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Visceral Leishmaniasis: Drug Carrier System Characteristics and the Ability to Clear Parasites from the Liver, Spleen and Bone Marrow in *Leishmania donovani* Infected BALB/c Mice

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Abstract—The efficacy of various sodium stibogluconate formulations against *Leishmania donovani* has been investigated using a BALB/c mouse model of visceral leishmaniasis. Only one therapy, multiple dosing with drug loaded sonicated vesicles, liposomes or niosomes, was found to be effective against parasites in the liver, spleen and bone marrow. Other treatments significantly reduced parasite liver burdens but either failed to effect spleen and bone marrow parasites, or were effective but toxic. Prophylactic treatment with sodium stibogluconate preparations, six days before infection, reduced parasite multiplication in the liver (free, niosomal and liposomal drug) and the spleen (sonicated, drug loaded niosomes only), but had no suppressive effect on bone marrow parasite burdens compared with controls. These results indicate that in-vivo sodium stibogluconate persists in some compartments at parasiticidal concentrations and that failure to reach this concentration at some sites of infection such as bone marrow, is the cause of treatment failure and relapse.

Treatment with drug loaded carriers has been suggested as a method of improving the chemotherapy currently in use for visceral leishmaniasis (reviewed by Alving 1986). Carriers such as liposomes are preferentially taken up by cells of the reticuloendothial system (RES) and thus target the drug directly to the parasitized host cells. However, most intravenously administered drug carrier preparations are cleared from the circulation by the liver thereby preferentially targeting this organ at the expense of other RES sites (reviewed by Gregoriadis & Allison 1980). *Leishmania donovani* parasites are predominantly found in the liver, spleen, and bone marrow, therefore, following carrier mediated chemotherapy, sparing of parasites in the spleen and bone marrow might be expected because of the avidity of the liver for exogenous particles. Results of our recent study using a mouse model have indeed shown this to be the case (Carter et al 1988). Treatment with liposomal, niosomal or free sodium stibogluconate reduced hepatic parasite burdens but not those of spleen and bone marrow. Clinical data also suggests that removal of parasites from tissues other than the liver may also be difficult in man. Wickramasinghe et al (1987) recovered *L. donovani* amastigotes from bone marrow aspirates of two of three patients who had received daily injections of sodium stibogluconate for two weeks. In addition, despite new treatment regimens, 2–8% of patients still relapse after stibogluconate chemotherapy (WHO 1984). Attempts to target liposomes to cells other than those in the RES by altering the size of the carrier (Roerdink et al 1981; Scherphof 1982; Scherphof et al 1983) or by RES blockade (Abra et al 1980; Abra & Hunt 1982; Proffitt et al 1983) although generally unsuccessful, modified the distribution of the particles within the RES. It was therefore decided to study the influence of carrier formulation and drug dosage schedule on spleen and bone marrow parasites.

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Materials and Methods

Materials

Sodium stibogluconate (Pentostam) equivalent to 0·32 mg Sb mg^{-1} was obtained from the Wellcome Foundation, UK. Synthetic (>99% pure) L-α-phosphatidylcholine (DPPC) and ash free cholestrol (CHOL) were obtained from Sigma. The single chain non-ionic surfactant (Surfactant 1, Baillie et al 1985) was obtained from L'Oreal, France. Liposomes and niosomes comprised 70% amphiphile (DPPC or non-ionic surfactant) and 30% CHOL, on a molar basis. The method of producing niosomes and liposomes has already been described (Baillie et al 1985). Briefly, multilamellar liposomes were produced by dissolving 150 μmol of DPPC/CHOL mixture in 10 mL chloroform in a 50 mL round-bottomed flask. The solvent was removed at room temperature (20°C), under reduced pressure and the resulting film hydrated with 5 mL drug solution at 50–60°C with gentle agitation. Sonicated liposomes were produced by probe sonicating the multilamellar preparation at 60°C for 3 min using an MSE 150W sonicator, fitted with a titanium probe, set at approximately 10–15% of maximum power output. Multilamellar niosomes were produced by dissolving 450 μmol surfactant/CHOL in 20 mL diethylether and injecting the mixture slowly (0·24 mL min^{-1}) through a 14 G needle into 4 mL of drug solution maintained at 60°C. Sonicated niosomes were produced by sonication of a multilamellar niosome suspension which was prepared as described above for multilamellar liposomes. Throughout the text, vesicles, liposomes and niosomes, unless described as sonicated, were unsonicated, multilamellar preparations.

