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Tuftsin-Bearing Liposomes as Rifampin Vehicles in Treatment of Tuberculosis in Mice†

ANSHU AGARWAL,[1] HEMA KANDPAL,[2] HARIDWARI P. GUPTA,[2] NEPAL B. SINGH,[2] AND CHHITAR M. GUPTA[1]*

Institute of Microbial Technology, Sector 39-A, Chandigarh-160014,[1] and Division of Microbiology, Central Drug Research Institute, Lucknow-226001,[2] India

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The antitubercular activity of rifampin was considerably increased when it was encapsulated in egg phosphatidylcholine liposomes. A further increase in the activity was observed when the macrophage activator tetrapeptide tuftsin was grafted on the surface of the drug-loaded liposomes. Intermittent treatments (twice weekly) with these preparations were significantly more effective than the continuous treatments. Rifampin delivered twice weekly for 2 weeks in tuftsin-bearing liposomes was at least 2,000 times more effective than the free drug in lowering the load of lung bacilli in infected animals. However, pretreatment with drug-free tuftsin-bearing liposomes did not render the pretreated animals resistant to the *Mycobacterium tuberculosis* infections, neither did it appreciably increase the chemotherapeutic efficacy of the liposomized rifampin. These results clearly demonstrate that liposome targeting to macrophages could considerably increase the antitubercular activity of liposomized drugs such as rifampin. Also, it shows that immunoprophylactic treatment with macrophage activators such as tuftsin does not afford any advantage in treatment of tuberculosis infections, presumably because of inactivation of the primed macrophages by the mycobacterial sulfatides.

Tuberculosis is an infection which results in the largest number of deaths worldwide; nearly 3 million people die of this infection every year (9). The causative organism, *Mycobacterium tuberculosis*, resides and proliferates primarily within mononuclear phagocytes, which normally serve as the first line of defense against infections (2). The most important factor in the treatment of tuberculosis is prolonged chemotherapy, which is often associated with serious and unwanted side effects (3, 20). Also, undesired effects may be caused by the high levels of antitubercular drugs in blood required to achieve an effective intracellular drug concentration. Keeping this in view, it is desirable to develop an approach which would allow the use of lower drug doses by delivering the agent to the infected cells, thereby improving efficacy and potentially reducing toxicity.

Tuftsin is a tetrapeptide (Thr-Lys-Pro-Arg) which is an integral component of the leukophilic immunoglobulin G (residues 289 to 292) and is released physiologically as the free peptide fragment after enzymatic cleavage (8). The peptide is known to bind specifically to macrophages/monocytes and also to potentiate the natural killer activity of these cells (8). Our earlier studies have shown that incorporation of this tetrapeptide in liposome bilayers, after its modification at the C terminus (17), increases not only its macrophage-activating property (16) but also liposome uptake by macrophages (17). The liposomes thus formed have been shown to be quite effective as drug vehicles in treatment of leishmaniasis (6) and aspergillosis (12). Also, pretreatment of animals with these liposomes has been found to render them resistant to a variety of infections (4, 6, 12). To further evaluate the usefulness of these liposomes as drug vehicles in the treatment of macrophage-based infections, we have now examined their suitability as rifampin (RFP) vehicles in treatment of tuberculosis in mice.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine was isolated and purified by published procedures (18). Tuftsin modified by attachment of a palmitoyl residue to its C terminus through an ethylenediamine spacer arm (Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-CO-$C_{15}H_{31}$) was prepared as described previously (17). RFP was bought from Sigma Chemical Co.

Liposomes. Egg phosphatidylcholine (62.5 μmol) and RFP (0.61 μmol), in the presence and absence of modified tuftsin (7 to 8% by egg phosphatidylcholine weight), were dissolved in chloroform, and the solution was dried in a glass tube under a slow jet of nitrogen, resulting in formation of a thin lipid film on the wall of the tube. Final traces of the solvent were removed by leaving the film in vacuo overnight at 4°C. To the tube was added 1.5 ml of phosphate-buffered saline (PBS; 10 mM phosphate containing 150 mM NaCl [pH 7.4]) containing 1.5 mg of RFP, and the mixture was vortexed at 35 to 40°C for 15 to 20 min. The lipid dispersion thus obtained was transferred to a cuvette and sonicated for 30 min under nitrogen, using a probe-type sonicator (W-220 F; Heat Systems). The sonicated preparation was centrifuged at 12,000 × *g* in an IEC refrigerated centrifuge for 30 min to remove titanium particles as well as undispersed lipids. The supernatant was carefully withdrawn and gel filtered through a Sephadex G-50 column (40 by 1.5 cm), with PBS as the eluant, to separate liposomized RFP (Lip-RFP) from free RFP. The Lip-RFP was eluted in the void volume. The liposome-rich fractions were pooled and concentrated in an Amicon Centriflo CF-25 cone. The outer diameter of liposomes, as determined by electron microscopy, ranged between 25 and 65 nm. About 28 to 32% of RFP was found to be incorporated in the liposomes.

