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Treatment of Experimental Cutaneous Leishmaniasis with Liposome-Intercalated Amphotericin B

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The therapeutic efficacy of a liposomal preparation of amphotericin B was evaluated in two murine models of cutaneous leishmaniasis. No significant decrease in tissue parasite density was observed in C57BL/6 mice after systemic treatment instituted seven days after footpad inoculation with *Leishmania tropica*; in contrast, BALB/c mice showed a modest response.

Leishmania spp. are pathogenic protozoa which can produce a spectrum of disease collectively referred to as leishmaniasis. The clinical manifestations vary from species to species of parasite and range from self-healing cutaneous ulceration (*Leishmania tropica* subsp. *major*) to erosive mucosal involvement (*Leishmania brasiliensis* complex) to fatal visceral dissemination (*Leishmania donovani*) (7). Although pentavalent antimonial agents remain the primary chemotherapeutic agents for all forms of human leishmaniasis (7), drug failure in some cases has prompted a search for other therapeutic measures and agents (13, 15). Because the aflagellate amastigote stage of *Leishmania* spp., which proliferate in mammalian hosts, is an obligate intracellular parasite of macrophages, one potential treatment strategy is the incorporation of therapeutic agents into liposomes which are preferentially taken up by tissue macrophages (15). This approach has been shown to improve the efficacy of antimonial agents in the treatment of experimental infections caused by *L. donovani* (1, 3, 12) and *L. tropica* (11).

Clinical experience has indicated that amphotericin B (AMB) may be useful in the treatment of visceral and mucosal leishmaniasis (7, 14). Since sterols, and specifically ergosterol, have been demonstrated in the plasma membranes of *Leishmania* spp. (2), it seems likely that the antileishmanial mechanism of AMB is similar to that which occurs with fungi (8). Systemic administration of AMB for fungal infection is frequently accompanied by severe adverse reactions (8). It has been shown that liposome intercalation reduces the toxicity of systemically administered AMB and improves survival in murine models of histoplasmosis (17), cryptococcosis (4), and disseminated candidiasis (6), presumably because higher doses of AMB are tolerated. In the only published study of the use of liposomal AMB for experimental leishmaniasis, New reported that a regimen of three daily injections of hydrogenated lecithin liposomes containing AMB at a dose of 2.5 mg/kg per day was more effective than the same dose of free AMB in clearing *L. donovani* amastigotes from the livers of NMRI mice (10).

We have developed a liposomal preparation of AMB composed of small unilamellar vesicles (C. Tremblay, M. Barza, C. Fiore, and F. Szoka, submitted for publication). Their small size (0.06 to 0.1 μm) is advantageous because it permits filter sterilization and may lessen the likelihood of pulmonary embolism in contrast to the larger liposomes used by other groups (4, 6, 10, 17). We now report the efficacy of this preparation against *L. tropica* footpad infection in two strains of mice which represent polar extremes in host defense against this parasite. In C57BL/6 mice, there is local proliferation of *L. tropica* at the site of inoculation, after which there is spontaneous cure and healing within 5 to 8 weeks (18), whereas in BALB/c mice, there is dissemination of the organism and ultimately death from infection (5). To evaluate efficacy, we determined the density of parasites in infected footpad tissue, a quantitative method not employed in previous reports in which liposomal drugs were studied in experimental leishmaniasis (1, 3, 10–12).

AMB was obtained from the Squibb Institute, Princeton, N.J. as the commercial preparation containing sodium desoxycholate. Small unilamellar vesicles with or without AMB were prepared from egg phosphatidylcholine (Avanti Polar Lipids, Birmingham, Ala.), cholesterol, and tocopherol succinate (both from Sigma Chemical Co., St. Louis, Mo.) in a molar ratio of 5:3:1 by previously published methods (16). The liposomes were sterilized by filtration through a 0.22-μm filter (Millipore Corp., Bedford, Mass.). The final drug concentration in the liposome dispersion was determined by a standard absorbance technique with a standard curve of solid AMB diluted in methanol. Both the commercial AMB and the liposomal preparations were freshly diluted each week in sterile distilled water to final concentrations such that each dose would be contained in 0.5 ml.

