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Aerosol Delivery of Liposome-Encapsulated Ciprofloxacin: Aerosol Characterization and Efficacy against *Francisella tularensis* Infection in Mice

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The aerosol delivery of liposome-encapsulated ciprofloxacin by using 12 commercially available jet nebulizers was evaluated in this study. Aerosol particles containing liposome-encapsulated ciprofloxacin generated by the nebulizers were analyzed with a laser aerodynamic particle sizer. Mean mass aerodynamic diameters (MMADs) and geometric standard deviations (GSDs) were determined, and the drug contents of the sampling filters from each run onto which aerosolized liposome-encapsulated ciprofloxacin had been deposited were analyzed spectrophotometrically. The aerosol particles of liposome-encapsulated ciprofloxacin generated by these nebulizers ranged from 1.94 to 3.5 μm, with GSDs ranging from 1.51 to 1.84 μm. The drug contents of the sampling filters exposed for 1 min to aerosolized liposome-encapsulated ciprofloxacin range from 12.7 to 40.5 μg/ml (0.06 to 0.2 mg/filter). By using the nebulizer selected on the basis of most desirable MMADs, particle counts, and drug deposition, aerosolized liposome-encapsulated ciprofloxacin was used for the treatment of mice infected with 10 times the 50% lethal dose of *Francisella tularensis*. All mice treated with aerosolized liposome-encapsulated ciprofloxacin survived the infection, while all ciprofloxacin-treated or untreated control mice succumbed to the infection ($P < 0.001$). These results suggest that aerosol delivery of liposome-encapsulated ciprofloxacin to the lower respiratory tract is feasible and that it may provide an effective therapy for the treatment of respiratory tract infections.

Ciprofloxacin is a potent and broad-spectrum fluoroquinolone that is effective against a number of microorganisms, particularly gram-negative bacteria. Ciprofloxacin has also been shown to demonstrate good in vitro bactericidal activities against a number of pathogens that cause respiratory infections, including *Mycobacterium tuberculosis* (10, 14), *Mycobacterium avium-M. intracellulare* (8), *Haemophilus influenzae* (22), *Pseudomonas aeruginosa* (24), and *Neisseria meningitidis* (3). Oral and intravenous forms of ciprofloxacin have been used clinically to treat respiratory tract infections. However, ciprofloxacin administered intravenously or orally has a relatively unfavorable pharmacokinetics profile in the lower respiratory tract, including a relatively short elimination half-life of 1.0 to 1.6 h and a low area under the concentration-time curve of 43 to 113 mg · h/liter (2).

We have recently described the development of a liposomal formulation for the encapsulation of ciprofloxacin (6, 30). When liposome-encapsulated ciprofloxacin was administered to mice via the intranasal route, it was found that the retention of the drug in the lungs was enhanced significantly from a half-life of 1 to 2 to one of 8 to 10 h (30). In addition, the efficacy of ciprofloxacin for the treatment of pulmonary infections caused by *Francisella tularensis* was enhanced severalfold by liposome encapsulation. *F. tularensis* is a facultative intracellular bacterium which can cause a potentially fatal human disease called tularemia. *F. tularensis* infection involves the reticuloendothelial system and leads to bacterial growth within tissues in the lungs, liver, and spleen. In order to develop a practical approach to delivering the liposome-encapsulated ciprofloxacin to the sites of infection in the lungs, the feasibility of developing an aerosol inhalation route for delivering liposome-encapsulated ciprofloxacin directly to the lungs was evaluated in this study. Toward this end, the aerosol characteristics of liposome-encapsulated ciprofloxacin generated from 12 different commercially available jet nebulizers were evaluated for pulmonary delivery. Of these, the nebulizer which generated the aerosols with the most desirable particle characteristics was selected and was subsequently evaluated in a study of the experimental treatment of respiratory *F. tularensis* infection in mice. The inhalation of aerosols directly into the lungs, the sustained release of drug from the liposomes, and the intracellular delivery of the drug by liposomes could significantly enhance the therapeutic efficacies of ciprofloxacin for the treatment of a number of respiratory infections.

