ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, June 1997, p. 1211–1214 Vol. 41, No. 6
0066-4804/97/$04.00+0
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Therapeutic Efficacies of Isoniazid and Rifampin Encapsulated in Lung-Specific Stealth Liposomes against *Mycobacterium tuberculosis* Infection Induced in Mice

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Received 17 October 1996/Returned for modification 3 January 1997/Accepted 21 February 1997

One recent promising development in the modification of drug formulations to improve chemotherapy is the use of a liposome-mediated drug delivery system. The efficacies of isoniazid and rifampin encapsulated in lung-specific stealth liposomes were evaluated by injecting liposomal drugs and free drugs into tuberculous mice twice a week for 6 weeks. Liposome-encapsulated drugs at and below therapeutic concentrations were more effective than free drugs against tuberculosis, as evaluated on the basis of CFUs detected, organomegaly, and histopathology. Furthermore, liposomal drugs had marginal hepatotoxicities as determined from the levels of total bilirubin and hepatic enzymes in serum. The elimination of mycobacteria from the liver and spleen was also higher with liposomal drugs than with free drugs. The encapsulation of antitubercular drugs in lung-specific stealth liposomes seems to be a promising therapeutic approach for the chemotherapy of tuberculosis.

The incidence of tuberculosis has increased significantly in the past decade due to the AIDS epidemic. *Mycobacterium tuberculosis*, the organism responsible for tuberculosis, can invade and replicate within macrophages. Clinical management of tuberculosis poses serious problems because the efficacy of chemotherapy has been reduced, which may be attributed to the degradation of drugs before reaching the target, the low level of cell permeability to drugs, or primary drug resistance. Other reasons for the failure of chemotherapy may be the difficulty in achieving adequately high drug concentrations at the infection site, inadequate penetration into macrophages, and low stability levels in cells. The standard chemotherapy includes isoniazid (INH), rifampin (RFP), streptomycin and pyrazinamide, which are given in combinations of two or three drugs. The prospect of finding newer and more effective drugs similar to the existing ones is small; therefore, attempts are being made to improve the therapeutic indexes of the existing drugs.

Liposomes have been used by many workers as carriers of drugs against many diseases such as leishmaniasis (1), fungal infections (9), and cancer (7). Liposomes can be part of the most effective form of chemotherapy against mycobacterial infections because they are avidly taken up by macrophages, release their contents intracellularly, and are effective against intracellular pathogens, e.g., *M. tuberculosis*. In animals infected with the *Mycobacterium intracellulare* complex, liposome-encapsulated streptomycin (20), amikacin (4), gentamicin (14), and RFP (19) exhibited greater efficacies than free drugs.

In our previous paper, we demonstrated that lung-specific stealth liposomes containing phosphatidylcholine, cholesterol (CH), *O*-stearyl amylopectin (*O*-SAP), dicetylphosphate (DCP), and distearoylphosphatidylethanolamine-polyethyleneglycol 2000 (DSPE-PEG 2000) were relatively stable (both in vitro and in vivo) and showed 40% accumulation in the lungs of normal and tuberculous mice (6). Further, INH and RFP encapsulated in these stealth liposomes exhibited less toxicity to macrophages and mice than free drugs (6). Based on our earlier observations, in this paper we report the comparative efficacies of free INH and RFP, as well as those of drugs encapsulated in lung-specific stealth liposomes given at their therapeutic concentrations and at lower concentrations, for mice infected with *M. tuberculosis* $H_{37}Rv$.

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MATERIALS AND METHODS

Chemicals and drugs. Egg phosphatidylcholine (ePC) was isolated in the laboratory and its purity was checked. CH, DCP, amylopectin (AP), PEG 2000, DSPE, INH, and RFP were purchased from Sigma Chemical Co., St. Louis, Mo. Kits for the estimations of levels of serum enzymes, e.g., glutamate pyruvate transaminase (SGPT) and serum alkaline phosphatase (ALP), were purchased from Ranbaxy, New Delhi, India. Kits for bilirubin estimation were purchased from Boehringer Mannheim Biochemicals.

