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Therapeutic Efficacy of Liposome-Entrapped Rifampin against *Mycobacterium avium* Complex Infection Induced in Mice

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Liposome-entrapped rifampin (RFP) was examined for therapeutic efficacy against experimental infection induced in mice by the *Mycobacterium avium* complex. Intraperitoneal injections (once daily, six times weekly) of liposome-entrapped RFP led to a greater reduction in bacterial growth in the lungs and spleen of infected mice than did free RFP alone. Liposome-entrapped RFP given to mice via the intramuscular or subcutaneous route failed to show such an increased therapeutic efficacy. RFP entrapped in the lipid layer of liposomal vesicles exhibited a level of therapeutic activity similar to that seen with RFP encapsulated in the inner solute of the vesicles. Entrapment of RFP in liposomal vesicles increased incorporation of the drug into host peritoneal macrophages and increased the activity of the agent against *M. avium* complex phagocytosed into the macrophages.

Mycobacterium avium complex infections are frequently present in immunocompromised hosts (20, 22), particularly patients with acquired immunodeficiency syndrome (20). Clinical management of *M. avium* complex infections presents serious problems, since most drugs are ineffective against them. There seems to be an intrinsic resistance of *M. avium* complex to most antimicrobial agents, presumably because of impermeability of the bacteria to these agents (4, 17, 21). The *M. avium* complex, one of the facultative intracellular parasites, can multiply primarily in phagocytes, leading to a reduction in the efficacy of antimicrobial agents that have poor ability to penetrate into phagocytic cells. This may account for the fact that the efficacy of rifampin (RFP) and its derivatives against *M. avium* complex infections is lower than would be expected on the basis of the in vitro antimicrobial activity of these drugs (6, 18).

Multilamellar liposomes and vesicles are widely used as carriers of antineoplastic and antimicrobial drugs (11). Since liposomes are preferentially taken up by reticuloendothelial cells (3, 9, 10), liposomal encapsulation of a given agent should result in greater availability of the agent to parasites present in phagocytic cells. The entrapment of certain antibiotics in liposomes increases their activity against bacteria phagocytosed into macrophages in in vitro culture systems (3, 5, 7). Moreover, liposome incorporation of ampicillin (2), cephalothin (8), streptomycin (19), 8-aminoquinoline (1), amphotericin B (12, 13), and primaquine (15) markedly increases the efficacies of these drugs against experimental infections induced by *Listeria monocytogenes*, *Salmonella typhimurium*, *Salmonella enteritidis*, *Leishmania* spp., fungi such as *Candida* spp., and plasmodia, respectively. In this study, we investigated the effect of liposome-entrapped RFP (Lip-RFP) against *M. avium* complex infection induced in mice.

MATERIALS AND METHODS

Animals. Five-week-old female ddY mice were purchased from the Shizuoka Central Animal Laboratory, Shizuoka, Japan. In some experiments, female CBA/JN mice (10 to 15 weeks old) obtained from Charles River Co., Kanagawa, Japan, were used.

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Organisms. *M. avium* complex 31F093, obtained from F. Kuze, Kyoto University, Kyoto, Japan, was cultivated in Dubos Tween-albumin medium (Eiken Chemical Co., Tokyo, Japan) at 37°C for 5 to 7 days. This strain is intermediately resistant to RFP (MIC, 6.25 μg/ml on 7H10 agar medium).

