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Tissue distribution and antileishmanial activity of liposomised Amphotericin-B in Balb/c mice[§]

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Abstract. Antileishmanial activity and organ distribution of the antifungal drug Amphotericin-B in free and liposomised form have been studied in Balb/c mice infected with *Leishmania donovani*. Results indicate that Amphotericin-B in the liposomised form is significantly more active than the free form. This increase in the activity is perhaps related to the reduced drug toxicity rather than the altered drug distribution at the site of infection.

Keywords. Amphotericin-B; antileishmanial activity; renal toxicity; liposomes; tissue distribution; drug delivery.

1. Introduction

Amphotericin-B (Amp-B) is a very potent antifungal drug which exhibits marked renal toxicity (Graybill and Craven 1983). However, this toxicity is dramatically reduced by incorporating the drug in liposomes (Alving 1986). The liposomised Amp-B is very effective not only against the fungal diseases (Graybill *et al* 1982; Lopez-Berestein *et al* 1983; Trembaly *et al* 1984) but also against the leishmania infections (New *et al* 1981; Berman *et al* 1986).

Earlier studies have shown that availability of Amp-B in various tissues is altered by the fungal infections (Lopez-Berestein *et al* 1984; Ahmad *et al* 1990). Also, altered tissue distribution patterns of this drug have recently been observed in animals suffering from diabetes (Wasan *et al* 1990). To examine whether the *Leishmania donovani* infection would also affect the drug availability in various tissues, we have studied the tissue distribution and antileishmanial activity of liposomised Amp-B in Balb/c mice.

2. Materials and methods

2.1 *Materials*

Fungizone, the lyophilized pharmaceutical preparation of Amp-B in deoxycholate, was obtained from Sarabhai Chemicals, Baroda, and was reconstituted in 5%

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Abbreviations used: Amp-B, Amphotericin-B; Chol, cholesterol; SPC, soya phosphatidylcholine; EPC, egg phosphatidylcholine; HPLC, high pressure liquid chromatography.

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dextrose before use. Amp-B and cholesterol (Chol) were procured from Sigma Chemical Co., USA. Soya phosphatidylcholine (SPC) was a kind gift from Dr G Maierhofer. Egg phosphatidylcholine (EPC) was prepared as described earlier (Gupta and Bali 1981). All other reagents used were of analytical grade.

2.2 *Animals*

Male Balb/c mice weighing 18–22 g were used in all the experiments.

2.3 *Parasites*

The strain of *L. donovani* (HOM/IN/80/Dd8), originally isolated from a Kala-azar patient from Bihar, was a kind gift from Professor P C C Garnham. The strain is being regularly maintained *in vitro* as promastigotes in NNN medium, and as amastigotes in golden hamsters.

2.4 *Infection*

Amastigote suspensions (about 10^8 amastigotes/ml) were prepared from the spleen of infected hamsters (> 1 month old) in Lock's solution (8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.3 g KH_2 PO_4 and 2.5 g glucose in 1 litre, pH 7.2). A measured aliquot (0.2 ml) of this suspension was administered intravenously to Balb/c mice. The establishment of the infection was confirmed by the presence of a number of amastigotes/500 host cell nuclei in liver and spleen.

2.5 *Liposomes*

Liposomes were prepared from EPC or SPC and Chol (PC/Chol molar ratio 7:3) as described earlier (Gupta and Bali 1981). Briefly, the lipids (total weight 45 mg) were dissolved in chloroform in a round-bottomed flask and to it 1 mg Amp-B in methanol was then added. The organic solvents were removed under reduced pressure, resulting m formation of a thin lipid film on the wall of the flask. The dried lipid mixture was dispersed in 150 mM saline and sonicated for 30 min under nitrogen using a probe-type sonicator. It was centrifuged at 12,000 g for 30 min (4°C). The supernatant was dialyzed against 150 mM saline to remove free Amp-B, and the amount of Amp-B incorporated in liposomes was determined spectrophotometrically (Mehta *et al* 1984). About 80–85% of Amp-B was found to be associated with the liposomes.

2.6 *Drug treatment*

About 3 weeks after infecting with *L. donovani,* the animals were divided into 4 groups. While the first group was given free Amp-B, the second group was administered Amp-B incorporated in EPC/Chol or SPC/Chol liposomes. The third group received empty liposomes (free of drug) whereas the fourth group was left untreated. The efficacy of the treatment was ascertained by monitoring the parasite burden in spleen and liver of the treated animals.

To monitor the parasite burden, the mice were sacrificed and impression smears of spleen and liver were prepared. Smears were fixed with methanol, stained with Giemsa, and the number of amastigotes/500 host cell nuclei ware counted. Per cent inhibition of the amastigote number was calculated, using untreated animals as controls, as follows:

$$\% \text{ Inhibition} = 100 - \left(\frac{X}{\bar{Y}} \cdot 100\right),$$

where X and Y denote actual and mean numbers of amastigotes/500 cell nuclei in treated and control animals, respectively.

2.7 *Tissue distribution*

Tissue distributions of Amp-B in various organs were determined essentially according to the method of Lopez-Berestein *et al* (1984). The detailed methodology has been described by us earlier (Ahmad *et al* 1989).

Both normal and infected mice were injected intravenously free or liposomised Amp-B, and 1 h or 24 h after the injection, the animals were sacrificed and various organs (*viz*., lung, liver, spleen and kidney) taken out, excised and frozen until analysis. The tissue samples from different animals were pooled together, and 0·5 g of each tissue homogenised in methanol. It was centrifuged at 10,000 *g* for 15 min and the supernatant injected into an HPLC column (μ C $_{18}$ reverse phase) for analysis. The drug concentration in various tissues was obtained after multiplying the observed values with the recovery factor. The recovery factor for various tissues was determined essentially as reported earlier (Ahmad *et al* 1989). The recovery of Amp-B from different tissues varied from 61 to 75%.

