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Drug targeting in *Leishmania donovani* infections using tuftsin-bearing liposomes as drug vehicles

P.Y. Guru, A.K. Agrawal, U.K. Singha, A. Singhal* and C.M. Gupta

Divisions of Membrane Biology and Parasitology, Central Drug Research Institute, Lucknow 226 001, India

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The efficacy of sodium stibogluconate against *Leishmania donovani* infections was markedly enhanced by encapsulating this drug in tuftsin-bearing liposomes. Also, pretreatment of the animals with these liposomes (free of drug) rendered them resistant to this infection, possibly by activating the host's macrophages. These results demonstrate that tuftsin-bearing liposomes besides delivering the drug to the target cells could also enhance the nonspecific resistance against infections, thus offering an additional advantage over the use of tuftsin-free liposomes as drug carriers in leishmania therapy.

Leishmania infection; Drug targeting; Liposome; Tuftsin; Immunomodulation; (Macrophage)

1. INTRODUCTION

Liposomes bearing cell-specific ligands on their surface may prove useful as vehicles for site-specific delivery of drugs in biophase [1,2], but their successful application in therapy is limited by their inherent tendency to concentrate in reticuloendothelial cells [3]. Since in the case of leishmaniasis, the causative organism is located within these very cells, the liposome-mediated delivery of drugs in treatment of this infection seems to be highly promising [4].

We have developed liposomes which specifically recognize the phagocytic cells, including macrophages [5]. These liposomes besides delivering their contents to the target cells [5] have also been shown to enhance the host's resistance against parasitic infections [6]. This property has been conferred to these liposomes by tuftsin, which is known to specifically bind macrophages/monocytes [7], and also potentiates the natural killer activity of these cells [7].

To evaluate further the usefulness of tuftsin-bearing liposomes in parasitic diseases, we have now examined the effect of these liposomes on *Leishmania donovani* infections in hamsters. Also, we have attempted to use these liposomes as vehicles for delivering the antileishmanial drug, sodium stibogluconate, to the infected animals. The data shown here clearly indicate that these liposomes not only enhance the resistance of hamsters to *L. donovani* infection, but are also very effective in increasing the efficacy of sodium stibogluconate in leishmaniasis treatment.

Correspondence address: C.M. Gupta, Division of Membrane Biology, Central Drug Research Institute, Lucknow 226 001, India

* *Present address:* Department of Radiation Oncology, Medical College of Virginia, Richmond, VA 23298, USA

2. MATERIALS AND METHODS

2.1. *Tuftsin*

Tuftsin was modified at the C-terminus (Thr-Lys-Pro-Arg-NH-$(CH_2)_2$-NH-$COC_{15}H_{31}$) to facilitate its incorporation in the liposome bilayer. Both tuftsin and modified tuftsin were prepared as in [5].

2.2. *Liposomes*

Liposomes were prepared from egg phosphatidylcholine (20 μmol) and cholesterol (10 μmol) with or without modified

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tuftsin (7–8% by phospholipid weight) in 1 ml Tris-buffered saline (10 mM Tris containing 150 mM NaCl, pH 7.4) by probe-type sonication, and fractionated by centrifugation [8]. For loading the liposomes with drug, sodium stibogluconate (about 410 μmol based on pentavalent antimony) was included in the above buffer, prior to sonication. Free and liposomised drugs were separated by gel filtration over Sephadex G-50. The amount of the liposome-encapsulated drug was determined by estimating pentavalent antimony [9]. The extent of incorporation of modified tuftsin in the liposome bilayer was measured following the published procedure [5]. Lower doses of drug encapsulated in liposomes were prepared by appropriate dilution of the preformed drug-loaded liposomes with suitable buffer.

2.3. *Animals*

Male golden hamsters (*Mesocricetus auratus*) and Balb/C mice (weight 18–22 g) were used in all the experiments.

2.4. *Parasites*

The *Leishmania donovani* strain (HOM/IN/80/Dd 8), originally isolated from a Kala-azar patient from Bihar (India), 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.

2.5. *Infection*

2.5.1. For chemotherapeutic studies

Amastigotes were isolated from the spleen of infected hamsters (with more than 2 months of infection). These were suspended in Locke's solution (8 g NaCl, 0.2 g KCl, 0.2 g $CaCl_2$, 0.3 g KH_2PO_4 and 2.5 g glucose in 1 l, pH 7.2) to about 10^8 amastigotes/ml. A measured portion (0.1 ml) of this suspension was administered intracardiacly (i.c.) to each hamster (weight 40–45 g). The animals developed 2+ infection (5–20 amastigotes/100 spleen cell nuclei) after 25–30 days, which was assessed by spleen biopsies. There was no effect of the operative trauma on the course of *L. donovani* infection, since we observed no difference in the intensity of infection determined by two independent methods, namely autopsy and spleen biopsy.