MATERIALS AND METHODS

Chemicals. The phosphatidylcholine and cholesterol used for the preparation of liposomes were purchased from Avanti Polar Lipids (Alabaster, Ala.). Ciprofloxacin (Bayer Corp. of Canada, Etobicoke, Ontario, Canada) was purchased through a local pharmacy.

Aerosol nebulizers. The 12 commercially available jet nebulizers used in this study were purchased from the suppliers, as follows: A1800 (ARS Vital Aire, Edmonton, Alberta, Canada), DVB7427 and DVB5601 (Devilbiss, Sommerset, Pa.), Microcirrus (DHD Medical Products, Canastota, N.Y.), Hosp3753 and Hosp952 (Hospitak, Lindenhurst, N.J.), HudTU, HudUD2, and HudMM (Hudson RCI, Temecula, Calif.), Int1112220E (Intertech, Bannockburn, Ill.), Marq (Marquest Medical Products Inc., Englewood, Colo.), and PurRD Raindrop (Puritan-Bennett, Lenexa, Kans.).

Animals. Six-week-old BALB/c female mice were obtained from the mouse breeding colony at the Defence Research Establishment Suffield (DRES), with breeding pairs purchased from Charles River Canada Ltd. (St. Constant, Quebec, Canada). The use of animals described in this study was approved by the DRES Animal Care Committee. The care and handling of animals described in this study followed guidelines set out by the Canadian Council on Animal Care.

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Bacteria. *F. tularensis* live vaccine strain ATCC 296684 (American Type Culture Collection, Rockville, Md.) was cultured on cysteine heart agar plates supplemented with 5% defibrinated rabbit blood (Remel Labs, Lenexa, Kans.) for 4 days in 5% CO_2 as described previously (9). Colonies were then selected for growth in modified Mueller-Hinton broth (Difco Laboratories) supplemented with ferric PP_i and IsoVitaleX (Becton Dickinson, Cockeysville, Md.). The broth cultures were incubated at 37°C for 4 to 5 days. The cultures were then aliquoted and frozen in 10% dimethyl sulfoxide (Sigma Chemical Company, St. Louis, Mo.). For determination of the 50% lethal dose, aliquots were thawed and diluted serially in sterile phosphate-buffered saline just prior to administration into animals.

Preparation of liposome-encapsulated ciprofloxacin. The liposomes used for the encapsulation of ciprofloxacin were prepared by the remote-loading procedure by using an ammonium sulfate gradient as described previously (21). The liposomes were made from egg phosphatidylcholine and cholesterol in a molar ratio of 1:1. This procedure was found to yield consistent and reproducible liposome-encapsulated ciprofloxacin preparations with a high drug entrapment rate of 90% ± 3.5%.

Generation and characterization of liposome-containing aerosols. Liposome-encapsulated ciprofloxacin (ciprofloxacin concentration, 30 mg/ml) at a volume of 3 ml was added to each jet nebulizer reservoir. Aerosols were generated by the nebulizers by using dry compressed air at 40 lb/in.^{2} with a flow rate of 4 or 6 liters/min. Aerosol particles were analyzed by using a laser aerodynamic particle sizer (APS) (model 3310; TSI Inc., St. Paul, Minn.) by using APS Advanced Software, version 2.9, purchased from TSI Inc. Aerosol particle analysis was initiated after 2 min of equilibration and was carried out continuously every 30 s until the end of each run. The aerosol particles generated by each nebulizer were characterized for their mass mean aerodynamic diameters (MMADs), geometric standard deviations (GSDs), and peak particle counts (PPCs). In addition, two aerosol samples were collected over 1 min each on glass sampling filters at 5 and 10 min into each run, and these were analyzed spectrophotometrically for drug contents as described below.

Determination of drug contents. The drug contents of the sampling filters onto which aerosolized liposome-encapsulated ciprofloxacin had been deposited were determined by using a spectrophotometer (UV-160U; Shimadzu Corp., Tokyo, Japan). The glass filters were quartered aseptically, placed in 5 ml of absolute ethanol (to disrupt the liposomes), and centrifuged at 1,200 × *g* for 20 min to remove glass fibers. The ciprofloxacin concentrations in the supernatant were determined at 276 nm, and values were extrapolated from a standard curve by using known ciprofloxacin standards.