The sonicated vesicular suspensions were sized by photon

correlation spectroscopy at a 90° scattering angle using a Malvern Instruments Type 7027, 60 channel correlator in conjunction with a He/Ne laser (Siemens), wavelength 632·8 nm, nominal power output 40 mW.

Animals

In-house bred 8–10 week-old female BALB/c mice (20–25 g) were used and in-house bred golden syrian hamsters (*Mesocricetus auratus*) which originated from the Bantim and Kingman colony (The Field Station, Aldborough, Hull) were used to maintain the parasite.

Parasite

Leishmania donovani, strain LV9, was harvested and maintained as described by Carter et al (1988). Mice were injected via the tail vein (without anaesthetic) with $1\text{-}2 \times 10^7$ *L. donovani* amastigote parasites in 0·2 mL.

Parasite distribution

The method of determining parasite burdens (numbers/1000 host cell nuclei) in the liver, spleen and bone marrow has been described by Carter et al (1988). The number of Leishman-Donovan units (LDU) was calculated per organ for the liver and spleen using the formula: LDU = number of amastigotes per 1000 host cell nuclei × the organ weight (g) (Bradley & Kirkley 1977).

Experimental design

Parasite suppression. In a typical experiment, infected mice were treated via the tail vein (without anaesthetic) on days 7 and 8 post-infection with 0·2 mL of one of the following: distilled water (controls); sodium stibogluconate solution (5, 45 or 100 mg antimony mL^{-1}); liposomal or niosomal drug (0·8 mg antimony mL^{-1}). On day 14 post-infection, parasite numbers in the spleen, liver and bone marrow of control and drug treated mice were determined. There were four variations on this standard protocol.

1. Animals, treated on days 7 and 8 post-infection with either 0·2 mL of water (controls) or free drug (5 mg antimony mL^{-1}), were killed on day 32 post-infection. After taking impression smears of spleen, liver and bone marrow for parasite burden determination, parasites were harvested using an identical method to that described by Carter et al (1988) for hamsters, from spleens pooled from these drug treated animals. These parasites were used to infect groups of mice which were then treated as the standard protocol above.

2. Before the injection of vesicular drug, mice were injected with 0·2 mL of "empty" vesicles which contained 300 mM glucose solution.

3. Mice were treated with either 2, 3, 4 or 5 daily drug doses, starting on day 7 post-infection, and then killed six days after the last injection.

4. Uninfected mice were treated on days 1 and 2 with 0·2 mL of one of the following: water (controls), free drug (5 mg antimony mL^{-1}) or, carrier forms of the drug (niosomal or liposomal drug, 0·8 mg antimony mL^{-1}). Six days later the mice were infected with $1\text{-}2 \times 10^7$ *L. donovani* amastigotes and their parasite burdens determined on day 14 post-infection.

Presentation and statistical analysis of data

Parasite burden of the spleen and liver was expressed as mean LDU/organ ± standard error, whereas the bone marrow counts were expressed as mean number of parasites/1000 host cell nuclei ± standard error. Parasite data were analysed using an independent *t*-test or a one way analysis of variance on the $\log_{10}$ transformed data.

Results

Dosing with either free sodium stibogluconate solution (equivalent to a total dose of 80–100 mg $Sb^V kg^{-1}$) or vesicular drug (equivalent to a total dose of 12·8–16 mg $Sb^V kg^{-1}$) significantly reduced parasite numbers in the liver ($P < 0.001$) but had little effect on those residing in the spleen and bone marrow (Fig. 1). At these dose levels the carrier forms of the drug caused a significantly greater reduction than the free drug in the liver parasite burden.

Treatment of mice infected with parasite numbers obtained from the spleens of drug-treated animals gave similar results, i.e. parasites numbers in the liver were greatly reduced but those in the spleen and bone marrow were unaffected (Fig. 2).

The supressive effect of free drug on parasites in all three sites was dose dependent (Fig. 3). However, even at the highest dose, equivalent to a total dose of 1600–2000 mg $Sb^V kg^{-1}$, which completely cleared parasites from the liver, amastigotes were still present in the spleen and bone marrow. Although the reduction in parasite numbers achieved at these two sites was significant compared with controls ($P < 0.001$ spleen; $P < 0.001$ bone marrow) this high Sb^V dose was toxic and caused a 66% mortality.