Animals. Naval Marine Research Institute mice 3 to 4 weeks old, originally obtained from the Central Research Institute, Kasauli, India, were used in this study. The animals were fed a standard pellet diet (Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Culture. The culture of *M. tuberculosis* $H_{37}Rv$ was originally obtained from the National Collection of Type Cultures, London, England, and was maintained on Youman's modified medium.

Preparation of liposomes. Multilamellar liposomes containing ePC, CH, DCP, and DSPE-PEG 2000 in a molar ratio of 2:1.5:0.2:0.2 were prepared as described previously (6). The *O*-SAP used in these liposomes for lung targeting was included at a ratio to ePC of 1:3 (wt/wt). The encapsulation of INH in liposomes was checked by incorporating 4 mg of glucosamine (structural analog of INH) per ml according to the method of Wasserman et al. (21). For RFP, the dried lipids were sonicated with phosphate-buffered saline (PBS) containing 2 mg of RFP per ml for 15 min in the dark at 4°C under reduced nitrogen pressure. RFP levels were estimated spectrophotometrically at 334 nm after the liposomes were lysed with Triton X-100.

Experimental infection and chemotherapy. Different groups of animals were infected with low doses (1.5×10^4 CFU/mouse) of *M. tuberculosis* $H_{37}Rv$ given intravenously. Fifteen days postinfection, the presence of mycobacteria was confirmed by acid-fast staining of tissue smears of liver, spleen, and lungs after sacrificing one or two animals. The animals were divided into various groups for different drug treatments. Groups 1 and 2 were injected with PBS or empty liposomes (without drug) and served as controls. Group 3 received 12 mg of free INH per kg of body weight in PBS. Group 4 received the same dose of INH encapsulated in lung-specific stealth liposomes. Group 5 received liposomal INH at a concentration of 4 mg/kg. Group 6 received 10 mg of free RFP per kg in PBS. Group 7 received the same dose of RFP encapsulated in liposomes. Group 8 received liposomal rifampin at a concentration of 3 mg/kg.

Each mouse received 12 intravenous doses of drugs, administered twice a week on Monday and Thursday. Seven days after the last injection the animals were sacrificed and the parameters discussed below were evaluated.

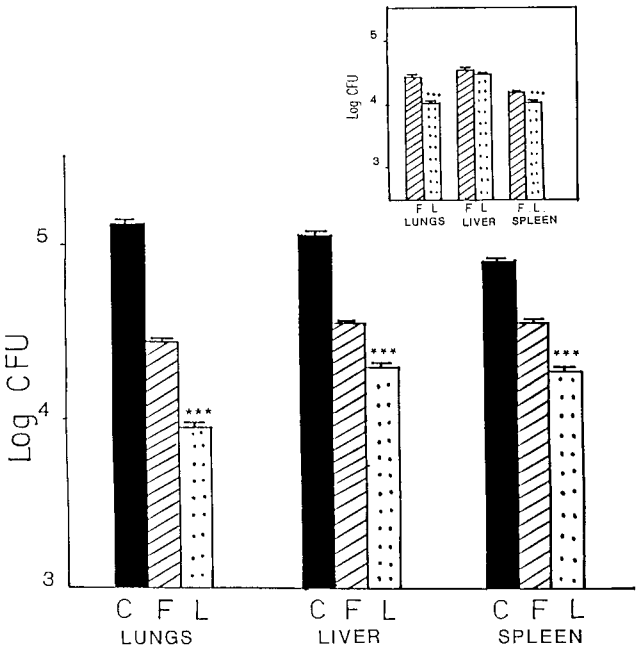

FIG. 1. CFU of *M. tuberculosis* in different organs of mice treated with free and liposomal INH. The concentration of both forms of the drug was 12 mg/kg. C, control; F, free drug; L, liposomal drug. The inset represents the CFU resulting from the same dose of free INH (12 mg/kg) and a lower dose of liposomal INH (4 mg/kg). The values are means ± standard deviations for 3 to 4 animals. ***, $P < 0.001$ (level of significance for liposomal drugs with respect to free drugs).