RFP and tuftsin estimation. We estimated the RFP concentration by measuring its A_{334}. We determined the amount of liposomized drug after lysing the liposomes with Triton X-100

* Corresponding author.

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(1%). The RFP A_{334} was found to remain unaffected by the presence of liposomes and detergent and was linear up to 100 μg of RFP. The amount of tuftsin incorporated in liposomes was estimated as described previously (17). The incorporation efficiency was found to be about 98%.

RFP localization in liposomes. To determine whether RFP is entrapped or intercalated in liposomes, we froze the liposome preparation in liquid nitrogen and immediately thawed it. The preparation was gel filtered again through the Sephadex G-50 column. RFP eluted under two different peaks, one corresponding to the Lip-RFP (about 40%) and the other corresponding to free RFP (about 60%).

Animals. Swiss albino mice weighing 20 ± 2 g were used in the study. The animals were given standard pellet diet (Hindustan Lever Ltd., Bombay, India) and water ad libitum.

Mycobacteria. *M. tuberculosis* $H_{37}RV$, equivalent to ATCC 25618-H37RV, was procured from the Trudeau Mycobacterial Culture Collection Centre, Trudeau Institute Inc., Saranac Lake, N.Y. It was maintained on Lowenstein-Jensen medium through routine bimonthly passage.

Infection. Mycobacterial growth (18 to 21 days old) maintained on Lowenstein-Jensen medium was harvested. It was weighed, and a 5-mg/ml suspension was prepared by homogenizing the culture in sterile saline containing 0.05% Tween 80. A 1-mg amount of culture was equivalent to about 10^9 CFU as determined from a sample of suspension plated to confirm the number of viable bacilli. Mice were infected intravenously by injection of about 10^9 CFU or 0.2 ml of the above suspension via the tail vein. Glass beads were added to the suspension to prevent clumping of the hydrophobic mycobacteria. Mice developed 3+ infection by 13 to 16 days and lost weight during progression of the infection. The animals in the control group were given saline by the same route.

Assessment of parameters. The number of bacilli in lungs, livers, and spleens of mice was quantitated by measuring CFU. Three animals from each group (8 to 12 animals per group) were sacrificed by cervical dislocation 3 days after the last dose. The spleens, livers, and lungs were aseptically removed and weighed. These organs from three different animals were separately pooled and homogenized in sterile saline. Serial twofold dilutions of the homogenates were prepared in sterile saline, inoculated onto duplicate set of slants of Lowenstein-Jensen medium, and incubated for 15 to 21 days at 37°C. After appearance of visible growth, the isolated colonies were counted and the number of live bacilli was calculated in the original dilutions.

Mean survival time (MST) was considered to be a parameter in all single treatments and prophylactic studies. The animals that were left (five to nine mice per group) after the CFU count were observed up to day 30 postinfection for calculation of MST. In the multitreatment studies, this parameter was not considered since no mortality was observed in the treated groups up to day 30 after the infection. Lung weight per 100 g of mice was considered to be a parameter in some experiments (5). The degree of lung damage was determined by the morphometric analysis as described by Weibel (19).

Treatment. In the first set of experiments, the infected mice were given a single intravenous dose of RFP, whereas in the second set, the animals were given four doses of RFP at 10 mg/kg/day either on consecutive days or twice weekly for 2 weeks. Treatment was begun 3 days after the infection in each case.

Statistical analysis. Statistical significance (P value) was determined by using F and t statistics. The means of two data sets were compared by using the two-sided F-ratio test and t test. The F-ratio test determined whether the standard deviations of two data sets were equal, and accordingly an appropriate t test was applied (7).