2.5.2. For prophylactic studies

Golden hamsters were challenged with 10^7 promastigotes (i.c.) in 0.1 ml Locke's solution. These promastigotes were derived from the in vitro cultures and washed with Locke's solution prior to use. In all the experiments, promastigotes were isolated from the infected spleen of hamsters in primary cultures, and used after 5–8 subcultures in vitro.

2.5.2. In in vitro macrophage cultures

Peritoneal macrophages of Balb/C mice were harvested in RPMI-1640 medium containing heparin, using standard procedures. These were washed twice and resuspended in complete RPMI-1640 medium supplemented with 10% fetal calf serum, gentamycin (40 μg/ml medium) and 40 mM 2-[tris(hydroxymethyl)methyl amino]-1-ethane sulfonic acid (Tes). The final suspension was made to about 10^6 macrophages/ml, and seeded into Leighton tubes with cover slips. On the following day, medium from each tube was removed and replaced with complete medium containing 2×10^6 promastigotes/ml. These promastigotes could infect the macrophages, and developed into amastigotes inside these cells. Cultures were incubated in special gas chambers with a gas mixture of 5% O_2, 5% CO_2 and 90% N_2.

2.6. *Treatment*

2.6.1. Chemotherapeutic treatment

Hamsters (weight 80–85 g) with 4–28 amastigotes/100 spleen cell nuclei, as assessed between day 25 and day 30 post-infection by spleen biopsy, were divided into four groups. Each group consisted of a large number of animals with 4–5 animals for each test dose. Animals in group I were given free sodium stibogluconate; group II, drug entrapped in tuftsin-free liposomes; and group III, drug entrapped in tuftsin-bearing liposomes. These preparations were administered i.c. only once, 3–4 days after the spleen biopsy. The fourth group was the control group, which was administered buffer only. On day 7 and day 28 post-infection, spleen biopsies were carried out again, and spleen smears after Giemsa staining were assessed for amastigote counts. Inhibition of amastigote multiplication was calculated using the following equation: $PI = 100 - (AN \times 100)/(INA \times TI)$ where AN, actual number of amastigotes/100 spleen cell nuclei after treatment; INA, initial number of amastigotes/100 spleen cell nuclei; TI, fold increase in the number of amastigotes in control animals on the corresponding day of the biopsy in treated animals; and PI, percentage inhibition.

2.6.2. Prophylactic treatment

Hamsters (weight 40–45 g) were divided into five groups, each having 3–7 animals. Group I was administered with a single dose (60 μg/animal, i.c.) of free tuftsin, group II with free tuftsin for 3 consecutive days (60 μg/animal per day, i.c.), group III with a single dose of liposomal tuftsin (60 μg/animal, i.c.), group IV with liposomal tuftsin for 3 consecutive days (60 μg/animal per day, i.c.), and group V, the control group, was injected (i.c.) with saline only. Seven days after the first injection all the animals were challenged with 10^7 promastigotes. Spleen biopsies were performed on day 30, day 45 and day 60 post-infection, and the percentage inhibition (PI) was calculated using the following equation: $PI = 100 - (NAE \times 100)/(MNAC)$ where NAE, number of amastigotes/100 spleen cell nuclei in experimental animals; and MNAC, mean number of amastigotes/100 spleen cell nuclei in control animals.

2.7. *Susceptibility of peritoneal macrophages of free and liposomised tuftsin-treated Balb/C mice to* L. donovani *infection*

Male Balb/C mice were used in 3 groups, each consisting of 4–5 animals. These were separately given saline, free tuftsin (75 μg/animal per day) and liposomised tuftsin (75 μg/animal per day) intravenously on 3 consecutive days. Seven days after the first injection, these animals were killed, and peritoneal macrophages were isolated, washed and seeded in Leighton tubes with cover slips, after suspending them in RPMI-1640 medium containing 10% fetal calf serum. Each tube contained about 10^6 macrophages/ml. On the following day, about 2×10^6 promastigotes were added to each tube. Cover glasses with attached macrophages were removed from the culture tubes on day 2 and day 5 post-infection, fixed with absolute methanol, stained with Giemsa and percentage of macrophages infected as well as average number of amastigotes/cell were determined.

