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Formulation and Efficacy of Liposome-Encapsulated Antibiotics for Therapy of Intracellular *Mycobacterium avium* Infection

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Mycobacterium avium **is an intracellular pathogen that can invade and multiply within macrophages of the reticuloendothelial system. Current therapy is not highly effective. Particulate drug carriers that are targeted to the reticuloendothelial system may provide a means to deliver antibiotics more efficiently to *M. avium*-infected cells. We investigated the formulation of the antibiotics ciprofloxacin and azithromycin in liposomes and tested their antibacterial activities in vitro against *M. avium* residing within J774, a murine macrophage-like cell line. A conventional passive-entrapment method yielded an encapsulation efficiency of 9% for ciprofloxacin and because of aggregation mediated by the cationic drug, was useful only with liposomes containing ≤50 mol% negatively charged phospholipid. In contrast, ciprofloxacin was encapsulated with >90% efficiency, regardless of the content of negatively charged lipids, by a remote-loading technique that utilized both pH and potential gradients to drive drug into preformed liposomes. Both the cellular accumulation and the antimycobacterial activity of ciprofloxacin increased in proportion to the liposome negative charge; the maximal enhancement of potency was 43-fold in liposomes of distearoylphosphatidylglycerol-cholesterol (DSPG-Chol) (10:5). Azithromycin liposomes were prepared as a freeze-dried preparation to avoid chemical instability during storage, and drug could be incorporated at 33 mol% (with respect to phospholipid). Azithromycin also showed enhanced antimycobacterial effect in liposomes, and the potency increased in parallel to the moles percent of negatively charged lipids; azithromycin in DSPG-Chol (10:5) liposomes inhibited intracellular *M. avium* growth 41-fold more effectively than did free azithromycin. Thus, ciprofloxacin or azithromycin encapsulated in stable liposomes having substantial negative surface charge is superior to nonencapsulated drug in inhibition of *M. avium* growth within cultured macrophages and may provide more effective therapy of *M. avium* infections.**

Mycobacterium avium infection is a common complication in AIDS (10, 38). For specimens taken from AIDS patients, *M. avium* could be cultured from 23 to 50% of blood cultures (37). *M. avium* can invade macrophages of the reticuloendothelial system (39), and intracellular organisms are able to withstand or evade macrophage antibacterial mechanisms. Furthermore, intracellular bacteria are shielded by the host cell from both immune surveillance and extracellular antibiotics.

For effective therapy of *M. avium* infection, drugs must penetrate the host cells in cytostatic or cytotoxic concentrations. However, many antibiotics lack the ability to traverse the cell membrane, are excluded from acidic compartments such as the phagocytic apparatus, or are severely toxic when administered in the high doses necessary to overcome their low cellular permeability (19, 36). To maximize the antimycobacterial effects of antibiotics while minimizing their toxicity, drug carriers to target drugs to *M. avium*-infected cells are under examination.

Of the drug delivery systems for which there has been significant human clinical trial experience (15), liposomes may be the most suitable to carry antimicrobial compounds to sites of intracellular *M. avium* infection. Liposomes are phospholipid vesicles that can encapsulate drugs of diverse physical characteristics either within the membrane bilayer or within the enclosed aqueous space, depending on the physicochemical properties of the compounds. Liposomes are taken up avidly by macrophages and are targeted naturally to the reticuloendothelial system (8), a major reservoir of *M. avium* infection in the body (5). Work presented elsewhere (29) showed that *M. avium*-infected cells can endocytose and process liposomes in a manner similar to that of uninfected cells, suggesting that the avid uptake of liposomes observed for normal macrophages is not hindered in infected cells.

A variety of antibiotics have been encapsulated in liposomes and have shown enhanced therapeutic effects against *M. avium* infection in vitro and in vivo (6, 7, 9, 12, 20, 22, 26, 28). Although such data suggest a therapeutic potential for liposomal antibiotics, few studies have examined the cellular uptake and retention of free or liposome-encapsulated antibiotics, or whether the intracellular distribution of liposomal antibiotics may differ from that of free antibiotics. In addition, concerns about low efficiency of drug entrapment and poor stability during storage have impeded development of some promising formulations for eventual human trial.

In the present work, we have developed liposomal formulations of ciprofloxacin and azithromycin, two antibiotics that have shown activity in the free form against isolated cultures of *M. avium* in vitro (13, 26) but for which stability, solubility, or cellular penetration hamper clinical application. In addressing pharmaceutical and cell-biological concerns, a "remote-loading" procedure (16, 27) was adapted to encapsulate ciprofloxacin in liposomes with almost quantitative efficiency and a

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lyophilization-rehydration method (31) was used to minimize the aqueous hydrolysis of liposome-encapsulated azithromycin during storage. We have also investigated the cellular uptake, intracellular distribution, and antimycobacterial effects of these liposomal antibiotics in macrophage-like cells infected with *M. avium.*

MATERIALS AND METHODS

Distearoylphosphatidylcholine (DSPC) and distearoylphosphatidylglycerol (DSPG) were purchased from Avanti Polar Lipids (Alabaster, Ala.). Cholesterol (Chol) was purchased from Sigma (St. Louis, Mo.) and was recrystallized thrice from methanol. Ciprofloxacin was from Miles Pharmaceuticals (West Haven, Conn.), and azithromycin was from Pfizer, Inc. (Groton, Conn.). RPMI 1640, fetal and newborn bovine serum, CO_2-independent medium, 7H9 broth, 7H10 agar, OADC (oleic acid-albumin-dextrose-catalase) enrichment, and dialysis tubing (molecular weight cutoff, 12,000 to 14,000) were from Fisher Scientific (Pittsburgh, Pa.). Sephadex G-75 and Enzyme-Free Cell Dissociation Solution were from Sigma.