Pre-dosing animals with "empty" liposomes before the injection of drug-loaded liposomes did not influence their efficacy against parasites in the spleen and bone marrow compared with controls ($P > 0.10$, Fig. 4).

Multiple dosing with large multilamellar niosomes had an additive effect on the parasite suppression in both the liver ($P < 0.001$) and spleen ($P < 0.01$) compared with controls (Fig. 5) but had no effect on bone marrow parasites

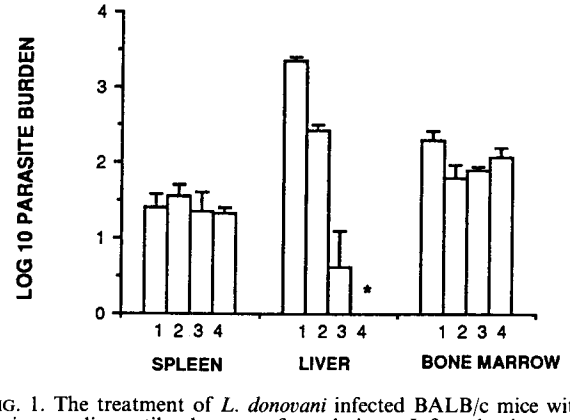

FIG. 1. The treatment of *L. donovani* infected BALB/c mice with various sodium stibogluconate formulations. Infected mice were treated on days 7 and 8 post-infection with one of the following: water, controls (1), free drug, equivalent to a total dose of 80–100 mg $Sb^V kg^{-1}$ (2), or multilamellar vesicular drug, equivalent to a total dose of 12·8–16 mg $Sb^V kg^{-1}$, in the form of niosomes (3) or liposomes (4). Animals were killed on day 14 post-infection and parasite burdens of the spleen, liver and bone marrow assessed. *no parasites found.

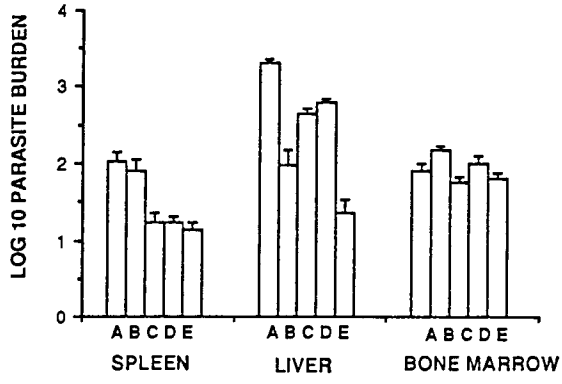

FIG. 2. Treatment of *L. donovani* infected BALB/c mice with free sodium stibogluconate. BALB/c mice were infected with amastigotes harvested from either hamster (A and B) or mouse (C, D, and E) spleens. C group mice were infected with parasites harvested from group A mice (water treated), whereas mice in groups D and E were infected with parasites harvested from group B mice (drug treated). Animals were treated on days 7 and 8 post-infection with either water, (A, C and D), or free drug, equivalent to a total dose of 80–100 mg Sb^v kg^{-1}(B and E). Parasite numbers were assessed on either day 14 (C, D and E) or day 32 (A and B) post-infection.

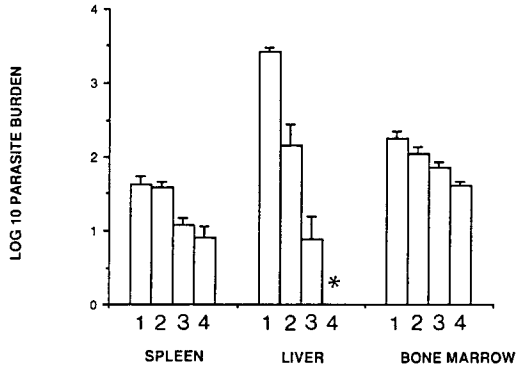

FIG. 3. The effect of free drug dose on *L. donovani* parasite suppression in BALB/c mice. Infected mice were treated on days 7 and 8 post-infection with either water, controls, (1) or free sodium stibogluconate equivalent to a total dose of 80–100 (2), 1200–1500 (3), or 1600–2000 (4) mg Sb^v kg^{-1}. On day 14 post-infection, mice were killed and parasite numbers assessed. *no parasites found.

