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Drug delivery system: targeting of pentamidines to specific sites using sugar grafted liposomes

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Different sugar-grafted liposomes were prepared and tested against experimental leishmaniasis *in vivo* using the classical drug pentamidine isethionate and its methoxy derivative. Both the drugs, when encapsulated in sugar-grafted liposomes were found to be more potent in comparison to normal liposome-encapsulated drug or to the free drug. Moreover, the mannose-grafted liposomes were adjudged to be the best in lowering of spleen parasite load in comparison with those bearing glucose or galactose. When encapsulated in mannose-grafted liposomes the therapeutic efficacy of pentamidine isethionate was found to be better than that of its methoxy derivative, although the latter seemed to be less toxic than the pentamidine isethionate itself.

Introduction

Targeting of drug directly to the macrophages, mediated by specific receptors is a challenge not only to counter leishmaniasis but also to control a few other diseases where the invading organisms can survive within macrophages. In an attempt to develop delivery systems acceptable to all macrophage-associated disorders, the uptake of glycoside-bearing liposomes by macrophages has been studied *in vitro*. Since, the uptape was found to be specific for the end sugar attached to the glycoside, the possibility was raised that glycoside-bearing liposomes might be used as systems to deliver drugs to macrophages *in vivo*. Using the antileishmanial drug urea stibamine, these delivery systems have been tested for therapeutic efficacy (Das *et al.*, 1990) and for toxicity (Medda *et al.*, 1993) against experimental leishmaniasis. Mannose-grafted liposomes were found to be most efficient in the lowering of spleen parasite load and least toxic when compared either to the ordinary liposomes or to the free drug. Our recently published report on hamycin intercalated in mannose-grafted liposomes also support these findings (Banerjee, Bhaduri & Basu, 1994). In the present paper we discuss the effective use of pentamidine isethionate and one of its derivatives in liposome-encapsulated form as well as in sugar-grafted liposome-encapsulated form against experimental leishmaniasis in hamsters. The efficacy and the toxicity of both drugs have been critically compared and analysed.

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Methods

Chemicals

Pentamidine isethionate (PI) and methoxy pentamidine isethionate (MPI) were prepared and their microbicidal properties were tested *in vitro* (Mahato *et al.*, 1993). Phosphatidylethanolamine (PE), cholesterol, dicetyl phosphate (DCP), different p-aminophenyl-α-D-glycosides, glutaraldehyde and sugar specific lectins were purchased from Sigma Chemicals (St. Louis, MO USA). All other reagents were of analytical reagent grade.

Preparation of liposome-encapsulated pentamidine isethionate (PI) and methoxy pentamidine isethionate (MPI)

The multilamellar liposomes were prepared using PE, cholesterol, DCP in the molar ratio 7:2:1 as described earlier (Gregoriadis & Ryman, 1972).

PI or MPI were suspended in 2 mL PBS, pH 7.4 and used for swelling the lipid film for 1 h at 37°C. The suspension was sonicated for 30 sec and centrifuged at 100,000*g* for 30 min in a Beckman Ultracentrifuge. The pellet was washed twice in an identical manner and suspended in a known volume of PBS. The supernatent was examined for free, unencapsulated drug by measuring absorbance at 265 nm (ϵ_{PI} = 12,000 $M^{-1}cm^{-1}$ and ϵ_{MPI} = 10,500 $M^{-1}cm^{-1}$). The amount of drug encapsulated in the liposomes was found to be 10% for PI and 15% for MPI, therefore the amounts of drug in the lipid film to provide a dose of 80 mg/kg were 300 mg/mL of PI and 200 mg/mL of MPI.

Coupling of p-aminophenyl-α-D-glycosides with liposomes

The liposome-encapsulated PI or MPI were suspended in 2 mL PBS. The coupling of the appropriate glycosides with amino group of PE-liposomes was done by using glutaraldehyde following the published protocols (Ghosh & Bachhawat, 1980). The presence of various sugars on the liposome surface was examined by agglutination test using sugar specific lectins (Surolia, Ahmed & Bachhawat, 1975).

Animal experiment

Our colony of golden hamsters (*Mesocricetus auratus*) originally from Haffkine Research Institute, Bombay was used to maintain *Leishmania donovani*, strain AG 83 from an Indian kala-azar patient, by intracardial passage every 6 weeks. Amastigotes were isolated from spleens by the method of Looker, Bernes & Marr, (1983) with some modification as described elsewhere (Das *et al.*, 1990). Each animal was infected intracardially with 2×10^6 amastigotes. A group of 24 hamsters of about 80 g body weight were infected at each time and the treatment was started after 30 days. The animals were distributed in six groups with four in each group for the testing of free drug and liposome-encapsulated drug with their appropriate controls: (i) the drug encapsulated in sugar-grafted liposomes; (ii) the drug encapsulated in regular liposomes without sugar; (iii) empty sugar-grafted liposomes; (iv) empty regular liposomes without sugar; (v) free drug (80 mg/kg); (vi) untreated controls.