Treatment study against respiratory tularemia in mice. For the treatment studies determining the efficacy of aerosolized liposome-encapsulated ciprofloxacin against respiratory tularemia, groups of mice were anesthetized with sodium pentobarbital (50 mg/kg of body weight given by the intraperitoneal route). When the animals were unconscious, they were intranasally infected with 10 times the 50% lethal dose of *F. tularensis*. The dose (50 μl/mouse) was gently applied into the nostrils with a micropipette. At 24 h postinfection, the animals were placed in a 24-port nose-only aerosol exposure chamber (In-Tox Products, Albuquerque, N.Mex.), where the animals were exposed for 20 min to a single dose of aerosolized liposome-encapsulated ciprofloxacin or free unencapsulated ciprofloxacin. These aerosols were generated with the PurRD Raindrop nebulizer and were characterized by using the APS described earlier. The infected animals were monitored daily for signs of symptoms and for deaths from the infection. At day 14 after infection, the number of mice which survived the lethal bacterial infection was recorded.

Bacterial determination of organ homogenates. To determine the bacterial load in the organs of control and treated mice, the lungs, spleens, and livers were aseptically harvested. The organs were then homogenized in 5 ml of sterile phosphate-buffered saline with a hand-held tissue grinder. The supernatants were then plated for growth in cysteine heart agar plates supplemented with 5% defibrinated rabbit blood. The inoculated plates were incubated at 37°C for 4 days, and the numbers of CFU of *F. tularensis* were determined.

Statistical analysis. The survival rates of the treated and nontreated control groups were compared by the Mann-Whitney unpaired nonparametric one-tailed test (In Stat, version 1.14; Graph-Pad Software, San Diego, Calif.). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Size characterizations and measurements of aerosolized liposome-encapsulated ciprofloxacin. The aerosol particle characteristics of each of the 12 nebulizers containing liposome-encapsulated ciprofloxacin are described in Tables 1 and 2. The MMADs, GSDs, and PPCs of the aerosol particles are characterized for each nebulizer. The MMAD is the aerodynamic diameter above which 50% of the total particle mass resides. The MMADs of aerosol particles generated by the 12 nebulizers containing liposome-encapsulated ciprofloxacin ranged from 1.94 to 3.84 μm (Tables 1 and 2). The MMADs of these aerosols generated by each nebulizer increased when the airflow was increased from 4 to 6 liters/min (Tables 1 and 2). The GSDs of the aerosol particles generated ranged from 1.47 to 1.70 μm and were independent of the airflow over a range of 4 to 6 liters/min.

TABLE 1. Aerosol particle characteristics of various clinical nebulizers containing liposome-encapsulated ciprofloxacin[a]

Nebulizer	MMAD (μm)	GSD (μm)	PPC (10^6)	Drug content (μg/ml)
DVB7427	1.94	1.66	1.20	5.5
A1800	2.62	1.58	2.62	13.1
HudTU	2.71	1.47	2.44	10.0
Marq	3.10	1.70	2.94	21.7
DVB5601	3.25	1.60	3.76	12.3
Hosp952	3.26	1.61	3.93	2.3
Hosp3753	3.31	1.61	3.50	25.5
HudUD2	3.31	1.57	4.46	26.1
PurRD	3.36	1.58	4.16	29.8
Micro	3.38	1.62	3.90	ND[b]
Int	3.46	1.63	4.08	10.9
HudMM	ND	ND	4.3	ND

[a] The flow rate was 4 liters/min.
[b] ND, not determined.

The PPCs of the aerosol particles were determined by the APS at approximately 4 min into each run. The PPCs generated by the different nebulizers varied from 1.2×10^6 (DVB7427) to 4.46×10^6 (HudUD2) particles. Increasing the airflow from 4 to 6 liters/min resulted in increases in PPCs for 9 of the 12 nebulizers.