RESULTS

Initially, experiments were done to optimize the RFP dose, after encapsulation in liposomes, for producing the maximum effect against the *M. tuberculosis* infections. For this purpose, different doses (3, 5, and 10 mg/kg) of Lip-RFP were intravenously delivered to *M. tuberculosis*-infected mice, and the antitubercular response was measured as described in Materials and Methods. The lung bacillus load, lung weight, MST, and percent lung damage were considered the main parameters to assess the antitubercular activity. At low doses (<10 mg/kg), the antitubercular response of Lip-RFP was not much different from that of free RFP, but at a 10-mg/kg dose of Lip-RFP, a significant reduction in the lung bacillus load and an increase in MST were observed (Table 1) compared with those in untreated ($P < 0.01$) or free-RFP-treated ($P < 0.01$) animals.

To further enhance the efficacy of Lip-RFP against the tuberculosis infections, we explored the effects of the route of administration, liposome surface charge, cholesterol content, and multiple treatments on the antitubercular activity. Changing the route of administration from intravenous to intraperitoneal, the liposome surface charge from neutral to positive, and the cholesterol content from 0 to 25 mol% did not further increase the RFP activity (data not shown). However, Lip-RFP appeared to be appreciably better ($P < 0.05$) than free RFP in lowering the lung bacillus load when up to four doses of this preparation were delivered on consecutive days (Table 2). Markedly better effects ($P < 0.01$) of Lip-RFP than free RFP were observed when the multiple treatment was given intermittently rather than continuously; both the lung bacillus load and lung weight were significantly reduced by administration of Lip-RFP twice weekly for 2 weeks (Table 3). That the intermittent treatment with Lip-RFP was more effective than the treatment with free RFP was further evident from the morphometric data (Table 3), which showed that the lungs were only 8% tuberculoid compared with 20% with free RFP.

Since grafting of tuftsin on the liposome surface is expected to increase liposome uptake by the macrophages, we evaluated the antitubercular response of RFP after encapsulating it in tuftsin-bearing liposomes (Tuft-Lip-RFP). Although the single treatment with Tuft-Lip-RFP was only marginally better than that observed with Lip-RFP (Table 4), Tuft-Lip-RFP given twice weekly for 2 weeks was considerably more effective ($P < 0.05$) than Lip-RFP in controlling tuberculosis (Table 5).

Because incorporation of tuftsin into the liposome bilayer increases the ability of tuftsin to increase the nonspecific resistance against infections and uptake by the macrophages, we thought it appropriate to study the effect of Tuft-Lip pretreatment on the establishment and course of *M. tuberculosis* infections. Pretreatment with Tuft-Lip (50 μg of tuftsin per day per animal) was administered on days 1 to 3 prior to the infection. Results of these experiments (not shown) revealed that the Tuft-Lip pretreatment did not significantly affect the establishment and course of the infection or the chemotherapeutic efficacy of Lip-RFP.

DISCUSSION

The high excretion rate is the major problem associated with the use of RFP as an antimycobacterial agent (15), because it

TABLE 1. Efficacy of Lip-RFP against *M. tuberculosis* infection in mice

Group and expt[a]	Log CFU[b] in:			MST (days)
	Lungs	Liver	Spleen	
Lip-RFP				
a	7.61	5.86	6.23	22.9
b	8.65	6.79	6.60	19.6
c	7.87	6.81	6.91	19.8
	(8.04 ± 0.44)	(6.48 ± 0.44)	(6.58 ± 0.27)	(20.76 ± 1.50)
Free RFP				
a	9.83	5.81	6.45	17.3
b	9.57	6.61	7.52	17.3
c	9.48	7.07	6.95	16.4
	(9.62 ± 0.15)	(6.49 ± 0.52)	(6.97 ± 0.44)	(17.00 ± 0.42)
Control				
a	11.30	7.03	6.86	13.5
b	10.85	6.23	8.32	14.6
c	9.60	7.23	8.92	14.1
	(10.58 ± 0.72)	(6.83 ± 0.43)	(8.03 ± 0.86)	(14.06 ± 0.45)
P value				
Lip-RFP vs free RFP	<0.01	>0.05	>0.05	<0.01
Lip-RFP vs control	<0.01	>0.05	<0.05	<0.01
Free RFP vs control	>0.05	>0.05	>0.05	<0.01

[a] a, b, and c denote three different experiments.
[b] The CFU in each experiment was determined by separately pooling different organs from three different animals. Values shown in parentheses are mean ± standard deviation.

necessitates daily administration of a high dose, which often leads to serious toxic side effects such as hepatotoxicity (14). Because liposomes have an inherent tendency to localize mainly in the cells (1) that also serve as the host for *M. tuberculosis*, encapsulation of RFP in liposomes may help to reduce the daily drug dose by concentrating the drug in these cells. That this may indeed be the case is well supported by our finding that Lip-RFP given at 10 mg/kg is appreciably more effective than the free drug in controlling the tuberculosis infections. It is also supported by results of an earlier study