Bacteria. *M. avium* ATCC 49601 was obtained from the American Type Culture Collection (ATCC). It was subcultured onto 7H10 agar plates and incubated at 37°C for 10 to 14 days. Individual transparent colonies of *M. avium* were then amplified in 7H9 broth supplemented with OADC.

Cell culture. The murine macrophage-like cell line J774 was maintained in RPMI 1640 medium supplemented with 5% heat-inactivated fetal serum and 5% newborn bovine serum. One day prior to experiments, cells were plated as monolayer cultures in 35-mm-diameter culture dishes or 24-well plates.

Preparation of ciprofloxacin-loaded liposomes. Ciprofloxacin was loaded into preformed liposomes at a high efficiency by modifying a remote-loading technique developed for doxorubicin (16, 27). Ten to 30 μmol of the appropriate phospholipid mixture was dried from chloroform and deposited as a thin film on the wall of a glass tube by using a rotary evaporator. The dried lipid film was hydrated with 250 mM ammonium sulfate that was adjusted to pH 2.5 with HCl. The final phospholipid concentration was 20 mM. The preparation was vortexed vigorously and extruded three times under argon pressure (200 lb/in^2) through a 0.2-μm-pore-size polycarbonate membrane filter (30). Unentrapped ammonium sulfate was removed from the liposome suspension by dialysis against 10% (wt/vol) sucrose (290 mosmol/kg) that was adjusted to pH 2.5 with HCl. In order to establish a transmembrane pH gradient, the liposome suspension was titrated to various pH values in the range of 4.5 to 7.5 by using 0.1 N KOH. The suspension was incubated at 60°C for 1 h (with intermittent vortexing) at a final concentration of 2 to 3 mM ciprofloxacin · HCl (formula mass, 367.81 Da). The pH was verified after the addition of drug. Residual ciprofloxacin was removed from the liposome formulation by dialysis against isotonic 10% sucrose that was adjusted to pH 5.0. Phospholipid concentrations were measured by the method of Bartlett (2). Ciprofloxacin concentrations were determined by using an SLM 8000-C spectrofluorometer (SLM, Chicago, Ill.), employing an excitation wavelength of 340 nm and an emission wavelength of 450 nm. The concentration of unknown samples was determined by comparison to a standard curve. Trapping efficiencies were calculated as follows:

$$\text{Trapping efficiency} = \frac{[\text{Ciprofloxacin}]}{[\text{phospholipid}]}\,\text{postdialysis}/\frac{[\text{Ciprofloxacin}]}{[\text{phospholipid}]}\,\text{predialysis}$$

We also investigated the encapsulation of ciprofloxacin by the method of Majumdar et al. (26), with minor modifications. In brief, ciprofloxacin was dissolved to a concentration of 14 mM in 10 mM acetate buffer (pH 5.5), and the osmolality of the solution was adjusted with sucrose to 290 mosmol/kg. Osmolality was measured with a vapor phase osmometer (Wescor Inc., Logan, Utah). Ten micromoles of the appropriate phospholipid mixture was dried from chloroform and deposited as a thin film on the wall of a glass tube. An aliquot of ciprofloxacin solution (0.5 ml) was added to the lipid film (to a final phospholipid concentration of 20 mM), the mixture was vortexed, and the liposomes were extruded as described above. Unencapsulated ciprofloxacin was removed from liposomes by gel filtration through Sephadex G-75 preequilibrated with isotonic sucrose containing 10 mM acetate buffer, pH 5.5.

Preparation of azithromycin-loaded liposomes. Small unilamellar vesicles containing azithromycin (Pfizer, Inc.) were prepared by a method adapted from Perez-Soler et al. (31) with a slight modification as described here. Azithromycin and phospholipids were mixed in organic solvent (chloroform and methanol) in the desired ratios, and a dried film was formed by rotary evaporation. The drug-lipid film was dissolved in tertiary butanol to a phospholipid concentration of 10 mM, shell-frozen in liquid nitrogen, and lyophilized overnight. The resulting fluffy white powder was rehydrated at the time of use to a concentration of 20 mM, by using NaCl (145 mM)-*N*-Tris (hydroxymethyl) methyl-2-amino ethane sulfonate (10 mM)-EDTA (0.1 mM) buffer, and was sonicated for 30 min under argon to produce small liposomes.

Liposomal azithromycin concentrations were determined by dissolving an aliquot of liposomes with Triton X-100 (to a final concentration of 1% detergent) and using a microbiologic assay (14) employing the indicator bacterium *Micrococcus luteus*. Control experiments verified that the concentration of Triton X-100 did not alter the performance of the assay. No purification step was included to separate liposome-entrapped from unentrapped drug. At neutral pH, azithromycin is poorly soluble in aqueous media; the apparent solubility was <0.003 mg/ml (data not shown). Therefore, formulations were examined for the presence of drug precipitates by phase-contrast or differential interference contrast microscopy, both before and after passage through 0.2-μm-pore-size filters. If drug precipitates were not observed prior to filtration, it was assumed that all drug present was incorporated within the liposome and that the concentration of unincorporated azithromycin was ≤0.003 mg/ml.

Antimicrobial effect of liposomes. J774 cells were harvested by using Enzyme-Free Cell Dissociation Solution (Sigma Chemical Co.) and plated at a density of 0.5×10^6 cells per well in 24-well tissue culture plates. After overnight incubation, J774 cells were overlaid with a bacterial suspension adjusted to yield a multiplicity of infection of 20 bacteria per cell. Cells were infected for 3 h and were washed three times with culture medium to eliminate unbound bacteria. *M. avium*-infected cells were refed with culture medium and then incubated with various concentrations of free or liposomal antibiotics. After 72 h, the cells were washed with culture medium to eliminate extracellular bacteria and lysed by addition of 1 ml of 0.25% (wt/vol) sodium lauryl sulfate in phosphate-buffered saline (PBS). The lysates were diluted serially, and 100-μl aliquots were dispersed on 7H10 agar plates. The CFU of *M. avium* were counted 2 weeks after incubation at 37°C.

