the liver (A), spleen (B), and kidneys (C) after the i.v. administration of free gentamicin (●) or gentamicin encapsulated in liposomes composed of either DPPC-chol (55/45; ■, □) or DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5; ▲, △) are shown. Data are means ± standard deviations for three animals. Error bars are plotted for all datum points; where error bars are not visible, they are smaller than the size of the symbol.

that these carriers were passively targeting fixed macrophages of these organs. As many intracellular infections are in macrophages, this represents an ideal mechanism for targeting of an antibiotic to infected cells. Consequently, the rapid removal of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) formulations from the circulation is not problematic since it is associated with rapid accumulation at the target disease site (Fig. 2A,B). Furthermore, similar amounts of drug accumulated in the spleens of infected mice after the administration of either the DPPC-chol or the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) liposomal formulations of gentamicin (Fig. 2B and Table 1). As described in the next section, we use this fact to directly compare the in vivo antibacterial activity of gentamicin delivered to the spleens of mice bearing *S. enterica* serovar Typhimurium infections by the use of nonfusogenic (DPPC-chol) and fusogenic (DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide; 35/30/30/5) formulations.

In vivo antibacterial efficacy. To evaluate the therapeutic benefit conferred by encapsulation of gentamicin in a pH-sensitive liposome, we have chosen an in vivo *S. enterica* serovar Typhimurium infection model (11). This model was used because it is a well-characterized model in which the infecting bacteria reside primarily in the macrophages of the liver and the spleen (11). Since the quantities of drug that accumulated in the spleens were similar for these nonfusogenic (DPPC-chol) and pH-sensitive (DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide) formulations (Fig. 2; Table 1), this represents an opportunity to distinguish between liposomal drug delivery to a diseased tissue and additional intracellular delivery to the infecting intracellular pathogen.

By administration of free gentamicin as single i.v. doses between 0.2 and 5.0 mg/kg 24 h after bacterial infection, the free gentamicin had no statistically significant antibacterial activity ($P = 0.05$) (Fig. 3). In contrast, the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposomal formulation of gentamicin was very effective at killing the intracellular pathogen (Fig. 3). That is, at doses between 0.2 and 5.0 mg/kg, the number of bacteria surviving in the spleens was decreased by approximately 10^3 (Fig. 3). At doses between 0.2 and 1.0 mg/kg, the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide formulation of gentamicin had a good dose-response relationship ($r^2 = 0.84$; Fig. 3). At all doses tested, the number of bacteria observed in the spleens of animals treated with the liposomal form of the antibiotic were significantly lower ($P < 0.05$) than those in the spleens of control and free gentamicin-treated mice (with the exception of the numbers after treatment with liposomal gentamicin at 5.0 mg/kg compared to those in no-treatment controls; $P = 0.081$).

To determine if this in vivo antibacterial efficacy could be attributed to pH-sensitive fusogenic drug delivery, an experiment with two types of nonfusogenic liposome control groups was performed (Fig. 4). At a dose of 5 mg/kg, the number of bacteria surviving in the spleens of mice treated with the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide formulation of gentamicin was 3×10^3-fold lower than the number in those treated with free drug (Fig. 4). This difference was statistically significant ($P < 0.05$). Enhanced antibacterial activity was also achieved in mice treated with the non-pH-sensitive (see next section) control formulation (dioleoylphosphatidylcholine [DOPC]–*N*-succinyl-DOPE–chol–PEG-ceramide) or with nonfusogenic DPPC-chol liposomes (Fig. 4), but only the result for the latter formulation was statistically significant compared to that from treatment with free gentamicin. None of the liposomal formulations achieved statistically significant differences in antibacterial activity when they were compared to each other (Fig. 4). A similar level of in vivo antibacterial killing was also observed when a pH-sensitive formulation (8) was evaluated in comparison to its corresponding non-pH-sensitive (DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide) control liposome (data not shown).