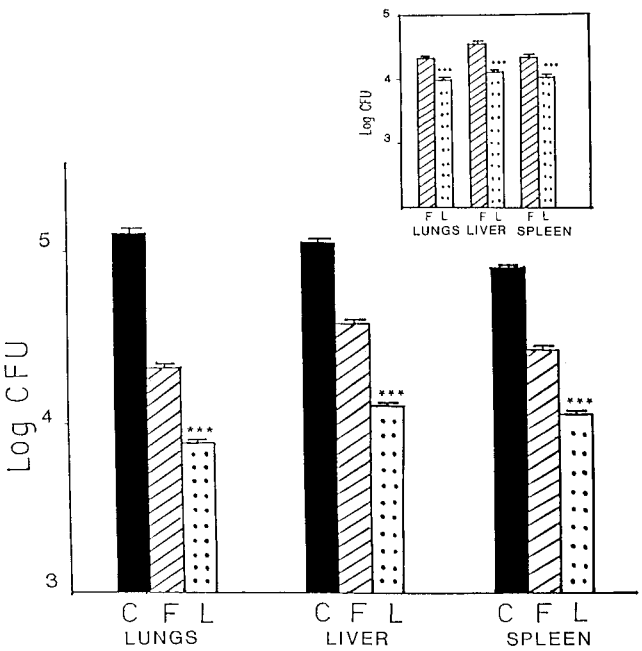

FIG. 2. CFU of *M. tuberculosis* in different organs of mice treated with free and liposomal RFP. The concentration of both forms of the drug was 10 mg/kg. C, control; F, free drug; L, liposomal drug. The inset represents the CFU resulting from the same dose of free RFP (10 mg/kg) and a lower dose of liposomal RFP (3 mg/kg). The values are means ± standard deviations for 3 to 4 animals. ***, $P < 0.001$ (level of significance for liposomal drugs with respect to free drugs).

Determination of CFU. Livers, lungs, and spleens were homogenized and cultured on plates containing Youman's medium supplemented with 1% bovine serum albumin for the enumeration of CFU (16). After 21 days, colonies were counted and CFU were calculated.

Toxicity studies. The levels of SGPT, ALP, and total bilirubin were measured before and on the 3rd day after the last therapeutic injection after bleeding the animals.

Evaluation of RSOW. The animals were killed after their weights were recorded. Their livers, spleens, and lungs were removed and weighed, and root specific organ weights (RSOW) were calculated (15) as follows:

$$\text{RSOW} = \sqrt{\frac{\text{organ weight (in milligrams)}}{\text{body weight (in grams)}} \times 10}$$

Histopathology. Tissues were fixed in 10% formalin solution, embedded in paraffin, and stained with H.E. and Fite's stains.

Statistical analysis. The data was analyzed by Student's paired *t* test.

RESULTS

Encapsulation of drugs. The encapsulation percentages for INH and RFP in the liposomes were 8 to 10 and 44 to 49, respectively.

Efficacies of free and encapsulated drugs given at their therapeutic doses for mice infected with tuberculosis. Animals infected with a low dose (1.5×10^4 CFU/mouse) of *M. tuberculosis* $H_{37}Rv$ exhibited acid-fast bacilli in tissue smears of livers, spleens, and lungs after 15 days of infection, confirming the presence of the disease.

(i) CFU in mice treated with free and encapsulated drugs. The effects of free drugs (INH and RFP) and drugs encapsulated in liposomes (containing ePC, CH, *O*-SAP, DCP, and DSPE-PEG 2000) in terms of elimination of mycobacteria from lungs, livers, and spleens were determined (Fig. 1 and 2). The free INH given at the therapeutic dose (12 mg/kg) reduced the CFU in lungs to about 4.5 log units, while the same dose of INH encapsulated in lung-specific stealth liposomes resulted in a decrease to 3.9 log units (Fig. 1), which was significantly ($P < 0.001$) lower than the free-drug value. The free-INH treatment of the mice decreased the number of CFU to 4.6 and 4.5 log units in the liver and spleen, respectively (Fig. 1), while liposomal INH reduced the CFU to about 4.2 and 4.3 log units, respectively (Fig. 1). The therapeutic dose of free RFP, i.e., 10 mg/kg, decreased the CFU in lungs to 4.3 log units (Fig. 2), and with the same dose of RFP encapsulated in the liposomes a reduction in the CFU in lungs to 3.8 log units was observed (Fig. 2), which was significantly ($P < 0.001$) lower than the free-drug value. The free-drug treatment decreased the CFU to 4.7 and 4.5 log units in the liver and spleen, respectively, while encapsulated RFP caused a reduction in CFU to 4.2 and 4.1 log units, respectively (Fig. 2). These observations revealed that encapsulation of drugs (INH and RFP) in lung-specific stealth liposomes resulted in significant ($P < 0.001$) increases in efficacy compared to those of free drugs (Fig. 1 and 2).