3. Results and discussion

Amp-B was intercalated into the egg PC/Chol or SPC/Chol liposomes bilayers by cosonicating this drug with a mixture of egg PC (or SPC) and Chol. The titanium particles and undispersed lipids were removed by centrifugation, and the free drug from the liposomised Amp-B was separated by extensive dialysis. The drug-loaded liposomes thus obtained were administered intravenously to Balb/c mice infected with *L. donovani,* and the efficacy of this treatment was assessed by measuring the parasite load in spleens and livers of the treated animals. Results given in table 1 shows that the parasite load in the spleens of infected animals was significantly reduced by incorporating the drug in EPC/Chol or SPC/Chol liposomes. However the drug incorporated in SPC/Chol liposomes appeared to be much less effective in controlling the liver infections as compared to that incorporated in EPC/Chol liposomes. Besides enhancing the drug efficacy, liposomisation of the drug also led to a marked decrease in the drug toxicity; all the animals died at 2 mg/kg dose of free Amp-B. These results are consistent with the earlier studies (New *et al* 1981; Berman *et al* 1986).

To determine the tissue distribution, free drug and liposomised drag were injected in both normal and infected animals at 1 mg/kg and 4 mg/kg dose respectively. Free drug dose of 1 mg/kg which was chosen as the LD $_{50}$ of free Amp-

Table 1. Activity of liposomised Amp-B against *L. donovani* infections in Balb/c mice.

Drug formulation	Drug dose (mg/kg)	Inhibition of infection (%)	
		Spleen	Liver
Liposomised (EPC/Chol)	5·0	70·8 ± 5·7 (10)	79·9 ± 9·6 (10)
	0·8	69·7 ± 7·3 (6)	78·5 ± 16·4 (6)
	0·4	62·5 ± 6·9 (7)	44·7 ± 14·1 (7)
Liposomised (SPC/Chol)	5·0	79·8 ± 3·7 (14)	49·8 ± 8·6 (14)
Free	0·8	32·7 ± 10·9 (10)	55·4 ± 15·0 (10)
EPC/Chol liposomes		21·2 ± 10·2 (7)	ND

Values shown are means ± SE, and have been calculated from the pooled data of 2 separate experiments, using 3-7 animals/group each time.
Numbers in parentheses denote the number of total animals used. At 5·0 mg/kg (or 2·0mg/kg) dose of free Amp-B, all the animals died spontaneously.
ND, Not determined.

Table 2. Organ concentration of Amp-B after injecting free and liposomised Amp-B in normal and *L. donovani-infected* Balb/c mice.

	Organ concentration (μg/g tissue)							
	Free Amp-B				Liposomised Amp-B			
	Normal		Infected		Normal		Infected	
Tissue	1 h	24 h	1 h	24 h	1 h	24 h	1 h	24 h
Liver	31·4	5·2	31·0	14·2	15·3	29·4	30·8	11·1
Spleen	26·0	7·2	20·4	8·4	7·0	8·1	4·6	11·7
Lung	8·8	6·3	2·5	4·8	9·0	6·5	10·5	6·8
Kidney	7·2	6·0	8·8	20·0	2·5	8·6	5·1	12·2

Liposomes used in these experiments were formed from SPC and Chol, Liposomal Amp-B was given at 4 mg/kg, while the free drug was administered at 1 mg/kg dose. However, for calculating the organ concentration, the free drug dose was adjusted to 4 mg/kg (for details see Lopez-Berestein *et al* 1984). Values shown are means of two independent determinations carried out on drug extracts obtained from pooled organs of 5 mice each time.

B is only about 1·2 mg/kg (Ahmad *et al* 1989). This low dose of the drug if administered in liposomes, can not be accurately detected in various tissues and hence a higher dose (4 mg/kg) of liposomised drug was used. The animals were sacrificed 1 h or 24 h after the drug administration, and various organs taken out and homogenised in methanol. The homogenates were centrifuged, and the organ concentration of Amp-B determined by HPLC as described in §2.

Table 2 shows that the drug concentration in livers of the infected animals was not influenced much by incorporating Amp-B in the liposomes. However, it was appreciably reduced in kidney under identical conditions. Moreover, the drug concentration in the case of the liposomal Amp-B in spleen was smaller, as compared to free Amp-B, at 1 h but not at 24 h after the injection. Besides the liposomisation, the drug distribution in various tissues was influenced also by the infection. This influence was more pronounced in the case of the liposomised drug, compared to the free drug (table 2). It would therefore seem that the Amp-B distribution in biophase is influenced by both the drug incorporation in liposomes and the *L. donovani* infections in animals.

These results indicate that liposomisation of Amp-B does not significantly alter its distribution in the macrophage-rich organs *(viz.,* liver, spleen and lung) of *L.* donovani-infected mice, but it does lead to a decrease in the drug concentration in the kidney of these animals. This is quite consistent with earlier studies (Lopez-Berestein *et al* 1984; Ahmad *et al* 1990). It may thus be suggested that the higher efficacy of the liposomised Amp-B observed here is at least partly due to the reduced drug concentration in kidney, as it should lead not only to the decreased drug toxicity (Graybill and Craven 1983) but also to an increased drug tolerance. This is quite consistent with our finding that the drug tolerance is significantly increased by delivering the drug in liposomes. It may thus be concluded that the increased efficacy of the liposomised Amp-B against *L. donovani* infections is perhaps largely due to the enhanced drug tolerance rather than the altered drug distribution at the site of infection.

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