3. RESULTS AND DISCUSSION

Maximum effect of sodium stibogluconate on *L. donovani* infection in hamsters was observed when this drug was given at 10 mg/kg per day for five consecutive days. This treatment produced over 90% inhibition of the infection on day 28 post-treatment. The extent of this inhibition was considerably reduced when the infected hamsters were treated with this drug at 250 μg or 500 μg/kg for one day only (table 1). However, single treatment by these reduced drug doses was very effective ($p < 0.01$) after encapsulating the drug in liposomes (table 1). At a 500 μg/kg dose, the drug encapsulated in tuftsin-bearing liposomes showed much better ($p < 0.01$) effects than that delivered in the tuftsin-free liposomes on day 28 post-treatment. But this difference was reduced ($p < 0.01$ on day 7 but >0.05 on day 28 post-treatment) by reducing the drug dose to 250 μg/kg, although at this dose the liposomised drug exhibited the maximum antileishmanial activity. Further decrease in the liposomised drug dose resulted in decreased biological activity and no differences were observed between the effects of drug encapsulated in tuftsin-free and tuftsin-bearing liposomes (data not shown).

These findings indicate that encapsulation of sodium stibogluconate in tuftsin-bearing liposomes significantly ($p < 0.01$) enhances the drug efficacy against *L. donovani* infection. Since the causative organism of this disease primarily resides within the macrophages which have been shown to possess on their surface the specific binding sites for tuftsin [7], it may be inferred that the observed enhanced efficacy of the drug encapsulated in tuftsin-bearing liposomes against *L. donovani* infection could be due to the enhanced uptake of these drug-loaded liposomes by the macrophages of infected hamsters. Here, it may be mentioned that our failure to observe the enhanced antileishmanial effect of the drug loaded in tuftsin-bearing liposomes, as compared to that in the tuftsin-free liposomes, at low drug doses (<250 μg/kg) is possibly due to dilution of the tuftsin-bearing liposomes below their optimal concentration required to observe their enhanced uptake by the host's macrophages [5].

Our previous studies [6] have shown that mice pretreated with the tuftsin-bearing liposomes strongly resist *Plasmodium berghei* infection. To ascertain whether similar treatment of hamsters would render them resistant to *L. donovani* infection, we studied the course of this infection in the animals pretreated with free and liposomal tuftsin. Fig.1 shows that pretreatments with both free and liposomal tuftsin provided protection against *L. donovani*. However, the inhibitory effect on this infection was greater ($p < 0.01$ on day 30 post-infection for single treatment) when tuftsin was incorporated in the liposome bilayer. Moreover, a single treatment with tuftsin or liposomal tuftsin was more effective than the multiple treatment. These results clearly show that the pretreatment of

Table 1

Effect of liposomised sodium stibogluconate on *L. donovani* infection in hamsters

Preparation[a]	Drug dose (μg/kg)	% inhibition of infection[b] (on day post-treatment)		Survival of animals (on day post-treatment)	
		7th	28th	7th	28th
F	500	35.8 ± 6.6	Dead	4/4	0/4
L	500	65.0 ± 6.1	61.5 ± 1.3	4/4	2/4
L-T	500	77.6 ± 1.8	85.0 ± 3.1	4/4	4/4
F	250	51.9 ± 4.7	Dead	4/4	0/4
L	250	74.8 ± 5.1	80.5 ± 11.6	5/5	4/5
L-T	250	86.5 ± 4.5	92.1 ± 2.7	4/4	4/4

[a] F, free drug; L, drug entrapped in tuftsin-free liposomes; L-T, drug entrapped in tuftsin-bearing liposomes