Preparation of liposomes. Entrapment of test agents into multilamellar vesicles consisting of lecithin, dicetyl phosphate, and cholesterol was done by two methods. First, 1 ml of chloroform containing 28 μmol of egg yolk lecithin (Kewpie Co., Tokyo, Japan), 8 μmol of dicetyl phosphate (Nakarai Chemical Co., Kyoto, Japan), 4 μmol of cholesterol (Wako Pure Chemical Industries, Osaka, Japan), and 2 mg (optimal dose, 5 mg) of RFP (Daiichi Pharmaceutical Co., Tokyo, Japan) was evaporated in a test tube (15 by 125 mm), and the resultant lipid layer was dispersed into 1 ml of phosphate-buffered saline (PBS) by sonication with an Ultrasonic Cleaner (model B220H; Branson Cleaning Equipment Co., Shelton, Conn.) at 37°C for 10 min. The liposome-entrapped drug thus obtained was designated Lip-RFP (lipid layer). In this case, 14 to 20% of the drug was entrapped, and 0.28 to 0.4 mg of RFP was encapsulated in liposome vesicles in 1 ml of the liposomal preparation. Second, the RFP-free lipid layer was prepared by the method just described and dispersed into PBS containing 2 mg of RFP per ml (optimal concentration) by sonication. The liposome-encapsulated RFP obtained by this method was designated Lip-RFP (inner solute). In this case, 11 to 24% of the drug was entrapped, and 0.22 to 0.48 mg of RFP was encapsulated in liposome vesicles in 1 ml of the liposomal preparation. The size distributions of liposome vesicles in the two preparations were nearly identical, showing peaks at 3.3 and 3.0 μm in diameter of liposomal vesicles in Lip-RFP (lipid layer) and Lip-RFP (inner solute) preparations, respectively, when measured by Coulter Counter (Coulter Electronics, Inc., Hialeah, Fla.) analysis.

Experimental infection and chemotherapy. A cell suspension (0.2 ml) of the organisms (2×10^6 to 6×10^6 cells) grown in Dubos Tween-albumin medium was given intravenously to mice. Free RFP (0.4 mg), liposome-entrapped RFP (0.4 mg of RFP in 6 mg of lipid), or drug-free liposome (6 mg of lipid) was given intraperitoneally (i.p.) to ddY mice once daily, six times weekly, from 7 days after infection to the end

of the experiment (for up to 8 weeks after infection) unless otherwise specified. At various intervals, the mice were killed and the visceral organs were observed for gross lesions. CFU of the organisms in the liver, spleen, lungs, and kidneys were counted by inoculating serial 10-fold dilutions of the homogenates of the visceral organs on 1% Ogawa egg medium (14).

Distribution of RFP in organs. At intervals after injection of free or liposome-entrapped RFP (three mice per regimen), the animals were killed and the concentrations of RFP in serum and visceral organs were measured as follows. Visceral organs (liver, spleen, lungs, and kidneys) were homogenized in 2 ml of physiological saline, using a glass homogenizer, and then centrifuged at 1,500 × *g* for 20 min. A paper disk (8 mm in diameter) was immersed in the resultant supernatant or serum, and the disk was placed on a heart infusion agar plate prepared by overlaying 5 ml of heart infusion agar containing ca. 5×10^4 spores of *Bacillus subtilis* (as an indicator bacterium) per ml onto 10 ml of a basal heart infusion agar layer. After overnight culture at 37°C, the concentration of RFP in the test solution was determined from the diameter of the resulting growth inhibition zone, using standard semilog plots of the agent at known concentrations. RFP standards were prepared in PBS.

Uptake of Lip-RFP by macrophages. Peritoneal exudate cells were collected from CBA/JN mice given i.p. injections of zymosan A (Sigma Chemical Co., St. Louis, Mo.) 4 days before harvest and washed twice with Hanks balanced salt solution (Nissui Pharmaceutical Co., Tokyo, Japan). The resultant cells were suspended in Eagle minimal essential medium (Nissui) containing 10% fetal bovine serum (FBS) (Whittaker M. A. Bioproducts Inc., Walkersville, Md.) at a concentration of 5×10^5 cells per ml. Then 10 ml of the cell suspension was poured onto a plate (90 mm in diameter), and the preparation was incubated at 37°C for 2 h in a CO_2 incubator (5% CO_2, 95% humidified air). The plate was then washed three times with a jet stream of Hanks balanced salt solution. To the resultant macrophage monolayer was added 10 ml of 10% FBS-minimal essential medium containing 100 μg of either free or liposome-entrapped RFP per ml, and the preparation was incubated at 37°C for 2 h in a CO_2 incubator. After being rinsed five times with PBS, the macrophage monolayer was treated with 0.5 ml of distilled water at 37°C for 20 min. The concentration of RFP in the resulting macrophage cell lysate was measured by a paper disk method on an assay plate seeded with *B. subtilis* as an indicator bacterium as described above.