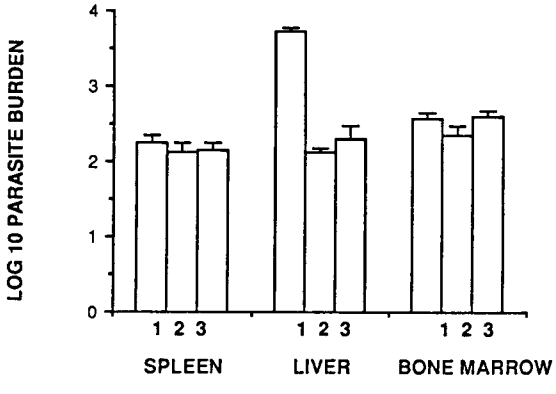

FIG. 4. The effect of pretreatment with empty liposomes (R.E.S. blockade) on liposomal stibogluconate therapy. Mice, infected with *L. donovani*, were treated on days 7 and 8 post-infection with one of the following: water, controls, (1), liposomal drug, equivalent to a total dose of 12·8–16 mg Sb^v kg^{-1}, preceded by a dose of "empty" liposomes (2) or liposomal drug only, equivalent to a total dose of 12·8–16 mg Sb^v kg^{-1} (3). Mice were killed on day 14 post-infection and parasite burdens assessed.

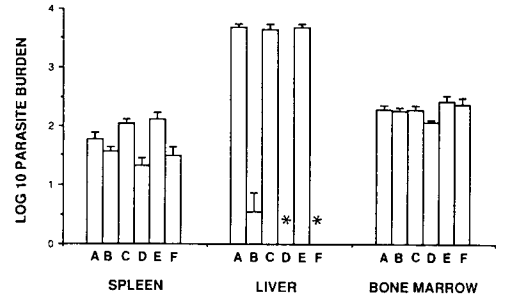

FIG. 5. The effect of multiple dosing with stibogluconate loaded, large, multilamellar niosomes on parasite suppression in *L. donovani* infected BALB/c mice. Infected mice were treated with three (A and B), four (C and D) or five (E and F) doses, one dose/day from day 7 post-infection, of either water, controls, (A, C and E), or a multilamellar niosomal drug suspension (B, D and F), equivalent to 6·4–8 mg Sb^v kg^{-1} day^{-1}. Mice were killed 6 days after their last injection and parasite burdens determined. *no parasites found.

($P > 0.05$). However, multiple dosing with sonicated liposomes and niosomes had an additive suppressive effect on parasite numbers in all three sites compared with controls (Fig. 6; $P < 0.001$ spleen, liver and bone marrow).

Pre-dosing with the free or carrier forms of the drug 6 days before infection (Fig. 7) caused a significant reduction in liver burdens compared with controls ($P < 0.001$) and in this respect, just as with the standard treatment protocol (Fig. 1),