The dose given to each animal was 80 mg/kg body weight. Routinely, 6.4 mg of PI or MPI in 5 mg lipid in 0.5 mL PBS was injected subcutaneously into each hamster every 3 days for a total of four doses over 10 days. For free PI or MPI the same amount

of drug (6.4 mg/0.5 mL PBS) was injected at each time. The animals were killed by cervical dislocation after 4 days of the last injection. Parasite load in the spleen was assessed from stained impression smears using the Stauber formula (Stauber Franchino & Grun, 1958):

$$\text{Total No. of amastigotes} = (\text{No. of amastigotes per host cell nucleus}) \times (\text{wt of spleen in mg}) \times 2 \times 10^5$$

Investigation on drug toxicity

The blood pathology, tissue histology and the specific enzyme levels related to normal liver function are some parameters that were chosen as the toxicity indices of the drug when tested *in vivo* in both free and liposome-encapsulated form. These parameters were analysed by established clinical procedures and the levels of specific enzymes e.g. alkaline phosphatase (AP) and serum glutamate pyruvate transaminase (SGPT) were assayed as described before (Medda *et al.*, 1993).

Results

Anti-leishmanial activity tested in vivo

The results of testing using free, liposome-encapsulated and sugar grafted liposome-encapsulated forms of PI and MPI in the hamster model are expressed in Table I. The free PI lowered the parasite load of the spleen by 18.5% whereas the liposome-encapsulated PI reduced the same by 46.6%. The reduction of spleen parasite load was found to be different for different sugar-grafted liposome-encapsulated PI. For mannose-grafted liposome-encapsulated PI the reduction in spleen parasite load was

Table I. Effect of sugar-grafted liposome-encapsulated PI and MPI for the treatment of experimental leishmaniasis on 30-day infected hamster model

Group	(Parasite load in the spleen) $\times 10^{-7}$ PI	MPI	% Suppression of spleen-parasite load PI	MPI
1. Infected control	13.5 ± 1.2	12.5 ± 1.2	—	—
2. Free-drug treated	11.0 ± 1.0	10.6 ± 0.8	18.5	15.2
3. Liposome-encapsulated drug treated	7.2 ± 0.4	7.8 ± 0.4	46.6	37.0
4. Sugar-grafted liposome-encapsulated drug treated				
(i) mannose	2.0 ± 0.1^a	3.6 ± 0.2^a	85.1	71.2
(ii) glucose	4.6 ± 0.3	ND	65.9	—
(iii) galactose	7.4 ± 0.4	ND	45.1	—

The values are expressed as mean $\pm$ S.D. ($n = 4$). The suppression of spleen-parasite load by empty liposomes (both ordinary and sugar-grafted) was about 10–12%.

Dose given to each animal each time was 80 mg/kg body weight. Routinely 6.4 mg of PI or MPI in 5 mg lipid in 0.5 mL PBS was injected into each hamster each time every 3 days for a total of four doses over 10 days. For free PI/MPI, the same amount of drug (6.4 mg/0.5 mL PBS) was injected at each time.

$^aP < 0.001$ compared with infected control.

Table II. The effect of sugar-grafted liposome-encapsulated drug(s) on specific enzymes level related to normal liver-function

Groups	Alkaline phosphatase[a] PI	Alkaline phosphatase[a] MPI	Serum glutamate pyruvate transaminase[b] PI	Serum glutamate pyruvate transaminase[b] MPI
1. Infected control	12.7 ± 3.0	8.8 ± 0.9	42.0 ± 4.0	40.2 ± 3.8
2. Free-drug treated	20.6 ± 5.9	10.6 ± 0.1	75.0 ± 5.7	48.3 ± 0.5
3. Liposome-encapsulated drug treated	14.0 ± 2.2	9.6 ± 0.4	55.2 ± 6.1	43.7 ± 1.7
4. Sugar-grafted liposome-encapsulated drug treated	13.6 ± 3.0	9.5 ± 0.5	43.7 ± 1.7	42.9 ± 2.6

Values are expressed as mean ± S.D ($n = 4$).

[a]μmol of p-nitrophenol released/min/dL sera. Normal level of alkaline phosphate was 8.8 ± 1.5.