[b] Values shown are mean of 4–5 animals ± SD

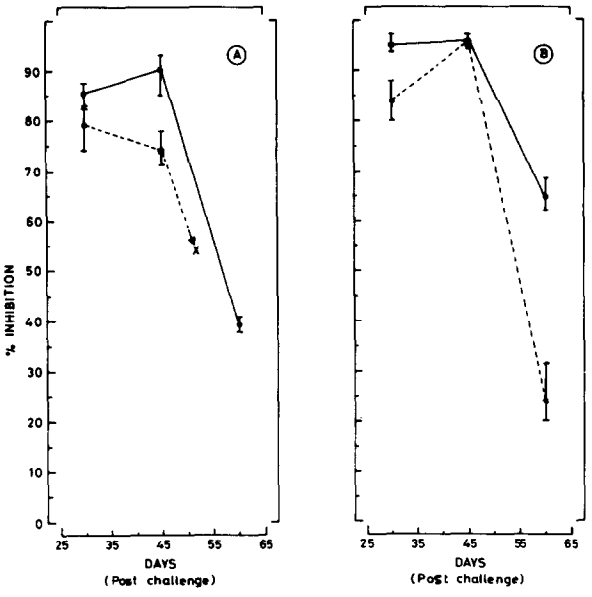

Fig.1. Prophylactic effect of tuftsin on *L. donovani* infection in hamsters. (A) Free tuftsin; (B) liposomal tuftsin. Solid and broken lines represent treatments with single and triple doses, respectively. Values are shown as both mean and range. For details, see section 2. All the animals survived up to day 45 in the treated groups, but on day 60 the survival in these groups was as follows: animals treated with single and triple doses of free tuftsin, 30% and 0% survival, respectively; animals treated with single and triple doses of liposomal tuftsin, over 60% survival. In the control group, only 50% and 30% of the animals survived on day 45 and day 60, respectively.

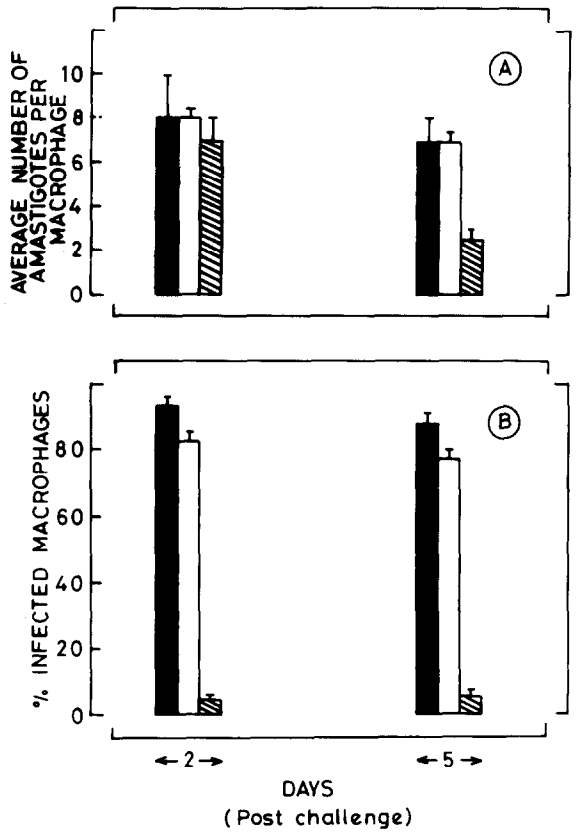

Fig.2. Susceptibility to *L. donovani* infection in vitro of macrophages derived from tuftsin-treated Balb/C mice. Values shown are mean of several determinations ± SD. (Solid bars) Macrophages derived from untreated animals; (open bars) macrophages derived from tuftsin-treated animals; (shaded bars) macrophages derived from liposomal tuftsin-treated animals.

animals with liposomal tuftsin considerably enhances their resistance to leishmania infection.

To analyse whether the increased resistance of the tuftsin-pretreated animals to the infection is due to the tuftsin-mediated activation of the host's macrophages [7], we examined the susceptibility of the peritoneal macrophages, derived from the pretreated animals, to *L. donovani* infection in vitro. Fig.2 shows that the infection of macrophages was markedly reduced when these cells were derived from the liposomal tuftsin-pretreated animals. Also, the parasite multiplication inside such macrophages was considerably decreased, as compared to the macrophages of untreated animals. These findings demonstrate that pretreatment of animals with tuftsin-bearing liposomes renders their macrophages refractory to *L. donovani* infections.

This study clearly shows that the efficacy of sodium stibogluconate against leishmania infection is greatly increased by encapsulating this drug in tuftsin-bearing liposomes. Also, it demonstrates that pretreatment of animals with tuftsin-bearing liposomes (free of drug) considerably enhances their resistance to this infection, possibly by activating the host's macrophages. It would therefore seem that these liposomes besides delivering the drug to the macrophages could also activate these cells for nonspecific killing of the pathogen, thus offering an additional advantage over the use of tuftsin-free liposomes as drug carriers in leishmania therapy [4].

Apart from *L. donovani*, several other pathogens, such as *Mycobacterium leprae, Mycobacterium tuberculosis*, human immunodefi-

ciency virus, etc., thrive inside the macrophages, which essentially results in an impairment of the host's nonspecific immune defence mechanisms against infections. In such situations, use of tuftsin-bearing liposomes as drug vehicles may prove highly advantageous, since these liposomes besides ensuring drug delivery to the target cells would also stimulate the killer activity of the phagocytic cells. We, therefore, conclude that these liposomes could find a useful application in macrophage-based infections, in general.

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