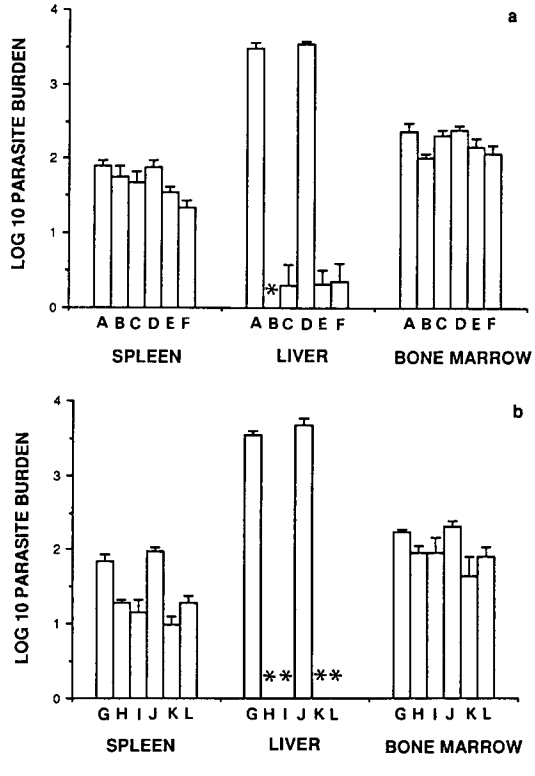

FIG. 6. The effect of multiple dosing with stibogluconate loaded, sonicated niosomes or liposomes on parasite suppression in *L. donovani* infected BALB/c mice. Infected mice were treated with (a), two (A, B and C) or three (D, E and F) or (b), four (G, H and I) or five (J, K and L) doses, one dose/day from day 7 post-infection, of either water, controls, (A, D, G and J) or sonicated vesicular drug, equivalent to 6·4–8 mg Sb^v kg^{-1} day^{-1}; niosomal (B, E, H and K) or liposomal (C, F, I and L). Parasite burdens were determined 6 days after the last injection. *no parasites found.

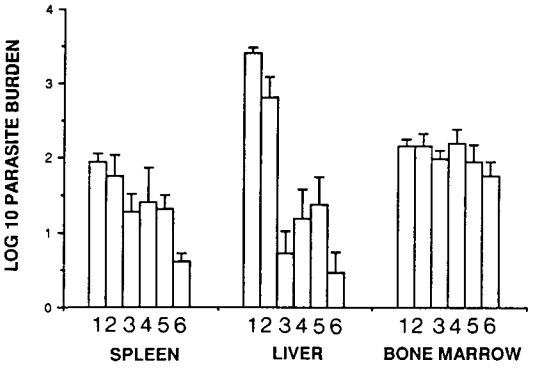

FIG. 7. The effect of pretreatment with various stibogluconate formulations on the parasite burdens of *L. donovani* infected BALB/c mice. Uninfected mice were treated on days 1 and 2 with one of the following: water, controls, (1), free drug, equivalent to a total dose of 80–100 mg Sb^v kg^{-1}, (2), or vesicular drug, equivalent to a total dose of 12·8–16 mg Sb^v kg^{-1}, in the form of multilamellar niosomes (3) or liposomes (4) or, sonicated niosomes (5) or liposomes (6). Mice were infected on day 8 and parasite burdens determined on day 14 post-infection.

the vesicular drug was more active than the free form, being more suppressive at a fifth of the dose. Only pre-treatment with sonicated niosomal drug significantly reduced splenic parasite numbers ($P < 0{\cdot}001$) and no pre-treatment reduced bone marrow parasite numbers.

The mean hydrodynamic diameters of the sonicated vesicles used in these studies were found to be: niosomes, 106 nm, polydispersity factor, 0·26; liposomes, 116 nm, polydispersity factor, 0·30. The mean vesicular hydrodynamic diameters in the unsonicated preparations, lying in the range 350–850 nm, were similar to those previously described (Carter et al 1988) for stibogluconate loaded, multilamellar niosomes and liposomes and were thus significantly larger than those of sonicated vesicles. Derived from the z average diffusion coefficient (Koppel 1972), all of these diameters are weighted towards the larger vesicles in the sample so that a large proportion of the sonicated vesicles will have diameters of < 100 nm.

Discussion

The results of this study demonstrate the difficulty of obtaining parasiticidal concentrations of sodium stibogluconate in the spleen and bone marrow. There is no reason to expect parasites in these sites to be stibogluconate resistant. The results of chemotherapy were identical in mice infected with parasites from either the spleens of hamsters or stibogluconate-treated mice. In addition, the spleen and bone marrow parasite suppression achieved by multiple dosing with vesicular drug or high dose free drug also indicated that parasites in these sites were stibogluconate sensitive. The evidence therefore suggests that the apparent resistance to Sb^v chemotherapy demonstrated by parasites in these two organs derives from anatomical features such that the drug simply did not reach them, or was present in sub-parasiticidal concentrations.

Increasing the free drug dose up to a total dose of 1600–2000 mg $Sb^v kg^{-1}$ did reduce spleen and bone marrow parasite burdens, although it did not completely clear parasites from these two sites, and was associated with a 66% mortality. This is an excessively high dose by human standards since the present guidelines suggest that patients should be treated with 20 mg Sb^v kg^{-1}, up to a maximum of 850 mg (WHO 1984). Higher doses are associated with feelings of malaise, vomiting, anorexia and electrocardiographic abnormalities (Bryceson 1987).