In vitro characteristics of liposomal gentamicin formulations. The pH dependence of the fusion of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5) formulation of gentamicin was confirmed by a resonance energy transfer assay (Fig. 5). In the presence of 20 mM Ca^{2+} and at pH values

TABLE 1. Biodistributions of free and liposomal gentamicin in liver, kidneys, and spleen of BALB/c mice infected with *S. enterica* serovar Typhimurium after i.v. administration[a]

Parameter and formulation	Organ		
	Liver	Kidney	Spleen
Gentamicin AUC (μg · h/g)[b]			
Free gentamicin	8.1	573	3.7
DPPC-chol	648	170	1,833
DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide	1,244	194	1,611
Lipid AUC (μg · h/g)[b]			
DPPC-chol	1.64×10^4	1.75×10^3	5.59×10^4
DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide	1.44×10^4	146	1.71×10^4
Liposomal AUC/free AUC			
DPPC-chol	80	0.30	497
DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide	153	0.34	437
Gentamicin AUC/lipid AUC			
DPPC-chol	0.040	0.097	0.033
DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide	0.086	1.33	0.094

[a] The overall effect of liposomal encapsulation on the accumulation of gentamicin in the liver, kidneys, and spleen is summarized by the liposomal AUC/free AUC ratio. The drug/lipid ratio for the organs, which is an indicator of the propensity of the tissue to accumulate free drug that has leaked from the liposomes (higher ratio) or intact liposomes (unchanged ratio), is presented as the gentamicin AUC/lipid AUC ratio. Note that the drug/lipid ratios upon i.v. administration were 0.04 and 0.10 for DPPC-chol and DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide, respectively.

[b] Linear-trapezoidal AUCs were calculated over the period from 0 to 24 h postadministration for liposomal gentamicin and over the period from 0 to 3 h postadministration for free gentamicin, as described in Materials and Methods.

between 6.9 and 4.0, a control formulation composed of DOPC–*N*-succinyl-DOPE–chol–PEG-ceramide (39.5/30/30/0.5) had no fluorescence dequenching indicative of membrane fusion. In contrast, the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide formulation (39.5/30/30/0.5) showed significant fusion activity, particularly in the pH range from 5.5 to 4.5 that is characteristic of late endosomes. These data are consistent with our previous report that a very similar liposomal gentamicin composition, DOPE–*N*-succinyl-DOPE–PEG-ceramide, undergoes a pH-dependent destabilization or membrane fusion event that facilitates intracellular delivery of liposomal contents (8). These results are also consistent with the report (3) that pH-sensitive liposomes containing up to 40 mol% cholesterol retain their pH-dependent membrane fusion activity.

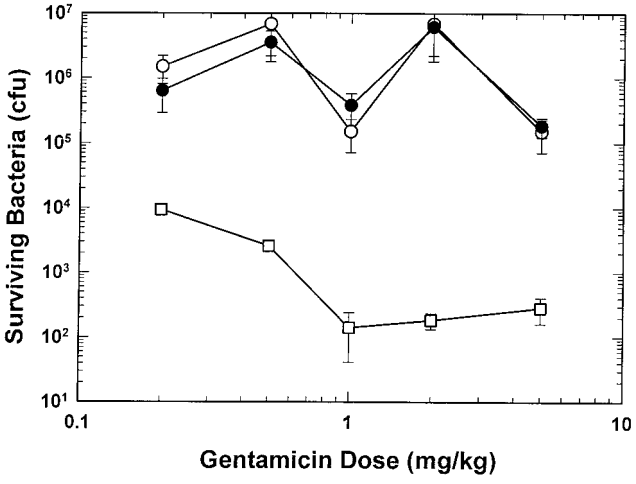

FIG. 3. In vivo antibacterial activity of free and liposomal gentamicin against an intracellular *S. enterica* serovar Typhimurium infection in the spleen. The numbers of bacteria surviving in the spleen are shown for no-treatment controls (○) or for mice receiving either free gentamicin (●) or gentamicin encapsulated in DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5 mol%; □) liposomes. Values represent the mean ± standard error numbers of CFU from duplicate assays with three or four mice per group. Results were analyzed by one-way analysis of variance on ranks and pairwise multiple comparison methods as described in Materials and Methods.

DISCUSSION

Gentamicin is an aminoglycoside antibiotic whose clinical utility against serious pathogens is limited by poor penetration of host cells to the location of the intracellular bacterial infection. In a previous study (8) we described a novel liposomal formulation of gentamicin that is capable of achieving efficient intracellular antibiotic delivery and improved antibacterial therapy. This formulation was designed to achieve intracellular drug delivery via the inclusion of lipids (*N*-succinyl-DOPE and DOPE) that destabilize the carrier membrane at endosomal pH values. It was the purpose of the present study to evaluate if this pH-sensitive liposomal delivery vehicle that demonstrates significantly enhanced intracellular delivery activity in vitro would also show increased antibacterial activity in vivo.