TABLE 2. Efficacy of four continuous doses of Lip-RFP given on days 3, 4, 5 and 6 postinfection against *M. tuberculosis* infection in mice

Group and expt[a]	Log CFU[b] in:		
	Lungs	Liver	Spleen
Lip-RFP			
a	6.48	4.48	4.10
b	6.64	5.97	6.11
c	6.48	4.30	5.60
	(6.53 ± 0.07)	(4.92 ± 0.75)	(5.27 ± 0.85)
Free RFP			
a	6.67	5.04	4.30
b	7.48	6.40	6.70
c	7.70	5.60	5.78
	(7.28 ± 0.44)	(5.68 ± 0.56)	(5.59 ± 0.98)
Control			
a	9.51	5.04	6.75
b	9.66	6.78	7.81
c	9.85	7.30	7.90
	(9.67 ± 0.14)	(6.37 ± 0.96)	(7.48 ± 0.52)
P value			
Lip-RFP vs free RFP	<0.05	>0.05	>0.05
Lip-RFP vs control	<0.01	>0.05	<0.01
Free RFP vs control	<0.01	>0.05	<0.05

[a] a, b, and c denote three different sets of experiments.
[b] CFU in each experiment was determined by separately pooling different organs from three different animals. Values shown in parentheses are mean ± standard deviation.

TABLE 3. Efficacy of intermittent treatment with Lip-RFP given twice weekly for 2 weeks against *M. tuberculosis* infection in mice

Group and expt[a]	Log CFU[b] in:			Lung wt (g/100 g of body wt)	Morphometric analysis of lung (vol/vol% lung parenchyma)[c]:	
	Lungs	Liver	Spleen		Normal	Tubercular
Lip-RFP						
a	7.00	4.30	5.60	2.10	91.5 ± 5.4	8.5 ± 5.4
b	6.95	4.78	5.00	1.13		
	(6.97 ± 0.02)	(4.54 ± 0.24)	(5.30 ± 0.30)	(1.61 ± 0.48)		
Free RFP						
a	8.60	5.20	6.08	2.22	81.0 ± 3.8	19.0 ± 3.8
b	8.66	5.28	6.18	1.27		
	(8.63 ± 0.03)	(5.24 ± 0.04)	(6.13 ± 0.05)	(1.74 ± 0.47)		
Control						
a	9.30	7.30	7.90	3.39	29.0 ± 2.3	71.0 ± 2.3
b	10.30	5.60	6.83	1.81		
	(9.80 ± 0.50)	(6.45 ± 0.85)	(7.36 ± 0.53)	(2.60 ± 0.79)		
P values						
Lip-RFP vs free RFP	<0.01	<0.05	<0.05	>0.05	>0.05	
Lip RFP vs control	<0.01	<0.05	<0.05	>0.05	<0.01	
Free RFP vs. control	<0.05	>0.05	<0.05	>0.05	<0.01	

[a] a and b denote two different sets of experiments.
[b] CFU in each experiment was determined by separately pooling different organs from three different animals. Values shown in parentheses are mean ± standard deviation.
[c] Morphometric analysis, for determining the lung damage, was carried out on three animals, and values are expressed as mean ± standard deviation.

(11), which showed significantly better antitubercular effects of a combination of isoniazid and RFP after their encapsulation in liposomes.

The better antitubercular response of Lip-RFP than of free RFP is perhaps due to the ability of liposomes to localize preferentially in macrophages/monocytes, leading to a high intracellular drug concentration. This is quite consistent with our finding that intermittent but not continuous treatment with