Drug deposition on sampling filters. The sampling filters onto which aerosol particles containing liposome-encapsulated ciprofloxacin were deposited were analyzed for their ciprofloxacin levels at the end of each run (Tables 1 and 2). The highest drug contents deposited on the sampling filters were obtained for aerosol particles generated with HudMM and PurRD Raindrop (40.5 and 39.0 μg/ml, respectively; 0.203 and 0.195 mg/filter, respectively). These two nebulizers produced aerosol particles with MMADs of 3.5 and 3.45 μm, respectively, and PPCs of 4.13×10^6 and 4.36×10^6, respectively. The two nebulizers (Marq and HudTU) which yielded the lowest level of drug deposition (12.7 and 24.5 μg/ml, respectively) generated particles with MMADs of less than 3.3 μm and PPCs of less than 3.5×10^6.

TABLE 2. Aerosol particle characteristics of various clinical nebulizers containing liposome-encapsulated ciprofloxacin[a]

Nebulizer	MMAD (μm)	GSD (μm)	PPC (10^6)	Drug content (μg/ml)
HudTu	3.16	1.65	3.37	24.5
DVB7427	3.21	1.63	3.41	34.3
Marq	3.23	1.84	3.42	12.7
PurRD	3.45	1.51	4.36	39.0
A1800	3.47	1.58	4.27	27.5
Int	3.48	1.62	4.25	33.5
Hosp3753	3.49	1.65	4.09	27.0
HudMM	3.50	1.53	4.13	40.5
Hosp952	3.52	1.59	4.21	34.5
DVB5601	3.52	1.58	4.22	27.5
Micro	3.74	1.71	3.50	ND[b]
HudUD2	3.84	1.57	4.12	30.0

[a] The flow rate was 6 liters/min.
[b] ND, not determined.

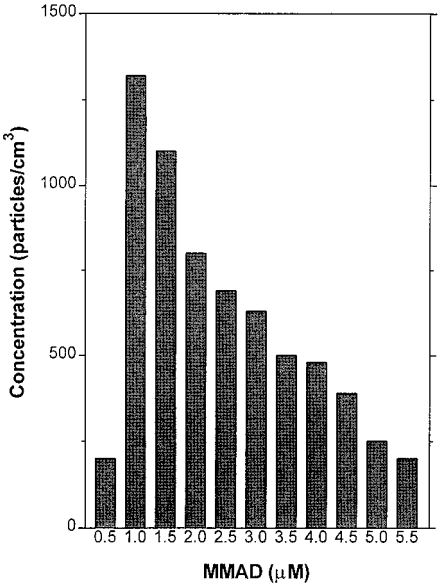

FIG. 1. Particle size distribution of aerosols generated by the PurRD nebulizer. Aerosols containing liposome-encapsulated ciprofloxacin were generated by using PurRD nebulizer at an airflow rate of 6 liters/min, and the particles were analyzed with a laser APS by using the APS advance software program.

Selection of nebulizers for in vivo efficacy study against tularemia infection in mice. Successful therapy of respiratory infection by using aerosol inhalation of liposome-encapsulated ciprofloxacin requires the selection of a nebulizer(s) which produces the best respirable aerosol characteristics and the highest level of drug deposition. On the basis of the criteria presented above, the nebulizers HudMM and PurRD Raindrop were considered to have met these requirements. These two nebulizers generated aerosol particles in the respirable size range, with MMADs of 3.4 to 3.5 μm, respectively, and the same GSD of 1.5 μm, and they yielded the highest levels of drug deposition. In addition, PurRD Raindrop was also found to generate higher PPCs than HudMM, and hence, it was subsequently selected as the nebulizer of choice for the aerosolization of liposome-encapsulated ciprofloxacin in the evaluation of efficacy against *F. tularensis* infection. A typical aerosol particle size distribution of liposome-encapsulated ciprofloxacin is presented in Fig. 1.

Treatment of mice against respiratory tularemia. The antibacterial efficacies of aerosolized, free, unencapsulated ciprofloxacin and liposome-encapsulated ciprofloxacin for the treatment of mice against a respiratory infection with *F. tularensis* were evaluated. Groups of mice were intranasally infected with 10 times the 50% lethal dose of *F. tularensis* and were then treated with aerosolized, liposome-encapsulated ciprofloxacin. The survival rates for these groups of mice at day 14 postinfection were compared (Fig. 2). Untreated control mice began to succumb to the infection as early as day 5 postinfection, and by day 9, all mice in the group were dead. Little or no protection was observed in mice treated with aerosolized, free, unencapsulated ciprofloxacin. All the mice in ciprofloxacin-treated group died by day 9 postinfection. Among the mice exposed to aerosolized liposome-encapsulated ciprofloxacin for 20 min, all the mice survived ($P < 0.01$ versus the control, ciprofloxacin-treated group). These results suggest that liposome-encapsulated ciprofloxacin delivered by aerosol inhalation is highly effective in the treatment of respiratory *F. tularensis* infection in mice.