On the basis of preliminary screening experiments, the formulations initially identified for in vivo efficacy studies (DOPE–*N*-succinyl-DOPE with 0.5 or 5.0% PEG-ceramide) based on in vitro experiments (8) were modified to include 30 mol% cholesterol. Significant improvements in the liposome circulation lifetime, drug retention within the liposomes, and drug concentrations in the circulation were observed with the inclusion of 30 mol% cholesterol in the DOPE–*N*-succinyl-DOPE-based vesicles (data not shown). Despite this improvement, a significant proportion of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposome population showed an initial clearance phase ($t_{1/2\alpha}$ = 0.22 h) that was associated with rapid liposome accumulation in the liver (Fig. 2A). It is likely that the clearance of these anionic vesicles was a result of protein opsonization and subsequent uptake by both fixed and circulating macrophages (5). Similarly, opsonization to these anionic formulations could promote their destabilization and subsequent drug release. This observation is consistent with

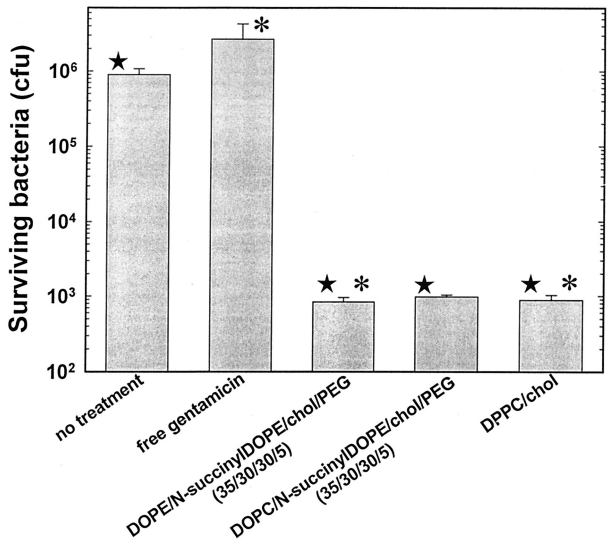

FIG. 4. In vivo antibacterial activity of free and liposomal gentamicin against a intracellular *S. enterica* serovar Typhimurium infection in the spleen. The numbers of bacteria surviving in the spleen are shown for no-treatment controls or for mice receiving a single administration of gentamicin at 5 mg/kg. Data are presented for free gentamicin or gentamicin encapsulated in liposomes of DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5 mol%), DOPC–*N*-succinyl-DOPE–chol–PEG-ceramide (35/30/30/5), or DPPC-chol (55/45 mol%). Values represent the mean ± standard error numbers of CFU from duplicate assays with three or four mice per group. Results were analyzed by one-way analysis of variance on ranks and pairwise multiple comparison methods as described in Materials and Methods. Statistically significant pairwise comparisons between each liposomal gentamicin formulation and either the no-treatment control (★) or the free gentamicin control (∗) for which P was <0.05 are indicated by matching symbols. For all other pairwise comparisons P was >0.05.

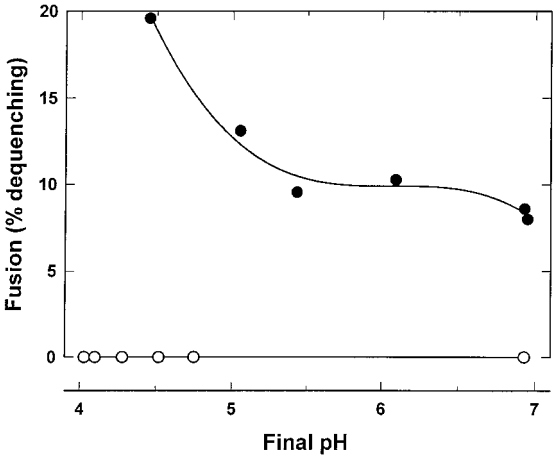

FIG. 5. pH dependence of the lipid demixing of DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide (39.5/30/30/0.5) (●) and DOPC–*N*-succinyl-DOPE–chol–PEG-ceramide (39.5/30/30/0.5) (○) liposomes. Liposomes encapsulating gentamicin and containing 1 mol% each NBD-PE and Rh-PE were mixed with a 10-fold excess of identical liposomes lacking fluorescent lipids and encapsulated drug at a final Ca^{2+} concentration of 20 mM and a pH of 6.93. The pH was adjusted and the percent dequenching was calculated at 2 min after the pH reduction as described in Materials and Methods.

previous reports that the presence of cholesterol increases the circulation longevity of liposomes and improves drug retention (5, 9). A remaining proportion of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposome population (<10%) had a $t_{1/2\beta}$ (8.69 h) very similar to the DPPC-chol $t_{1/2\lambda}$ (8.49 h). It is possible that this small proportion of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposome population may have had a negligible surface charge and consequently acquired clearance characteristics comparable to those of a neutral liposomal carrier such as DPPC-chol (5).