Intracellular killing of *M. avium* complex. The macrophage monolayer was prepared by seeding zymosan A-induced peritoneal exudate cells (10^6 cells per 16-mm culture well; Corning Glass Works, Corning, N.Y.) in 1 ml of 10% FBS-minimal essential medium and incubating the preparation for 2 h at 37°C. Then 1 ml of 20% FBS–RPMI 1640 medium (Nissui) containing 10^7 *M. avium* 31F093 was added, and the preparation was incubated at 37°C for 2 h in a CO_2 incubator. The macrophage monolayer was then rinsed three times with 1% FBS-Hanks balanced salt solution, 1 ml of 20% FBS–RPMI 1640 with or without RFP (20 μg/ml) in the free or liposome-entrapped form was added, and the preparation was incubated for an additional 72 h at 37°C in a CO_2 incubator. The resultant macrophage monolayer was rinsed five times with PBS and treated with distilled water at 37°C for 20 min to lyse the cells. CFU in the cell lysate was counted by inoculating serial 10-fold dilutions onto Middlebrook 7H10 agar plates.

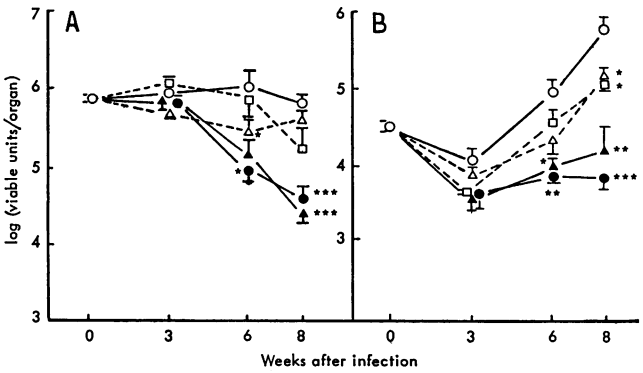

FIG. 1. Efficacy of Lip-RFP against *M. avium* complex infection in mice as determined by numbers of organisms in spleen (A) and lungs (B) after infection. Mice were given saline (○), free RFP (△), liposome without RFP (□), Lip-RFP (lipid layer) (●), or Lip-RFP (inner solute) (▲) as described in the text. *, **, and ***, Statistical difference from solute control value at $P < 0.05$, $P < 0.01$, and $P < 0.005$, respectively. At week 8, differences between results for free RFP and Lip-RFP were statistically significant for RFP versus Lip-RFP (lipid layer) in panel A ($P < 0.05$), RFP versus Lip-RFP (inner solute) in panel A ($P < 0.005$), and RFP versus Lip-RFP (lipid layer) in panel B ($P < 0.05$). The difference between results for RFP versus Lip-RFP (inner solute) in panel B was not significant ($0.05 < P < 0.1$).

RESULTS

Efficacy of Lip-RFP against *M avium* complex infection. Figure 1 shows the in vivo activity of two types of Lip-RFP preparations, Lip-RFP (inner solute) and Lip-RFP (lipid layer), given at a dose of 0.4 mg of RFP (in 6 mg of lipid) per mouse per injection, against *M. avium* complex infection. Both preparations enhanced elimination of the organisms in the spleen (Fig. 1A) and inhibited bacterial growth in the lungs (Fig. 1B) of infected animals. Both of the Lip-RFP preparations were significantly more effective than was free RFP. Liposome preparations free of RFP also exhibited a considerable therapeutic effect, possibly because of stimulation of some cellular functions of host macrophages of the reticuloendothelial system, which ingested the liposomal vesicles.

Table 1 shows organ weights and the incidence of macroscopic pulmonary lesions in mice 8 weeks after infection, with or without treatment with Lip-RFP, observed in the experimental infection shown in Fig. 1. In mice given either Lip-RFP (inner solute) or Lip-RFP (lipid layer), some increase in spleen weight was noted, although liposomes alone produced no such results. Pulmonary lesions were completely suppressed upon administration of both Lip-RFP preparations, whereas a 60% reduction was observed in mice given free RFP. We also noted a suppressive effect of free liposome on the incidence of pulmonary lesions.