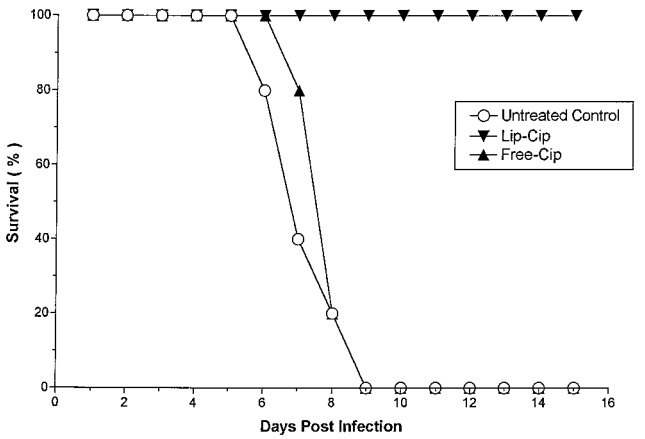

FIG. 2. Survival curves of *F. tularensis*-infected mice treated with aerosolized ciprofloxacin or liposome-encapsulated ciprofloxacin. Mice were intranasally infected with 10 times the 50% lethal dose of *F. tularensis*. At 24 h postinfection, the mice were treated with aerosolized ciprofloxacin (Free-Cip) or liposome-encapsulated ciprofloxacin (Lip-Cip).

Bacterial load of organs from infected and treated mice. The spleens, livers, and lungs from the untreated and treated mice were isolated at days 7 and 14 postinfection, respectively. These organs were homogenized and assayed for the presence of *F. tularensis* growth in cysteine heart agar plates (Table 3). All tissue homogenates from the mice treated with aerosolized liposome-encapsulated ciprofloxacin, including those of lungs, spleens, and livers, were found to be devoid of bacteria. In contrast, homogenates of all three organs from untreated control mice contained very high numbers of *F. tularensis* organisms. These results suggest the potency of aerosolized, liposome-encapsulated ciprofloxacin in the eradication of *F. tularensis* from these tissues which are normally primary infection sites.

DISCUSSION

Since the respiratory tract is the most common route of entry and the primary site of infection for a number of airborne pathogens, including *M. tuberculosis*, *M. avium*, *P. aeruginosa*, *H. influenzae*, and *F. tularensis*, aerosol delivery of antibiotics to the lower respiratory tract is becoming an increasingly important and rational approach for the treatment of various pulmonary infections. This approach may potentiate high sustained therapeutic levels of the drugs in the lungs without causing a systemic burden in the unaffected organs. However,

TABLE 3. CFU determinations of *F. tularensis* from organs of control mice and mice treated with aerosolized liposome-encapsulated ciprofloxacin

Group	Organ	CFU
Untreated control[a]	Lung	4×10^7
	Spleen	4×10^6
	Liver	3×10^7
Treated[b]	Lung	0
	Spleen	0
	Liver	0

[a] The numbers of CFU were determined at approximately 7 days postinfection, before mice were moribund from infection.
[b] The numbers of CFU were determined at day 14 postinfection.

when ciprofloxacin is directly delivered by intranasal administration to the lungs of mice, it is rapidly absorbed and cleared from the lungs (30). The use of a liposomal drug delivery system to cause a gradual, sustained release of drug may significantly enhance the pulmonary retention of ciprofloxacin and may therefore improve its therapeutic efficacy. Liposomes have been shown to be effective for the pulmonary delivery of various therapeutic agents (5, 25, 29).