TABLE 4. Efficacy of Tuft-Lip-RFP against *M. tuberculosis* infection in mice

Group and expt[a]	Log CFU[b] in:			MST (days)	Lung wt (g/100 g of body wt)
	Lungs	Liver	Spleen		
Tuft-Lip-RFP					
a	6.60	6.59	6.97	25.6	0.97
b	6.04	5.45	5.60	26.7	1.27
	(6.32 ± 0.28)	(6.02 ± 0.57)	(6.28 ± 0.68)	(26.15 ± 0.55)	(1.12 ± 0.15)
Lip-RFP					
a	7.41	6.38	6.13	25.0	1.18
b	7.15	5.89	5.90	27.8	1.33
	(7.28 ± 0.13)	(6.13 ± 0.24)	(6.01 ± 0.11)	(26.40 ± 1.40)	(1.25 ± 0.07)
Tuft-Lip					
a	9.00	7.02	6.76	17.1	1.24
b	8.60	5.99	6.01	20.1	1.45
	(8.80 ± 0.20)	(6.50 ± 0.51)	(6.38 ± 0.37)	(18.60 ± 1.50)	(1.34 ± 0.10)
Control					
a	9.08	8.08	7.20	15.7	1.30
b	9.60	5.83	6.34	18.2	1.56
	(9.34 ± 0.26)	(6.95 ± 1.12)	(6.77 ± 0.43)	(16.95 ± 1.25)	(1.43 ± 0.13)
P value					
Tuft-Lip-RFP vs Lip-RFP	<0.05	>0.05	>0.05	>0.05	>0.05
Tuft-Lip-RFP vs Tuft-Lip	<0.01	>0.05	>0.05	<0.01	>0.05
Tuft-Lip-RFP vs control	<0.01	>0.05	>0.05	<0.01	>0.05
Lip-RFP vs Tuft-Lip	<0.01	>0.05	>0.05	<0.01	>0.05

[a] a and b denote two different sets of experiments.
[b] CFU in each experiment was determined by separately pooling organs from three different animals. Values shown in parentheses are mean ± standard deviation.

TABLE 5. Efficacy of intermittent treatment with Tuft-Lip-RFP given twice weekly for 2 weeks

Group and expt[a]	Log CFU in[b]:			Lung wt (g/100 g of body wt)
	Lungs	Liver	Spleen	
Tuft-Lip-RFP				
a	7.73	4.85	5.70	2.26
b	6.85	4.30	5.08	2.00
	(7.29 ± 0.44)	(4.57 ± 0.27)	(5.39 ± 0.31)	(2.13 ± 0.13)
Lip-RFP				
a	9.28	6.26	6.60	2.63
b	8.48	5.30	5.90	2.35
	(8.88 ± 0.40)	(5.78 ± 0.48)	(6.25 ± 0.35)	(2.49 ± 0.14)
Control				
a	13.08	7.85	8.48	5.67
b	11.40	7.60	7.87	4.75
	(12.24 ± 0.84)	(7.72 ± 0.12)	(8.17 ± 0.30)	(5.21 ± 0.46)
P value				
Tuft-Lip-RFP vs Lip-RFP	<0.05	>0.05	>0.05	>0.05
Tuft-Lip-RFP vs control	<0.01	<0.01	<0.01	<0.01
Lip-RFP vs control	<0.01	<0.01	<0.01	<0.01

[a] a and b denote two different sets of experiments.
[b] CFU in each experiment was determined by separately pooling different organs from three different animals. Values shown in parentheses are mean ± standard deviation.

Lip-RFP was considerably more (about 50-fold) effective than that with free drug under identical conditions (Table 3). Also, it suitably explains our present observation that incorporation of tuftsin in the liposome bilayer leads to a further increase (about 40-fold) in the antitubercular activity of Lip-RFP (Table 5).

Orozco et al. (10) have determined the distribution of Lip-RFP in mice, which showed a marked difference after infection of the animals with tuberculosis. A relatively higher liposome concentration in lungs, kidneys, and blood was observed in tuberculosis-infected mice, whereas the maximum concentration in the uninfected animals was in the liver and spleen. Furthermore, these authors observed a longer retention of liposomes by the infected tissue. These findings could explain our observation of the greater efficacy of Lip-RFP in the lungs, since in all our experiments the lungs were the organs showing tubercle formation with the highest bacillus load.

Tuftsin potentiates the natural killer activity of the macrophages and other phagocytosing cells and has been shown to enhance the nonspecific resistance of the host against parasitic and fungal infections (4, 6, 12). However, in the present study the immunoprophylactic treatment with Tuft-Lip did not prove very effective against tuberculosis infections. The observed failure of this treatment in providing resistance against tuberculosis infections could be attributed primarily to the presence in the mycobacterial cell wall of some sulfolipids which are known to inhibit macrophage activity (13).

In summary, we have demonstrated that Lip-RFP is more effective than free RFP in controlling *M. tuberculosis* infections in mice. This increased antitubercular response of Lip-RFP is further increased by incorporating tuftsin in the liposome bilayer. From these results, we conclude that homing of liposomes to macrophages may considerably improve the therapeutic efficacy of the liposomized antitubercular drugs.

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