Electron microscopy. J774 cells were cultured on glass coverslips and infected with *M. avium* as described above. At intervals, cells were rinsed three times with complete culture medium (containing 10% serum) and three times with PBS to remove extracellular bacteria. Cells were fixed with 4% glutaraldehyde in 0.1 M Na cacodylate buffer (pH 7.2). After fixation, cells were rinsed thrice with 0.1 M Na cacodylate and postfixed with 1% osmium tetroxide in 0.1 M sodium cacodylate. Specimens were dehydrated in a series of graded ethanols and embedded in Epon-Araldite. The coverslips were dissolved in 52% hydrofluoric acid, and sectioned to a thickness of 10 nm. Sections were stained in 2% uranyl acetate and lead citrate and examined by using a Hitachi H500 transmission electron microscope.

Light microscopy. Aliquots of liposome suspensions were examined with a Nikon Diaphot microscope. Digital images of liposomes were acquired by using a charge-coupled device camera and image processor (Quantex, Woburn, Mass.).

Uptake of ciprofloxacin by J774 cells. Approximately 10^6 cells in 35-mm-diameter culture dishes were incubated in triplicate with free or liposomal antibiotics for various intervals at 37°C. At the end of the incubation, cells were washed three times each with complete culture medium and with PBS to remove extracellular antibiotics. Cells were counted with a hemocytometer or Coulter Counter model ZM (Coulter, Hialeah, Fla.) and then lysed with 0.25% sodium lauryl sulfate. The amount of cell-associated ciprofloxacin was measured by fluorescence and was normalized according to the cell count.

Video fluorescence microscopy. J774 cells were cultured on glass coverslips and were incubated with free or liposome-encapsulated ciprofloxacin. After various intervals, each coverslip was rinsed three times each with complete culture medium and then PBS. Coverslips were then placed in a custom holder, and live cells were observed under PBS by using an inverted fluorescence microscope. Ciprofloxacin was detected by fluorescence microscopy by using a 320- to 360-nm bandpass excitation filter, a 400-nm dichroic mirror, and a 430- to 470-nm bandpass emission filter. Images were acquired by using a silicon-intensified charge-coupled device camera and image-processing hardware and software (Quantex).

RESULTS

Formulation of ciprofloxacin liposomes. Ciprofloxacin has been encapsulated in liposomes previously by hydration of a thin lipid film with drug-containing aqueous media (26). Such passive encapsulation techniques result in the incorporation of a small fraction of the drug added, as well as a low drug/phospholipid ratio. In our hands, hydration of a thin lipid film yielded an encapsulation efficiency of 9% for liposomes composed of DSPG-DSPC-Chol (molar ratio, 5:5:5), corresponding to a final drug/phospholipid ratio of approximately 0.225 mol/mol (or ≈0.09 g/mol). In contrast, a remote-loading procedure, in which potential or pH gradients were used to drive drug into preformed liposomes, resulted in 94% encapsulation efficiency for liposomes of the same composition (data not shown) (cf. Table 1). The final drug/phospholipid ratio achieved was approximately 0.52 mol/mol, approximately 2.3-fold higher than achieved by hydration of a thin lipid film. Given the pharmaceutical and pharmacological advantages likely with high entrapment efficiency and a high drug/phospholipid ratio, parameters affecting the production of ciprofloxacin-containing liposomes were examined in greater detail.

TABLE 1. Efficiency of ciprofloxacin encapsulation by remote loading

Liposome composition	Molar ratio	Encapsulation efficiency ± SE (%)
DSPC-Chol	10:5	91.6 ± 7.3
DSPG-DSPC-Chol	3:7:5	91.1 ± 3.5
DSPG-DSPC-Chol	5:5:5	94.6 ± 1.6
DSPG-DSPC-Chol	7:3:5	89.3 ± 3.9
DSPG-Chol	10:5	94.2 ± 1.3

The efficiency of ciprofloxacin encapsulation by remote loading was dependent on the pH of the bulk external medium. Figure 1 shows that the fraction of ciprofloxacin encapsulated nearly doubled as the external pH was increased over the range of pH 2.5 to pH 7.5. The highest loading efficiency achieved was 94% of the added drug and was observed when the external pH was adjusted to pH 7.5, approximately the isoelectric pH of ciprofloxacin.

Because of the electrostatic charge borne by ciprofloxacin, drug-lipid interactions limited the range of lipid compositions in which drug could be encapsulated. Previous data support the rationale for testing liposomes of high negative charge for enhanced cellular delivery (17, 34). However, extensive and irreversible aggregation of liposomes occurred upon hydration of lipid films composed of more than 50 mol% negative charge (e.g., added as DSPG), possibly resulting from the complexation of the cationic ciprofloxacin with the anionic DSPG. In contrast, liposomes containing up to 100 mol% DSPG (i.e., DSPG-Chol, 2:1) could be prepared by the remote-loading process. When the external pH was adjusted to 7.5, large aggregates of liposomes initially were observed by light microscopy (data not shown). However, the aggregates dissipated during the loading process and a fine liposomal suspension was observed after 1 h (data not shown). Table 1 shows that the efficiency of ciprofloxacin entrapment by remote loading was independent of the lipid composition.