(ii) Comparison of toxicities induced by free and liposomal drugs. Toxicity studies were performed by monitoring the levels of total bilirubin, SGPT, and ALP before and on the 3rd day after the last therapeutic injection. Table 1 shows that the encapsulation of INH and RFP in liposomes reduced the total bilirubin levels compared to those observed for free drugs. The differences in the levels induced by free and liposomal drugs (INH and RFP) were highly significant ($P < 0.001$).

The SGPT levels were significantly ($P < 0.001$) lower in mice treated with liposomal INH and RFP than in free-drug-treated and control mice (Table 1). The mice treated with liposomal drugs showed significantly ($P < 0.001$) lower levels of ALP than animals treated with free drugs.

The levels of total bilirubin and hepatic enzymes (SGPT and ALP) demonstrated that encapsulation of INH and RFP in

TABLE 1. Levels[a] of total bilirubin, SGPT and ALP in mice

Group[b]	Total bilirubin (mg/100 ml)	SGPT (IU/liter)	ALP (IU/liter)
Control	0.35 ± 0.03	117.0 ± 1.1	45.70 ± 1.5
Free INH	0.97 ± 0.07	161.3 ± 1.6	115.7 ± 1.9
Liposomal INH_1	0.49 ± 0.02***[c]	134.6 ± 2.1***	73.70 ± 1.5***
Liposomal INH_2	0.41 ± 0.01***	125.3 ± 1.0***	61.4 ± 1.0***
Free RFP	2.39 ± 0.36	193.4 ± 1.6	138.1 ± 1.4
Liposomal RFP_1	0.55 ± 0.01***	136.9 ± 1.7***	76.10 ± 1.9***
Liposomal RFP_2	0.42 ± 0.16***	128.1 ± 3.2***	58.4 ± 1.0***

[a] Values are means ± standard deviations for five or six mice.
[b] INH_1, INH dose of 12 mg/kg; INH_2, INH dose of 4 mg/kg; RFP_1, RFP dose of 10 mg/kg; RFP_2, RFP dose of 3 mg/kg.
[c] ***, $P < 0.001$ (level of significance of liposomal drugs with respect to free drugs).

liposomes reduced their toxicities significantly ($P < 0.001$) compared to those of free drugs.

(iii) Organomegaly. The organomegaly for all the animals in the different groups was determined by measuring RSOW. The tuberculosis infection results in an increase in organ weight because of the presence of mycobacteria and increases in the concentrations of macrophages and lymphocytes which get activated because of the presence of tubercle bacilli. Increases in RSOW were observed in the infected animals compared to uninfected mice. The groups treated with lung-specific stealth liposomes containing drugs demonstrated significant ($P < 0.001$) reductions in RSOW of lungs compared to the free drug groups (Table 2). The RSOW of livers and spleens for the group treated with liposomal INH were 23.1 ± 0.95 and 7.3 ± 0.8 compared to 30.4 ± 1.3 and 9.3 ± 0.3 for the group treated with free INH. Values of RSOW for the respective organs treated with liposomal RFP were 24.6 ± 0.12 and 8.6 ± 1.1 in comparison to 26.3 ± 0.9 and 9.2 ± 1.6 for the free-drug-treated group.