Table 2 compares the efficacies of Lip-RFP (inner solute) given via the i.p., intramuscular (i.m.), or subcutaneous (s.c.) route at a dose of 0.4 mg of RFP (in 6 mg of lipid) per mouse per injection. Only when the drug was given i.p. was there a significant reduction in CFU of *M. avium* complex in the liver and spleen ($P < 0.005$; Student *t* test); there was also a moderate decrease in the lungs ($0.05 < P < 0.1$) at 8 weeks after the challenge. Thus, i.m. or s. c. injection of Lip-RFP did not seem to be as efficacious as i.p. injection against *M. avium* complex infection. The three RFP preparations (free RFP, free liposome, and Lip-RFP) given to mice i.m. as well as s.c. and, in some cases, i.p. caused a

TABLE 1. Organ weights and pulmonary lesions in five mice infected with *M. avium* complex, with or without Lip-RFP treatment[a]

Agent	Mean body wt (g) ± SEM	Organ wt (g)[b]				No. with pulmonary lesions
		Lungs	Liver	Spleen	Kidneys	
Solute control	35 ± 4	0.35	1.69	0.30[c]	0.41	5
Liposome alone	41 ± 6	0.31	1.64	0.33	0.41	0[d]
Free RFP	41 ± 6	0.36	1.72	0.22	0.44	2
Lip-RFP						
Lipid layer	41 ± 5	0.47[e]	1.71[f]	0.45	0.38	0[d]
Inner solute	44 ± 3	0.35	1.75	0.41	0.41	0[d]

[a] Details are as described in the legend to Fig. 1. Mice were killed 8 weeks after infection.
[b] Mean values; standard errors were less than 10% of means except as indicated.
[c] Standard error was 27% of mean.
[d] Significantly different from solute control value ($P < 0.01$; χ^2 test with Yates modification).
[e] Significantly different from solute control value ($P < 0.05$; Student t test).
[f] Standard error was 12% of mean.

significant increase in the number of CFU recovered from kidneys compared with the number recovered from the solute control. Although the precise reason for this enigmatic result is unknown, it may be attributed in part to differences in antimicrobial functions of macrophages resident in the kidneys and those in the other three organs.

Distribution in organs of RFP injected i.p. in free and liposome-entrapped forms. Figure 2 shows the distribution in organs of RFP (0.4 mg per mouse) given i.p. in free and Lip-RFP (inner solute) forms. Distributions of the two preparations were virtually identical; no statistically significant differences were detected.

Uptake of RFP in free and liposome-entrapped forms by macrophages. Entrapment of RFP into liposomal vesicles considerably enhanced incorporation of the agent in peritoneal macrophages (Fig. 3); the difference in results for free RFP and Lip-RFP (inner solute) was statistically significant ($P < 0.05$; Student t test). The rate of uptake of Lip-RFP (lipid layer) by macrophages was also much higher than that of free RFP, but the difference was statistically insignificant ($0.05 < P < 0.1$) because of a somewhat large variation in the data. Lip-RFP (lipid layer) was incorporated into macrophages more readily than was Lip-RFP (inner solute), but the difference was not significant ($0.1 < P < 0.25$).