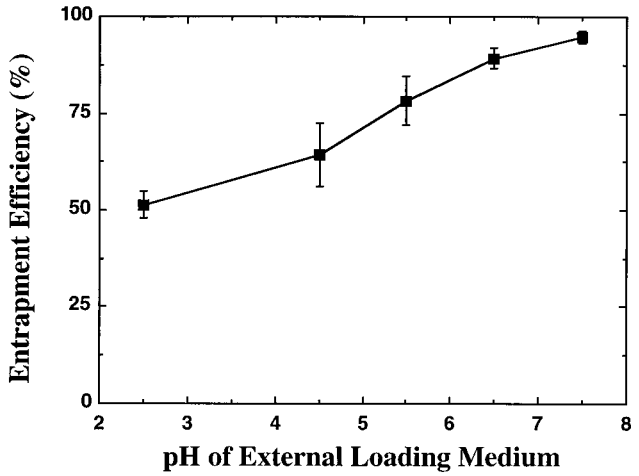

FIG. 1. pH dependence of ciprofloxacin encapsulation by remote loading. Ciprofloxacin-containing liposomes composed of DSPG-DSPC-Chol (molar ratio, 5:5:5) were prepared by remote loading of drug into preformed liposomes, as described in Materials and Methods. After removal of unencapsulated ciprofloxacin by dialysis, drug and phospholipid concentrations were determined by fluorescence and phosphorus assay, respectively. Encapsulation efficiencies were calculated as the ciprofloxacin/phospholipid ratio after dialysis divided by the ciprofloxacin/phospholipid ratio before dialysis. Each value represents the mean ± standard error (error bars) ($n \geq 3$ replications).

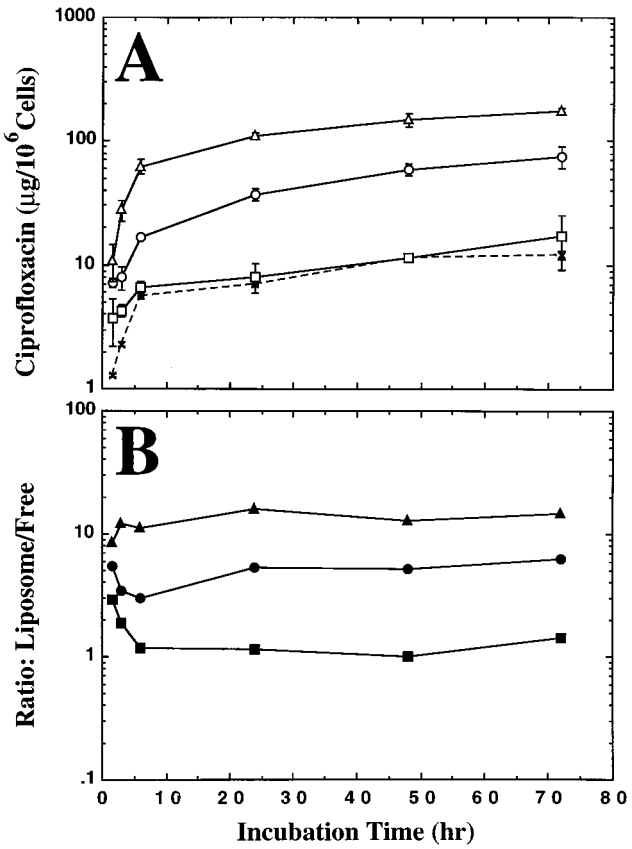

FIG. 2. Uptake of ciprofloxacin by J774 cells. Monolayers of J774 cells were incubated with fresh growth medium containing 0.95 mM ciprofloxacin, either free or encapsulated in liposomes, as described in Materials and Methods. At the times indicated, cells were washed to remove unincorporated ciprofloxacin or liposomes and the amount of cell-associated ciprofloxacin was measured by fluorescence spectroscopy. Drug concentrations are normalized according to cell number. Squares, DSPC-Chol (10:5) ciprofloxacin-containing liposomes; circles, DSPC-DSPG-Chol (5:5:5) ciprofloxacin-containing liposomes; triangles, DSPG-Chol (10:5) ciprofloxacin-containing liposomes; crosses, free ciprofloxacin. Each value represents the mean ± standard error (error bars) ($n = 3$ replications). (A) Quantity of cell-associated ciprofloxacin as a function of time; (B) ratio of liposome-mediated drug uptake to free drug uptake as a function of time.

Because of the high drug/lipid ratio attained, the high efficiency of encapsulation, and the ease of preparation, liposomes prepared by remote loading were used in the studies with encapsulated ciprofloxacin that are described below.

Uptake of ciprofloxacin by cells in culture. The cellular uptake rates for liposome-encapsulated and free ciprofloxacin were compared in order to identify formulations and conditions resulting in optimal antibiotic delivery. The quantity of free ciprofloxacin associated with J774 macrophage tumor cells increased over the first 6 h of incubation and reached a plateau of 11 $\mu g/10^6$ cells (Fig. 2A). Cellular accumulation of ciprofloxacin in DSPC-Chol (10:5) liposomes was similar to that of free drug with incubation times of ≥5 h. At early times, a slightly greater amount of antibiotic was associated with cells when liposomes were used, but that differential equalized after 5 h of incubation (Fig. 2B).