It would appear that reducing the size of the carrier and RES blockade may, in combination, suppress *L. donovani* infection in spleen and bone marrow. RES blockade alone seemed ineffective since on the basis of parasite suppression, pre-dosing with "empty" liposomes did not cause a "spill over" effect of drug loaded carrier from liver to spleen and bone marrow parasites compared with controls. However there was the possibility that the pre-dose was insufficient to saturate the phagocytic capabilities of the Kupffer cells. Similarly, simply reducing vesicle size did not improve chemotherapy since two doses of small drug loaded liposomes (equivalent to 6·4–8 mg Sb^v kg^{-1} $dose^{-1}$) did not consistently reduce parasite burdens in the spleen and bone marrow, although a significant reduction in liver burdens was obtained. Multiple dosing with vesicles, which may partially blockade the RES, did target drug to the spleen (large and small vesicles) and bone marrow (small vesicles only). The latter observation implies that for access to bone marrow parasites, vesicular size is also important. The nature of the carrier vesicle, phospholipid or non-ionic surfactant, is known not to exert any great influence on ability to deliver drug to the liver (Hunter et al 1988) and apparently also exerts little influence on delivery to the less accessible tissues of the RES.

It has been shown that large vesicles (> 100 nm) are unable to pass through the fenestrations in the liver's sinusoidal epithelium (Scherphof et al 1983) thus increasing the probability of their uptake by Kupffer cells. Small vesicles (< 100 nm) have a higher chance of circulating to the spleen as they can pass through these fenestrations although it has been shown that small particles are also taken up by liver endothelial cells (Spanjer et al 1986). Increasing the number of doses of vesicular drug may simply saturate the phagocytic capabilities of the liver allowing access to other organs in the RES. It has been suggested that different hepatic binding sites exist for small and large liposomes (Abra & Hunt 1982). The suppressive effect on splenic parasite numbers of multiple dosing with large or small drug loaded vesicles suggests that the two types of binding sites in the liver become saturated at similar rates. If by analogy, there are different small and large binding sites in the spleen, the suppressive effect on bone marow parasite burdens achieved only by multiple dosing with small drug loaded vesicles suggests that either, binding sites for small particles are more easily saturated in the spleen, or that there is a size limit on particles reaching the bone marrow.

Pre-dosing animals with free and vesicular drug six days before infection suggested that parasiticidal quantities of drug persisted in the liver (free, drug loaded, multilamellar and sonicated vesicles) and spleen (sonicated niosomal drug), since parasite burdens in pre-dosed animals were lower than in the controls. It has been shown that after dosing with vesicular stibogluconate, antimony persists in murine liver for much longer periods than after free drug

administration (Baillie et al 1986) and the greater efficacy of the carrier form of the drug against liver parasites can be explained on a pharmacokinetic basis (Dolan et al 1988).

The apparent persistence of free drug described here is at variance with its rapid urinary excretion (Rees et al 1980) but pharmacokinetic data obtained in man (Pamplin et al 1981) and more recently by Chulay et al (1988) provide evidence for antimony accumulation. After administration of stibogluconate or meglumine antimonate, the plasma concentration of antimony is best described by a two compartment model with a rapid elimination phase, $t_{\frac{1}{2}}$ = 2 h but more importantly a slow elimination phase where $t_{\frac{1}{2}}$ = 76 h (Chulay et al 1988). If the drug that persisted after pre-dosing here was located in the lysosomal compartment, relatively low quantities would be sufficient to inhibit incoming parasites. The greater efficacy of pre-dosing with vesicular drug is not inconsistent with lysosomal accumulation.

Cases of relapse of visceral leishmaniasis in man following chemotherapy is perhaps a result of parasite persistence in deep sites such as the bone marrow (Wickramasinghe et al 1987). The results of this study using the BALB/c mouse as a model of visceral leishmaniasis showed that it is possible to attain parasiticidal concentrations of stibogluconate in the spleen and bone marrow by multiple dosing with small, drug loaded vesicles. Most of the extant literature on the liposomal therapy of experimental visceral leishmaniasis describes the use of large multilamellar vesicles and in view of the apparent inability of such vesicles to reach the bone marrow, any such therapy in man should address this problem by using small vesicles as a component of the delivery system.

Acknowledgements

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