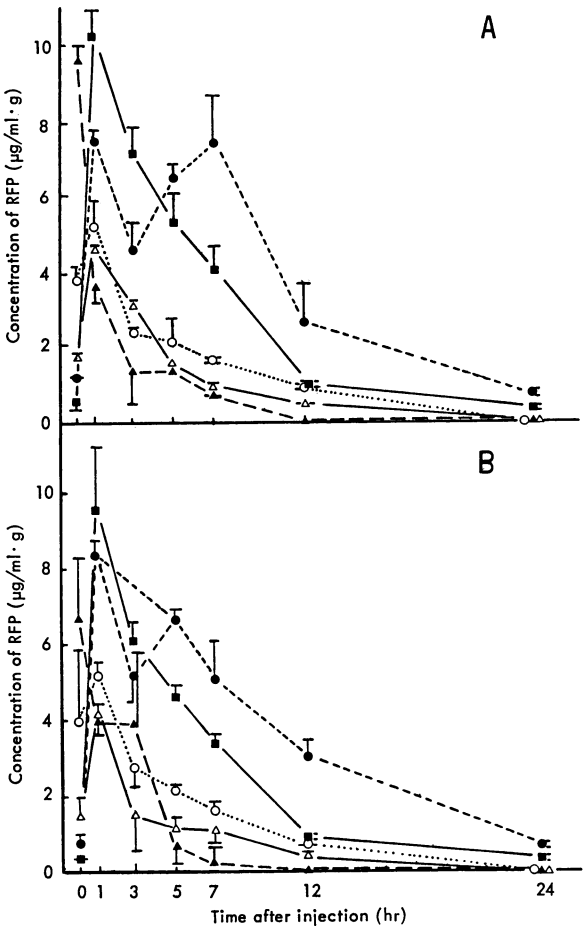

FIG. 2. Distribution in lungs (○), kidneys (△), spleen (▲), liver (●), and serum (■) of free RFP (A) and Lip-RFP (inner solute) (B) given i.p. at a dose of 0.4 mg per mouse. Each datum point indicates mean ± standard error.

Microbicidal activity of free and Lip-RFP against *M. avium* complex ingested in macrophages. Table 3 shows the intracellular survival of *M. avium* complex during 72 h of incubation in 20% FBS–RPMI 1640 medium, with or without addition of either free RFP or Lip-RFP (lipid layer), after

TABLE 2. Efficacy of Lip-RFP (lipid layer) against *M. avium* complex infection after different routes of administration[a]

Route	Agent	Mean log (viable units/organ) ± SEM			
		Lungs	Liver	Spleen	Kidneys
i.p.	Solute control	4.3 ± 0.3	5.9 ± 0.1	6.2 ± 0.1	1.9 ± 0.2
i.p.	Free RFP	4.5 ± 0.3	5.5 ± 0.1	6.0 ± 0.3	2.5 ± 0.2
	Liposome alone	4.2 ± 0.3	4.7 ± 0.1[b]	5.3 ± 0.3[c]	2.2 ± 0.2
	Lip-RFP	3.6 ± 0.2	4.5 ± 0.1[b]	4.9 ± 0.1[b]	2.7 ± 0.2[c]
i.m.	Free RFP	4.4 ± 0.1	5.4 ± 0.1[c]	5.8 ± 0.2	2.7 ± 0.2[c]
	Liposome alone	4.4 ± 0.1	5.3 ± 0.0[c]	6.0 ± 0.3	2.9 ± 0.2[c]
	Lip-RFP	5.1 ± 0.3	6.7 ± 0.0[b]	6.6 ± 0.4	3.6 ± 0.2[b]
s.c.	Free RFP	4.6 ± 0.1	5.5 ± 0.1	6.2 ± 0.2	2.8 ± 0.2[c]
	Liposome alone	4.7 ± 0.4	6.3 ± 0.3	6.6 ± 0.4	3.6 ± 0.5[c]
	Lip-RFP	4.5 ± 0.4	5.7 ± 0.4	5.9 ± 0.2	3.1 ± 0.1[b]

[a] Five mice were killed 8 weeks after infection with *M. avium* complex N-260, and the number of viable units per organ was determined.
[b] Significantly different from solute control value ($P < 0.005$; Student t test).
[c] Significantly different from solute control value ($P < 0.05$; Student t test).

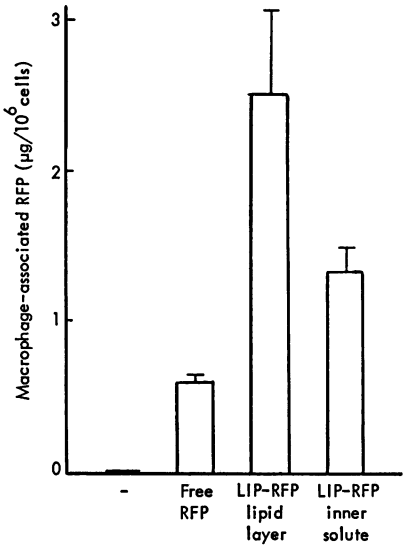

FIG. 3. Uptake of Lip-RFP by macrophages. The macrophage monolayer was incubated in medium with or without 100 μg of RFP per ml in free or liposome-entrapped (3 mg of lipid per ml) form, and the amount of macrophage-associated RFP was determined as described in the text.

phagocytosis by peritoneal macrophages. Although RFP in both forms showed activity against intracellular organisms, the activity of Lip-RFP was significantly higher than that of free RFP ($P < 0.05$; Student t test).