In contrast, ciprofloxacin in negatively charged liposomes showed a time-dependent increase in uptake over a 72-h period (Fig. 2A); at all time points, liposomes of DSPG-DSPC-Chol (5:5:5) gave levels of cell-associated ciprofloxacin higher than those obtained with free drug (Fig. 2B). Liposomes of high negative charge resulted in the largest cellular accumula-

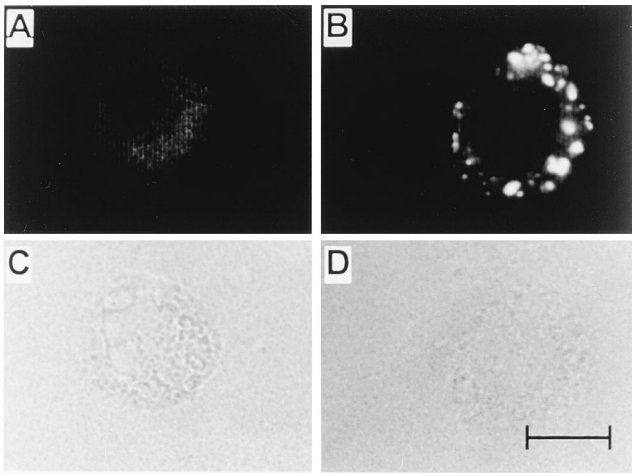

FIG. 3. Cellular distribution of ciprofloxacin after 1 h of exposure. J774 cells were grown on glass coverslips and were exposed for 1 h at 37°C to 0.95 mM ciprofloxacin, either free or in liposomes. Cells were washed to remove unassociated drug and examined by fluorescence microscopy, as described in Materials and Methods. (A) Fluorescence image of cells exposed to free drug; (B) fluorescence image of cells exposed to ciprofloxacin in DSPG-Chol (10:5) liposomes; (C) phase-contrast image of cell shown in panel A; (D) phase-contrast image of cell in panel B. Bar, ~5 μm.

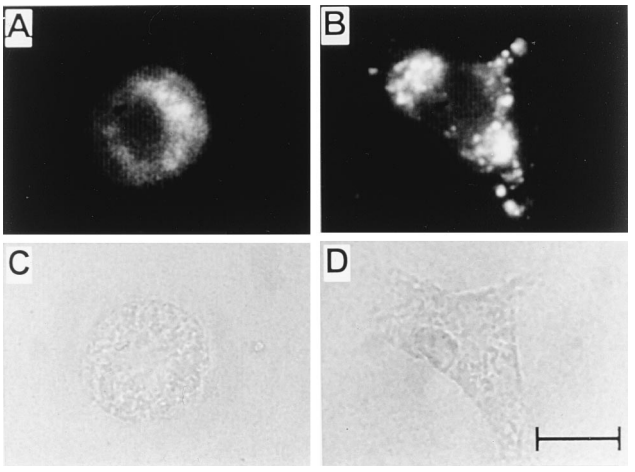

FIG. 4. Cellular distribution of ciprofloxacin after 24 h of exposure. J774 cells were grown on glass coverslips and were exposed for 24 h at 37°C to 0.95 mM ciprofloxacin, either free or in liposomes. Cells were washed to remove unassociated drug and examined by fluorescence microscopy, as described in Materials and Methods. (A) Fluorescence image of cells exposed to free drug; (B) fluorescence image of cells exposed to ciprofloxacin in DSPG-Chol (10:5) liposomes; (C) phase-contrast image of cell shown in panel A. (D) phase-contrast image of cell shown in panel B. Bar, ~5 μm.

tion of ciprofloxacin; DSPG-Chol (10:5) liposomes showed continuous uptake by J774 cells throughout the 72-h experiment (Fig. 2A), and at all times, the level of cell-associated drug was approximately 10-fold higher than it was when free ciprofloxacin was used (Fig. 2B). Compared with the area under the exposure curve (AUC) for free ciprofloxacin, the AUC for DSPG-DSPC-Chol (5:5:5) liposomes was 5-fold greater, while the AUC for DSPG-Chol (10:5) liposomes was 14-fold higher than that for free drug (data not shown).

Intracellular distribution of ciprofloxacin. *M. avium* and a variety of other intracellular pathogens reside within cellular vesicles subsequent to phagocytosis by host macrophages. For effective therapy, antibiotics not only must accumulate in cells, but also must penetrate to the intracellular site of infection. To evaluate the intracellular distribution of liposome-delivered antibiotics within cells, we employed video fluorescence microscopy, exploiting the intrinsic fluorescence of ciprofloxacin.

After 1 h of incubation with free drug, dim fluorescent accretions were observed in J774 cells (Fig. 3A). In contrast, exposure of cells to ciprofloxacin-loaded liposomes of DSPG-Chol for 1 h resulted in much more intense fluorescence that was confined spatially (Fig. 3B), suggesting localization within cellular structures. Similar accumulation patterns for other fluorescent probes have been taken as evidence for endocytosis of liposomes and sequestration of their contents in endocytic vesicles (cf. reference 35). Ciprofloxacin fluorescence was not sensitive to pH in the range of pH 5.0 to 7.0 (data not shown), suggesting that cell-associated fluorescence corresponds to the intracellular distribution of drug, and was not biased by pH variations in intracellular compartments such as lysosomes.

After 24 h of exposure to free ciprofloxacin (Fig. 4A), cells showed more intense antibiotic fluorescence than was observed after 1 h of drug exposure (Fig. 3A). Some diffuse fluorescence was observed, but the intracellular pattern of fluorescence suggested that the majority of the intracellular drug was confined and not free to diffuse throughout the cytoplasm. For example, ciprofloxacin fluorescence appeared to be excluded from the nucleus and was not distributed uniformly in the cytoplasm. In cells exposed to ciprofloxacin-loaded liposomes for 24 h, highly intense, perinuclear punctate accretions of fluorescence coexisted with less-intense, diffuse deposits (Fig. 4B), possibly signifying localization in secondary lysosomes or endocytic vesicles.

Antimycobacterial effects of free and liposome-encapsulated ciprofloxacin. To investigate the relationship between the observed cellular ciprofloxacin content and antimycobacterial activity, we compared the effects of free and liposome-encapsulated drugs on the survival and replication of *M. avium* within J774 cells. To ensure that the majority of bacteria were harbored within J774 cells at the time of treatment, rather than exposed at the cell surface to extracellular antibiotics, J774 cells were examined by electron microscopy 1 h after infection. Figure 5 shows abundant intracellular bacteria within membrane-delimited cellular vacuoles. After the extensive postinfection washing, few extracellular bacteria were observed, either free in the extracellular medium or bound to the plasma membrane.