(iv) Histopathological studies. The lungs of the control animals showed characteristic histopathological lesions, which consisted of collections of foamy histocytes containing small pyknotic nuclei and abundant foamy cytoplasm. The presence of diffuse sheets or nodular aggregates around the bronchi was also observed. In addition, the formation of focal abscesses and peribronchial lymphocyte infiltrate was observed in the control preparations. The staining for acid-fast bacilli showed the presence of many mycobacteria. The lungs of the animals treated with free RFP and INH demonstrated reductions in the numbers of granulomas. The presence of small focal collections of foamy histocytes and of extra alveolar macrophages was observed, in addition to peribronchial and interstitial lymphocytic infiltrate. However, no acid-fast bacilli were observed by acid-fast staining. The histopathology results for the animals treated with liposomal drugs (INH and RFP) revealed that the animals showed normal morphology of the lungs, except for small foci of histocytes and peribronchial lymphocytic infiltrate observed in the lungs from one animal. However, no acid-fast bacilli were observed by acid-fast staining, indicating clearance of bacilli from the lungs.

TABLE 2. Values of RSOW for lungs of mice treated with liposomal and free drugs

Group[a]	RSOW[b]
Control (uninfected)	8.66 ± 0.03
Control (infected)	14.00 ± 0.62
Free INH	13.60 ± 0.56
Liposomal INH_1	9.90 ± 0.02***[c]
Liposomal INH_2	9.90 ± 0.09***
Free RFP	11.30 ± 0.15
Liposomal RFP_1	9.0 ± 0.31***
Liposomal RFP_2	10.30 ± 0.2[NS][c]

[a] INH, INH_2, RFP_1, and RFP_2 are as defined for Table 1.
[b] Values are means ± standard deviations for three or four mice.
[c] ***, $P < 0.001$ (level of significance of liposomal drugs with respect to free drugs); NS, not significant.

Comparative studies of groups treated with therapeutic doses of free drugs versus low doses of liposomal drugs. The Fig. 1 inset shows a comparison of the CFU in lungs resulting from a lower concentration of INH (4 mg/kg) encapsulated in lung-specific stealth liposomes with the CFU resulting from therapeutic doses of free drug (12 mg/kg). The liposomal drug significantly ($P < 0.001$) reduced the numbers of CFU in the lungs to about 4 log units, which was lower than the number of CFU observed for free drugs (4.4 log units). The reduction in CFUs in the liver was the same with liposomal INH at the lower concentration as with the free drug; however, in the spleen a reduction in CFU to 4.1 log units was observed with encapsulated INH, a value lower than that observed for free drug (Fig. 1, inset). The decrease in CFU in the lungs observed with a low dose of RFP (3 mg/kg) encapsulated in liposomes was observed to be to a value of 4 log units, which was lower than that observed for free rifampin (10 mg/kg) (Fig. 2, inset). The reduction in CFU in liver and spleen was to 4.2 and 4.1 log units, respectively, for encapsulated RFP, values lower than those for the free drug. Thus, these observations indicate that liposomal drugs even at one-third of their therapeutic concentrations were more effective than free drugs at their therapeutic concentrations (Fig. 1 and 2).

The levels of hepatic enzymes (SGPT and ALP) and total bilirubin in groups treated with lower concentrations of INH and RFP encapsulated in liposomes were significantly ($P < 0.001$) different from the levels induced by the free drugs given at the therapeutic concentrations (Table 1). Thus, encapsulated drugs in lower concentrations induced lower toxicities and showed greater efficacies than free drugs.

The RSOW of lungs of mice treated with the lower concentrations of encapsulated INH and RFP were compared to the values of RSOW for lungs of free-drug-treated animals (Table 2). There was a significant ($P < 0.001$) difference in the RSOW for lungs of mice treated with liposomal drugs in comparison to those of the free-drug groups. The values of RSOW for livers and spleens in mice treated with encapsulated INH at the lower concentration were 24.2 ± 0.6 and 7.6 ± 0.2, respectively, in comparison to 30.4 ± 1.3 and 9.3 ± 0.3, respectively, for free INH. Encapsulated RFP at one-third the concentration of free drug resulted in RSOW values of 24.2 ± 0.48 and 8.2 ± 0.59 in liver and spleen, respectively, compared to 26.3 ± 0.9 and 9.2 ± 1.6, respectively, for free drug.