The respiratory infection-causing bacteria *M. tuberculosis*, *F. tularensis*, *M. avium-M. intracellulare*, *Pseudomonas pseudomallei*, and others are intracellular pathogens which reside and/or multiply within phagocytic cells including pulmonary macrophages. Liposomes as drug carriers are effective in the intracellular delivery of antibiotics to these infected cells, particularly to sites inside the phagolysosomes, where these microorganisms are known to reside. The use of liposome-encapsulated antibiotics for the treatment of infectious diseases caused by intracellular parasites are well established (17, 18, 30). Aerosol delivery of liposome-encapsulated ciprofloxacin to the lower respiratory route, the primary site of infection for respiratory infections, may therefore provide an effective combination for the treatment of these infections, especially but not exclusively those caused by intracellular pathogens. The use of aerosolized liposomes has been reported for a number of antimicrobial and other therapeutic agents including enviroximine (11), amphotericin B (12), amikacin (28), and beclamethasone dipropionate (27).

To generate aerosol particles with proven respirable particle characteristics, commercially available jet nebulizers were evaluated in this study. They have been used to deliver clinical aerosols containing drugs such as ribavarin (16), rimantadine (1), pentamidine (19, 20), bronchodilators (4), a mucolytic agent (23), and carbenicillin and gentamicin (15). In the present study, the MMADs of the aerosol particles generated by the nebulizers containing liposome-encapsulated ciprofloxacin at an airflow rate of 6 liters/min ranged from 3.1 to 3.8 μm. This is consistent with MMADs of 3.2 to 3.7 μm for the aerosols containing liposomes generated by air-jet nebulizers reported by others (7, 26) and appears to be independent of the drug entrapped. It is generally accepted that a particle size range of 1 to 3 μm in MMAD with a GSD of 2.0 μm is ideal for pulmonary delivery (27). It is suggested that aerosol particles in this size range are optimal because they are predicted by a computer model of the lung to deliver the largest doses to peripheral lung sites (27). However, the liposome-containing aerosol particles generated by these nebulizers differed in their PPCs as well as in the amounts of aerosolized liposome-encapsulated ciprofloxacin deposited on the sampling filters. On the basis of the data presented in Tables 1 and 2, it would appear that the amounts of aerosolized liposomal drug deposited by each nebulizer correlated more to the PPCs generated than to the MMADs.

The aerosol delivery of liposome-encapsulated ciprofloxacin described in this study provided very effective postexposure treatment against *F. tularensis* infection, while aerosol delivery of unencapsulated ciprofloxacin at equivalent doses provided little or no protection. The enhanced efficacy achieved by aerosol delivery of liposome-encapsulated ciprofloxacin may be due in part to the intracellular delivery of ciprofloxacin provided by liposomes and enhanced pulmonary targeting and retention of the drug to the lower respiratory tract. Since conventional liposomes are naturally taken up by pulmonary alveolar macrophages (13), intracellular delivery of ciprofloxacin may therefore partly account for the effective eradication of the intracellular bacteria from the lungs. In addition, preliminary pharmacokinetic studies of mice with aerosolized liposomal and free ^{14}C-ciprofloxacin indicate that pulmonary retention of ciprofloxacin in mice was significantly enhanced in the liposomal ciprofloxacin group compared to that in the free drug group. In addition, therapeutic levels of ciprofloxacin were also found in the liver and spleen following aerosol administration to the lungs. These observations are consistent with the data obtained with intranasal administration of the liposomal drugs in our previous studies (29, 30). On the basis of these results, it is reasonable to conclude that sustained therapeutic doses of ciprofloxacin in the lower respiratory tract can be achieved with aerosol delivery of the liposome-encapsulated ciprofloxacin. Recently, two major toxicology studies looking at the effect of chronic exposure (daily exposures for 6 weeks) of mice to aerosolized liposome-encapsulated ciprofloxacin, sham liposomes, and free ciprofloxacin were completed. The results from histopathology evaluation did not show any significant abnormal pathological features in the lungs of these mice (unpublished data). Since ciprofloxacin is a potent and broad-spectrum antibiotic that is effective against a number of causative agents of pulmonary infections, aerosol delivery of liposome-encapsulated ciprofloxacin may provide a valuable and safe approach to the prevention and treatment of these infectious diseases.

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