Infected J774 cells were treated with antibiotics, either free or encapsulated in liposomes. As a control, cells were incubated with liposomes prepared as described above, except no drug was included in the loading step. With a 3-day exposure to free ciprofloxacin, the drug concentration reducing the number of cell-associated bacteria by 50% relative to values for untreated controls (IC_{50}) was 4.35 μM (Table 2). DSPC-Chol (molar ratio, 10:5) liposomes containing ciprofloxacin were as potent as free drug in inhibiting intracellular *M. avium* growth, and progressively greater potency was observed for liposomes of higher negative charge (Fig. 6). Control liposomes containing no ciprofloxacin did not inhibit *M. avium* growth (data not shown). Ciprofloxacin-containing liposomes of DSPG-Chol (molar ratio, 10:5) were 43-fold more potent than free drug in reducing intracellular *M. avium* growth, with an IC_{50} of 0.102 μM (Table 2).

Formulation of azithromycin in liposomes. Like ciprofloxacin, azithromycin shows activity against isolated *M. avium*. However, efficacy is reduced by poor aqueous solubility and a tendency to undergo aqueous hydrolysis (32). A lyophilized

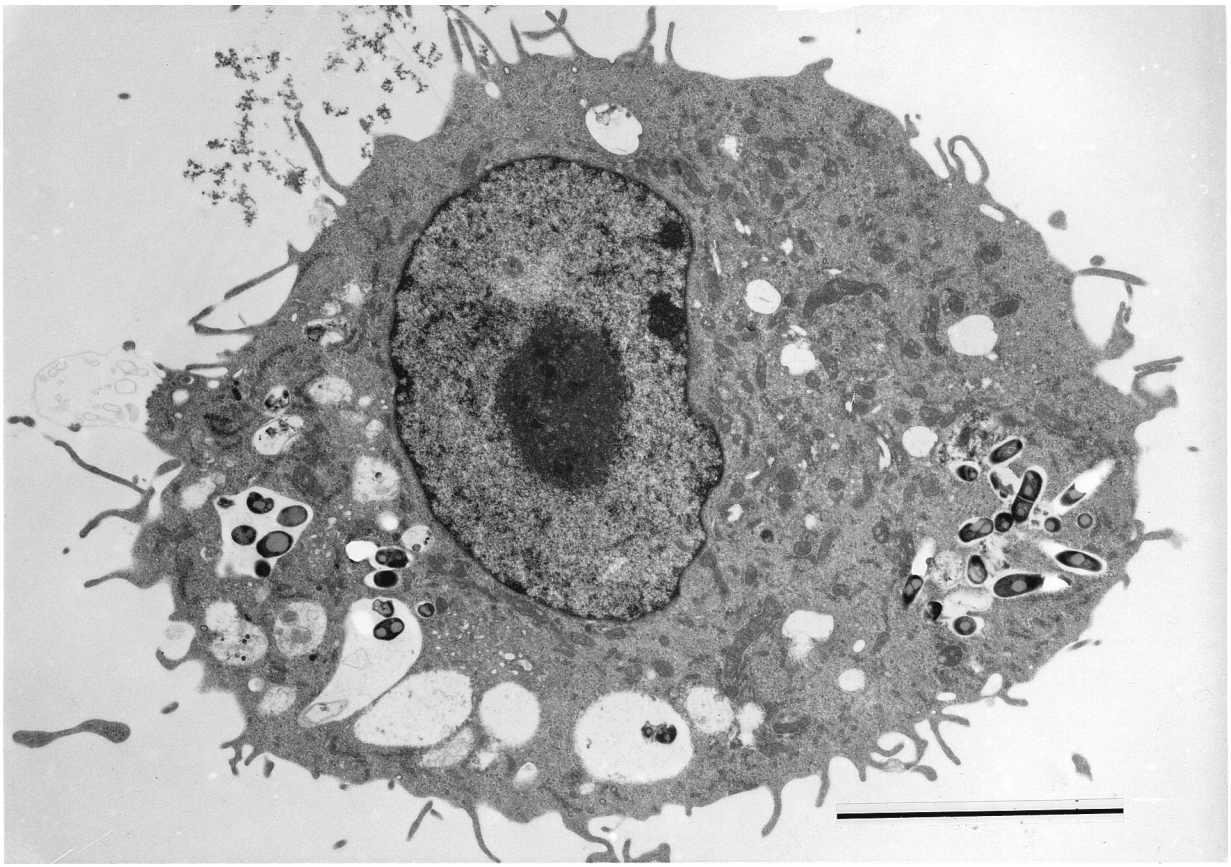

FIG. 5. Internalization of *M. avium* by J774 cells. J774 cells were propagated and infected with *M. avium* as described in Materials and Methods. The multiplicity of infection was 20 bacteria per cell. After 1 h, cells were washed extensively, fixed, embedded, stained, and sectioned for electron microscopy. *M. avium* cells are visible as highly electron-dense structures within electron-lucent cellular vesicles. Bar, 5 μm.

drug-phospholipid formulation was prepared to avoid hydrolysis during storage and to allow administration without a cosolvent-containing vehicle. Small liposomes were prepared by sonication after rehydration of the lyophilized drug-lipid powder in buffered saline. Sonicated liposomes composed of DSPG-Chol (2:1) and containing ≤33 mol% azithromycin (with respect to phospholipid) appeared as a fine suspension (data not shown), and aggregation or precipitation of drug was not observed with rehydrated samples after 1 month of storage at 4°C. Liposomes containing 43 mol% azithromycin (with respect to phospholipid) were unstable, and polygonal crystals of azithromycin were observed in the liposome suspension within 1 day of rehydration (data not shown).