[b]μmol of sodium pyruvate released/min/L sera. Normal value of SGPT was 36.3 ± 2.0.

found to be 85.1% whereas for glucose grafted liposomes, it was 65.9%. However, for galactose-grafted liposome encapsulated PI the reduction in spleen parasite load was about 45.1% i.e. almost the same as found for PI encapsulated in ordinary liposomes. However, for MPI, only mannose-grafted liposomes were used. The free MPI reduced the spleen parasite load by 15.2%, whereas the liposome encapsulated MPI, gave a reduction of around 37.0%. The reduction of parasite load using mannose-grafted liposome encapsulated MPI was around 71.2%.

Assay of two specific enzymes related to normal liver function

The levels of alkaline phosphatase and glutamate pyruvate transaminase were assayed under different conditions. The results are summarised in Table II. For both PI or MPI, the level of alkaline phosphatase increased on treatment with free drug but the increase was much more significant for the PI, thus showing a comparatively higher drug toxicity than MPI. For liposome-encapsulated or sugar grafted liposome-encapsulated PI (or MPI) the level of alkaline phosphatase reduced further and reached the normal level.

The level of SGPT increased from the normal level in the free drug-treated group for both PI and MPI. But the increase was almost twice with PI, whereas for MPI, the increase was only 1.3 times, showing again the reduced drug toxicity in case of MPI. For liposome-encapsulated or sugar-grafted liposome-encapsulated MPI, the level of SGPT came down almost to the normal level. However, for PI the level remained a little higher than normal when it was encapsulated in ordinary liposomes but came to normal level when sugar-grafted liposomes were used.

Histological studies

Histological examination of the spleen was made after staining with eosin and heamatoxylin. Although some histological changes were observed for the free PI treatment, no detectable changes were noticed for either liposomal or mannose-grafted liposomal PI. On the contrary, no such changes could be noticed for any of the three MPI formulations.

Discussion

Antimonials, which are notorious for their toxic effects, have remained the mainstay for the treatment of leishmaniasis, and the second-line drugs (e.g. amphotericin B and pentamidines) are too toxic to be used as first-line therapy on a large scale. But, very recently, many cases clinically resistant to antimonials have been found, an alarming situation (Banerjee *et al.*, 1994). Meanwhile, there have been reports of the successful use of pentamidines in the treatment of antimony-resistant leishmaniasis (Sands, Kron & Brown, 1985). Thus, an attempt was made to search for a suitable delivery system which could not only reduce the toxicity of the drug but also would be able to increase its efficacy. The potential of using liposomes as a delivery system is strengthened by the fact that both the liposomes and the leishmania parasites are taken up by the same reticuloendothelial cells, creating an ideal situation for studying drug-parasite interaction. Moreover, if appropriate ligands are covalently attached to liposomes, so that they could be easily recognized by the macrophage receptors, then these modified liposomes could possibly be used very efficiently as vehicles for site-specific delivery. Very recently, we have synthesized several pentamidine analogues and have already screened them for their antileishmanial activity *in vitro* (Mahato *et al.*, 1993).

Pentamidines are not only known for antileishmanial activity (Chance, 1980), but also for their antiviral, tumoricidal, antibacterial and fungistatic properties (Bell *et al.*, 1990). Pentamidine analogues have very recently been used against *Plasmodium falciparum* and *Pneumocystis carinii* pneumonia (Bell *et al.*, 1990; Jones *et al.*, 1990). The exact mechanism by which pentamidine and its analogues exert various microbicidal activities is not known, although their possible active role in the inhibition of DNA/RNA synthesis can not be ruled out (Jones *et al.*, 1990).

The results presented in this paper show the efficacy of both pentamidine isethionate and its methoxy derivative when tested against reversible visceral leishmaniasis in hamsters in the free, liposome-encapsulated and sugar-grafted liposome-encapsulated forms. The best efficacy in terms of lowering of spleen parasite load was found when sugar-grafted liposomes were used as the delivery vehicles. Mannose-grafted liposomes were found to be the most efficient in comparison with either glucose or galactose-grafted ones, the latter being the least effective. Judging from the fact that mannose and glucose are specifically involved in the recognition of the parasites or appropriate ligands on the macrophage surface and the galactose was least involved in the binding process (Mukherjee Ghosh & Basu, 1988), our results, in relation to the lowering of parasite loads using different sugar-grafted liposomes (Table I), might be expected. However a comparison of pentamidine isethionate and its methoxy derivative, showed the former to be more potent at equivalent drug concentration—free, liposome-encapsulated or mannose-grafted liposome-encapsulated form, although the methoxy derivative was found to be less toxic and thus, if delivered in appropriate doses in such tailor-made delivery vehicles, may possibly have better applications in clinics.

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