DISCUSSION

We examined the therapeutic effect of RFP entrapped in liposomes against *M. avium* complex infection in mice. Lip-RFP given i.p. was more efficacious against *M. avium* complex infection than was free RFP, as judged by elimination or growth inhibition in the target organs. Presumably, this result can be attributed to facilitation of the microbicidal efficacy of RFP molecules against *M. avium* complex located in phagosomal vesicles of host macrophages. Indeed, we noted that Lip-RFP was more readily taken up by macrophages than was free RFP (Fig. 3). Although RFP can easily penetrate the cell membrane of macrophages because of its hydrophobic nature, it is thought that RFP-entrapped liposomal vesicles may be incorporated much more rapidly than free RFP into macrophages by phagocytosis and pinocytosis. Indeed, the importance of phagosome-pinosome fusion is implicated in expression of the chemotherapeutic efficacy of antimicrobial agents (16). This difference seems to be important for expression of the activity of RFP against organisms in phagosomal vesicles of host macrophage cells. We found that Lip-RFP exhibited greater activity than did free RFP against *M. avium* complex engulfed in macrophage cells (Table 3). Similar results regarding the microbicidal effects of liposome-entrapped antimicrobial agents such as ampicillin (3) and cephalothin (7) against *L. monocytogenes* and *S. typhimurium*, respectively, have been reported.

TABLE 3. In vitro microbicidal activity of peritoneal macrophages against *M. avium* complex in the presence of Lip-RFP

Incubation time (h)	Addition[a]	Mean no. of viable organisms/macrophage ± SEM[b]
0		5.40 ± 0.11
72	None	12.7 ± 3.0
72	Free RFP	3.30 ± 0.19
72	Lip-RFP (lipid layer)	2.05 ± 0.08

[a] *M. avium*-ingested macrophages were incubated in medium containing 20 μg of RFP per ml in free or liposome-entrapped form.
[b] Two incubations.

Liposomes are preferentially trapped by phagocytic cells in the reticuloendothelial organs such as the liver and spleen (3, 9, 10). However, in this study we observed no such greater distribution of Lip-RFP than of free RFP in the liver, although some prolonged retention of Lip-RFP in the spleen was noted. The peak values of concentration in the liver and serum of RFP given in liposomal form were 8.5 and 9.5 μg/ml, respectively; these levels were higher than the MIC for *M. avium* complex 31F093 (6.25 μg/ml on 7H10 agar medium). Lip-RFP given via the i.m. or s.c. route failed to produce the significant therapeutic activity that was noted when the drug was given i.p. (Table 2), apparently because administration by the former two routes results in a lower rate of transfer of the drug to sites of infection than does i.p. injection.

The liposome preparation free of RFP was found to inhibit the growth of *M. avium* complex in the lungs and spleen to some extent (statistically significant in some cases) (Fig. 1). Thus, it is possible that liposome vesicles alone can increase host resistance, presumably through an immunostimulatory effect. Indeed, it is well known that some types of lipids, such as lipid A of bacterial lipopolysaccharide, have potent macrophage-activating functions. Therefore, it is likely that the greater efficacy seen with Lip-RFP could be related to some beneficial effect of the liposome vesicles themselves.

In separate experiments, we observed that Lip-RFP failed to increase activity against *Mycobacterium kansasii* infection in comparison with results for free RFP, presumably because of the high susceptibility of this species to RFP. Moreover, we observed no enhancement of the effect of ofloxacin against *M. fortuitum* infection. These observations may suggest that liposome-entrapped antimicrobial agents can be effective only against persistent infections, such as that induced by *M. avium* complex.

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