TABLE 2. Antimycobacterial effects of ciprofloxacin and azithromycin

Liposome composition or drug status	Molar ratio	IC_{50} (μM) (± SE)	
		Ciprofloxacin	Azithromycin
DSPC-Chol	10:5	2.84 ± 0.19	32.00 ± 9.01
DSPG-DSPC-Chol	3:7:5	2.84 ± 0.19	11.00 ± 1.80
DSPG-DSPC-Chol	5:5:5	0.28 ± 0.06	3.80 ± 2.93
DSPG-DSPC-Chol	7:3:5	0.176 ± 0.02	ND[a]
DSPG-Chol	10:5	0.102 ± 0.02	0.44 ± 0.09
Free drug		4.35 ± 0.77	18.00 ± 3.60

[a] ND, not determined.

Antimycobacterial effects of free and liposome-encapsulated azithromycin. In order to test the relationship between antimycobacterial activity and liposome negative charge, the DSPG/DSPC ratio was varied incrementally from 0:10 to 10:0 and the effect on intracellular *M. avium* growth was compared with that of free azithromycin. The lipid component of azithromycin-loaded liposomes consisted of phospholipid-cholesterol in a molar ratio of 2:1, and azithromycin was incorporated at 33 mol% (with respect to the phospholipid content). Drug encapsulated in liposomes composed of DSPC-Chol (10:5) showed activity against *M. avium* that was comparable to that of free azithromycin (Table 2 and Fig. 6). Antimycobacterial activity increased as the mole fraction of negatively charged phospholipid increased (Fig. 6). The highest potency was obtained with liposomes composed of DSPG-Chol (10:5) (Table 2), which showed 41-fold greater potency than did free azithromycin.

DISCUSSION

The goal of eradicating infectious organisms that reside intracellularly presents a number of unusual challenges. Organisms such as *M. avium* are protected within host macrophages and are shielded both from immune surveillance and from antibiotics present in the extracellular medium. A wide range of antibiotics have been found to be active against isolated *M. avium* but have been disappointing in their activity against intracellular organisms, leading to failure of clinical trials with these otherwise-promising compounds. By several mecha-

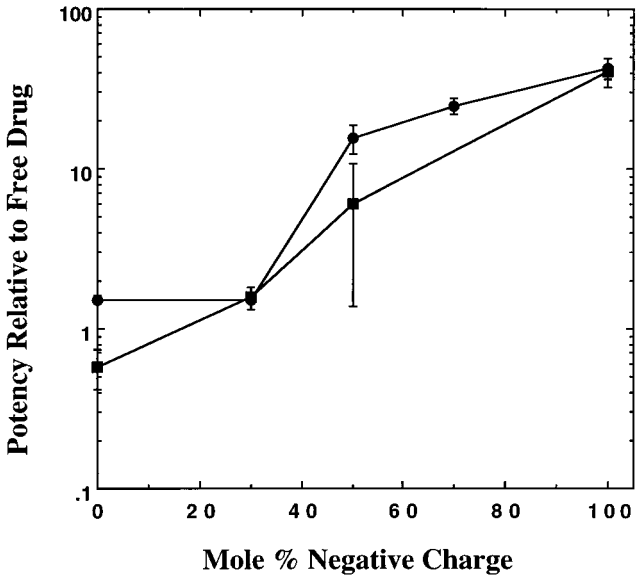

FIG. 6. Antimycobacterial activities of free and encapsulated ciprofloxacin and azithromycin. Ciprofloxacin was encapsulated in extruded liposomes composed of DSPG-DSPC-Chol (molar ratio, *x*:*y*:5) by a remote-loading procedure as described in the text. Azithromycin was incorporated into liposomes of similar composition by lyophilization-rehydration. The ratio of DSPG/DSPC (*x*:*y*) for each preparation was varied from 0 to 100%, as indicated on the abscissa. J774 cells were infected with *M. avium*, washed extensively to remove extracellular bacteria, and then treated for 72 h with free or liposome-encapsulated drug. Cells were then lysed with detergent, and viable *M. avium* cells were enumerated. Potency was calculated by comparing the IC_{50}s; the data are expressed as

$$\text{relative potency} = \frac{IC_{50}\,(\text{liposomes})}{IC_{50}\,(\text{free drug})}.$$

Circles, ciprofloxacin-containing liposomes; squares, azithromycin-containing liposomes. Each datum point represents the mean ± standard error (error bars) (n = 3 replications).

nisms, drug carriers may provide the means to overcome specific limitations of existing drugs and thereby may contribute to the effectiveness of therapy. In the present work, we have focused on two promising agents for *M. avium* therapy and have devised liposome-based formulations to address specific pharmaceutical and cell-biological limitations of these drugs.

Ciprofloxacin has been investigated for therapy of *M. avium* infection, and liposome-based formulations have been described previously (26). In this study, we have developed approaches to overcome two limitations of existing liposomal formulations of ciprofloxacin. First, ciprofloxacin is a water-soluble drug that is captured at low efficiency by conventional passive encapsulation techniques. Not only does the low efficiency of capture pose a potential manufacturing problem, but it also results in a low drug/phospholipid ratio and thereby increases the amount of liposomal lipid that must be administered. Second, little information on how liposome formulation parameters may alter the antimycobacterial effect of ciprofloxacin-loaded liposomes was available.

We have developed a remote-loading technique that can encapsulate ciprofloxacin in liposomes with >90% efficiency. Remote loading provides a means by which to accumulate weakly basic drugs and peptides into preformed liposomes with a transmembrane chemical or potential gradient (16, 25). The principle of these techniques is that the protonated form of weakly basic or amphoteric compounds may permeate into the internal aqueous phase of liposomes, driven by the transmembrane gradients, and subsequently may be trapped by complexing with highly concentrated ions inside the liposomes.