The histopathology of lungs of mice treated with the lower concentrations of encapsulated INH and RFP showed normal morphology; no lesions were observed. However, the lungs of mice treated with free drugs at their therapeutic doses showed lesions with focal collections of foamy histocytes and extra-alveolar macrophages. These observations indicated that the encapsulated drugs even at lower concentrations provided better clearance of acid-fast bacilli than free drugs at their therapeutic concentrations.

DISCUSSION

Liposomes are biocompatible, biodegradable microvascular systems ideal for drug delivery. The use of liposomes for improving drug therapy relies on two rationales, viz., controlled

drug release and site-directed delivery. In our previous study, the surfaces of the stealth liposomes (reticuloendothelial system avoiding) were modified by tagging *O*-SAP for lung targeting (7). In this study the efficacies and toxicities of free drugs and drugs encapsulated in lung-specific stealth liposomes were investigated. The efficacies of free and liposomal drugs were evaluated in terms of the elimination of mycobacteria from the organs of infected mice. The reductions of CFU in the lung, liver, and spleen with liposomal INH and RFP were significantly ($P < 0.001$) greater than those with free drugs (Fig. 1 and 2). Earlier studies have also reported that liposomal encapsulated isoniazid (15), rifampin (19), and amikacin (4) showed greater efficacies than free drugs against murine tuberculosis and infections caused by the *Mycobacterium avium* complex. However, in these studies, the extent of clearance of CFU in the lungs was less than that in the livers and spleens. The reductions in CFU in the lungs, livers, and spleens by liposomal drugs at one-third of their therapeutic doses yielded efficacies that were even greater than those of free drugs given at their therapeutic dosages (Fig. 1 and 2, inset). Majumdar et al. (10) have earlier reported that lower concentrations of liposomal streptomycin and ciprofloxacin had greater activity than higher concentrations of free drugs; these results are consistent with our observations. The increased efficacies of liposomal drugs suggest that due to phagocytosis and pinocytosis of liposomes, the delivery of encapsulated drugs to macrophages was more rapid than that of free drugs. The importance of the phagosome-pinosome in the expression of chemotherapeutic efficacy of antimicrobial agents was earlier explained by Rastogi and David (18).

The values of RSOW for the lungs of infected mice increased to 13 and 15 for the two groups of control mice, as compared to 8.7 for the lungs of normal mice (Table 2). The higher values are due to the presence of mycobacteria and the activation of macrophages, lymphocytes, and other immune system cells. These observations were in accordance with the results of Aoki et al. (2), who showed the presence of extensive tuberculous lesions in organs having RSOW values of more than 12. There was a decrease in the RSOW of different organs of mice treated with liposomal INH and RFP as compared to the RSOW resulting from treatment with free drugs. These results are in accordance with the observations of Orozco et al. (15), who reported that the use of encapsulated drugs resulted in RSOW values that were decreased compared to RSOW values for a free-drug group. Similarly, histopathological studies of the targeted organ, i.e., the lungs, of mice treated with liposomal INH and RFP at therapeutic and lower concentrations showed normal morphology compared to the lungs of mice in the free-drug groups treated at therapeutic dosages, consistent with the results of Orozco et al. (15).

Most of the antitubercular drugs, such as INH and RFP (3), pyrazinamide (17), and streptomycin (5), have been shown to induce hepatotoxicity when used alone or in combination (5). Levels of SGPT, ALP, and total bilirubin were monitored as an index of hepatotoxicity, and lowered values ($P < 0.001$) for liposomal drugs were observed (Table 1), indicating the lower toxicity of the liposomal drugs. These results are comparable with earlier reports dealing with the encapsulation of drugs such as doxorubicin (8), amphotericin B (9), nystatin (12), urea stibamine (11), hamycin (13), and gentamicin (14).

This study clearly demonstrates that antitubercular drugs encapsulated at and below therapeutic concentrations in lung-specific stealth liposomes have enhanced efficacies against tuberculous infection in mice and nominal hepatotoxicities. Further, our results suggest that antitubercular drugs encapsulated in liposomes may provide therapeutic advantages over the existing chemotherapeutic regimen for tuberculosis.

ACKNOWLEDGMENT

This project was financed in part by a grant (58/13/92-BMS-II) from the Indian Council of Medical Research, New Delhi, India.

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