The results presented in Fig. 2 demonstrate that the DOPE–*N*-succinyl-DOPE-based formulations effectively precluded drug accumulation in the kidneys and redirected the antibiotic to the liver and spleen. While the accumulation of drugs in the liver and spleen is well known for liposomal formulations, the rate of the DOPE–*N*-succinyl-DOPE–chol–PEG-ceramide liposomal form of gentamicin accumulation in these organs was substantially faster than that for neutrally charged DPPC-chol liposomes. These data are consistent with those that were reported previously and that demonstrated the extensive accumulation of empty 200-nm egg PC–chol–*N*-succinyl-DOPE liposomes in the livers of mice (10). pH-sensitive liposomes composed of DOPE-oleic acid have also been observed to be removed rapidly from the circulation and to accumulate to high levels in the livers, lungs, and spleens of mice (2). The data reported by those investigators also suggested rapid leakage of the entrapped marker after i.v. administration and subsequent marker distribution to organs different from those to which the injected carrier was distributed (2). In contrast, the drug/lipid ratios presented in Table 1 demonstrate that the liposomes localized in the liver and spleen contained encapsulated gentamicin, while the kidney appeared to be accumulating gentamicin that had been released from liposomes in the circulation. The blood circulation times for these pH-sensitive formulations are not as long as those reported for sterically stabilized pH-sensitive liposomes composed of cholesterol hemisuccinate (13). However, the rapid accumulation of these carriers in the target organs (the liver and spleen) makes these pH-sensitive carriers ideal for in vivo evaluation in an antibacterial efficacy model.

The in vivo antibacterial activity of the pH-sensitive liposomal gentamicin carriers was excellent, achieving 10^3- to 10^4-fold reductions in the numbers of *S. enterica* serovar Typhimurium organisms residing in the spleen (Fig. 3) and liver (data not shown). Similar decreases in bacterial load have been reported in rats bearing pulmonary *Klebsiella pneumoniae* infections and treated with liposomal gentamicin (1), and substantial increases in survival have been reported in mice bearing *S. enterica* serovar Typhimurium infections that were treated with liposomal gentamicin (14). The previous results, achieved with nonfusogenic liposomes based on egg PC or egg PC and chol mixtures, are consistent with the results presented in Fig. 4. That is, the antibacterial effects observed with the pH-sensitive DOPE–*N*-succinyl-DOPE-based formulations (Fig. 4) were also achieved with two different nonfusogenic control formulations of gentamicin. There are two primary reasons why this might occur: (i) the pH-sensitive formulation is rendered nonfusogenic upon systemic administration, possibly by the adsorption of serum proteins, or (ii) after liposomal drug accumulation at the disease site (Fig. 2), the therapeutic benefits that are achieved by fusogenic carrier-mediated intracellular delivery of gentamicin are outweighed by those benefits that arise from the substantial increases in the AUC for the antibiotic at the infection site (Table 1) for both fusogenic and nonfusogenic carriers. The latter explanation is more likely, given the observation that these liposomal formulations retain their pH-sensitive behavior in the presence of fetal bovine serum (8) (data not shown). It is pertinent that the antibacterial activities of the liposomal gentamicin formulations were poorly correlated to the pharmacokinetic parameters. Rather,

antibacterial activity was closely associated with the AUC for drug accumulation in the target organs, the liver and spleen, irrespective of the liposomal formulation used to deliver the antibiotic.

It should be noted that the numbers of bacteria that survived in the spleens of mice treated with liposomal gentamicin were similar to those that were present at the start of gentamicin therapy (11). Therefore, it cannot be excluded that the liposomal formulations may be exerting cytostatic rather than cytotoxic effects. However, since the infection is exclusively present intracellularly, these data demonstrate that the use of these liposomal formulations enhanced the intracellular delivery of the drug to increase either the cytotoxic or the cytostatic activities of the drug compared to those achieved with free gentamicin.

This is the first report of a study that has characterized the in vivo efficacy of an antibiotic encapsulated in a pH-sensitive fusogenic liposome. It is anticipated that the ability of pH-sensitive fusogenic liposomal carriers to significantly improve in vivo therapeutic activity will be seen with the optimum combination of encapsulated drug, disease target, and carrier pharmacodynamics.

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