Ciprofloxacin is an amphoteric quinolone antibiotic having an acidic pK_a at 6.0 and a basic pK_a at 8.8 (3). When an ammonium sulfate (potential) gradient alone was employed to drive ciprofloxacin into liposomes, we found an encapsulation efficiency of 51%, considerably lower than that observed with doxorubicin, which was captured at nearly 100% efficiency under similar conditions (27). Such a difference may be explained by the observation that the application of a single driving force (such as a transmembrane pH gradient alone) can result in variable trapping efficiencies, depending on characteristics such as the pK_a, lipophilicity, and mass of the molecule being encapsulated (25). To improve the efficiency of loading ciprofloxacin into liposomes, we increased the gradient-derived driving force by combining a transmembrane potential gradient with a pH gradient. Although we did not ascertain whether the pH or potential gradients individually had any other driving forces as components, we observed that the application of combined gradient systems provided greater and more consistent encapsulation of ciprofloxacin. Under optimal conditions, the efficiency of ciprofloxacin entrapment increased to 94%.

The remote-loading technique described for ciprofloxacin could be advantageous from several perspectives. First, the technique increases the drug/phospholipid ratio, thereby allowing liposomes to carry and deliver larger amounts of antibiotics to infected cells. Second, it is more efficient than passive encapsulation methods and minimizes the potential loss of expensive drugs during the formulation process. Third, by loading ciprofloxacin into preformed liposomes at the time of use, the remote-loading technique could reduce concerns about chemical or physical stability during prolonged storage (27).

Azithromycin, a promising azalide antibiotic (32) also active against isolated mycobacteria, has limitations different from those of ciprofloxacin. Azithromycin is poorly soluble in water and thus requires the use of a cosolvent for subcutaneous administration (4). One cosolvent investigated is polyoxyethylated castor oil (Cremophor EL). Although Cremophor EL has been a frequently used solubilizer and emulsifier in the pharmaceutical industry, there are reports describing hepatotoxic effects (33), clastogenic activity (1), and well-known histamine-releasing effects (11, 24) that underlie life-threatening anaphylactoid reactions observed clinically (21). Furthermore, azithromycin undergoes hydrolysis in water, which limits the storage period to less than a few days. Finally, azithromycin shows extensive distribution throughout the body and has a large volume of distribution, 27 liters/kg (32). Encapsulation of the drug in liposomes may offer the potential to confine the drug to the circulatory system and target the reticuloendothelial system, the reservoir of *M. avium* in the body. We have developed a lyophilized drug-lipid mixture that forms liposomes spontaneously upon hydration, thereby overcoming the low solubility and instability of azithromycin in water while retaining or enhancing antimycobacterial activity. The storage properties of a lyophilized drug-lipid formulation are expected to be good.

Although ciprofloxacin and azithromycin are somewhat disparate chemically, the formulations developed showed several similarities in terms of their antimycobacterial effects. For both antibiotics, encapsulation within liposomes of the appropriate composition resulted in increased potency against intracellular *M. avium*. For liposomes having little or no electrostatic charge, anti-*M. avium* activity was approximately equal to that of free drug. In contrast, both drugs showed 40-fold greater

activity than free drug when encapsulated in liposomes having a high fraction of negative charge. For one of the two drugs (ciprofloxacin), we determined that changes in formulation that enhanced antibiotic effect also increased the deposition of drug within cells. We were unable to perform similar experiments with azithromycin because of the lack of a sufficiently sensitive and accurate assay for intracellular distribution.

We observed that negatively charged liposomes containing ciprofloxacin are superior to neutral liposomes with respect to both cellular uptake and antimycobacterial potency of the antibiotics. The results are consistent with the previous findings that the uptake rate of negatively charged liposomes by cells (17, 18), including J774 cells (23), is severalfold higher than that of neutral liposomes and that negatively charged liposomes had stronger affinity for unknown liposome binding sites on the cells than did neutral liposomes (23). Negatively charged liposomes containing the liposome-dependent anticancer drug phosphonacetyl-L-aspartate (PALA) exerted greater in vitro cytostatic activity than did neutral liposomes, and the potency of PALA was augmented as the negative-charge fraction of liposomes increased (17, 34). Interestingly, the magnitude of charge-mediated enhancement of PALA cytostatic activity (34) was greater than the enhancement observed in this study for the activity of encapsulated antibiotics; the potency of PALA increased approximately 1,500-fold as the DSPG/DSPC ratio was increased above a molar ratio of 5:5 (34), while the antimycobacterial effect increased approximately 40-fold over a comparable range of liposome negative charge. An explanation of the difference between liposome-enhanced delivery of an anticancer drug and delivery of an antibiotic may rest in part with different requirements for drug disposition once liposomes have entered the target cell.

Although a correlation between cell-associated drug concentration and antimycobacterial activity might appear to be anticipated from tangentially related reports (17, 34), the previous work focused on the activity of cytostatic anticancer agents, for which the objective was delivery to the cytoplasm from liposomes contained within endocytic vesicles. In contrast, the effective delivery of antibiotics to an intracellular organism that resides within a membrane-delimited vacuole may depend on formulation criteria other than high cell-associated drug levels, because drug may be ineffective if released into the cytoplasm, sequestered in intracellular or surface-bound liposomes, or accumulated within endocytic vesicles other than those in which the bacteria reside. The results presented here show enhanced cellular delivery of liposome-encapsulated antibiotics and suggest that the efficiency of intracellular targeting is sufficient to mediate enhanced antimycobacterial effects.

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