Amastigotes of *L. tropica*, NIH S strain (9), were freshly harvested from infected footpads of BALB/c mice by previously described methods (12a). Separate groups of female C57BL/6 and BALB/c mice (18 to 20 g; both obtained from Charles River Breeding Laboratories, Wilmington, Mass.) were infected in one footpad with a subcutaneous inoculum of 2×10^5 live parasites. Seven days later, groups of five to seven mice were given one of the following regimens: (i) sterile distilled water; (ii) empty liposomes; (iii) commercial AMB, 1 mg/kg; (iv) liposomal AMB, 1 mg/kg; or (v) liposomal AMB, 5 mg/kg. All agents were administered by intraperitoneal injection every 3 days for a total of six doses. Twenty-four hours after the last dose, the animals were killed, and the nodular footpad lesions were excised. The specimens were weighed, minced, and processed through a no. 50 stainless steel screen into 2 ml of Hanks buffered salt

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TABLE 1. Parasite densities and total counts of *L. tropica* in footpads of mice treated with various formulations of AMB[a]

Treatment group	Mean ± SEM amastigotes (log_{10}) in following mouse strain:			
	BALB/c		$C57B_1/6$	
	Per mg of tissue	Per biopsy	Per mg of tissue	Per biopsy
Distilled water	5.39 ± 0.10	7.4 ± 0.13	5.45 ± 0.14	7.0 ± 0.12
Empty liposomes	5.48 ± 0.13	7.2 ± 0.10	5.62 ± 0.10	7.2 ± 0.14
AMB				
Commercial (1 mg/kg per dose)	4.84 ± 0.12	6.8 ± 0.17	5.36 ± 0.13	6.8 ± 0.15
Liposomal (1 mg/kg per dose)	4.72 ± 0.16	6.5 ± 0.22	5.49 ± 0.11	7.0 ± 0.14
Liposomal (5 mg/kg per dose)	4.62 ± 0.18	6.4 ± 0.25	5.44 ± 0.10	6.8 ± 0.10

[a] Data are the means ± standard errors of the mean of five to seven values. Footpads were examined after 18 days of treatment.

solution. Subsequent passage of the tissue suspension through a 25-gauge needle disrupted the macrophages and released the free parasites. The suspensions were mixed with equal volumes of a known concentration of chicken erythrocytes. The ratio of parasites to 500 nucleated erythrocytes was determined by examining fixed slides stained with Wright-Giesma (Diff-Quik, Dade Diagnostics, Inc., Aquada, P.R.), and the number of parasites was calculated from this ratio. Parasite density was expressed as log_{10} of the number of amastigotes per milligram of tissue.

Seven days after infection, moderate swelling and erythema were detectable in the footpads of both C57BL/6 and BALB/c mice. During the subsequent 18-day treatment period, footpad lesions progressed, but no deaths occurred in any of the groups. Table 1 outlines the mean ± standard error of the mean of the log_{10} of the number of amastigotes per milligram of footpad specimen as well as the mean ± standard error of the mean log_{10} of total amastigotes in the entire tissue lesion excised from animals of each treatment group. In the C57BL/6 strain, there was no significant effect of any of the AMB regimens on either of these indices compared with controls. In the BALB/c mice, however, each of the AMB preparations produced a mild to moderate antileishmanial effect, reducing the parasite densities by 0.6 to 0.8 log_{10}/mg of tissue and the total parasite counts by 0.6 to 1.0 log_{10}. The parasite densities observed in BALB/c footpads after treatment with liposomal AMB at 5 mg/kg (regimen v) were significantly different from control values (regimen i) by the unpaired *t* test when either logarithmic values ($P < 0.001$) or natural numbers ($P < 0.05$) were compared. Although ingestion of empty liposomes might theoretically activate macrophages to kill intracellular parasites, drug-free liposomes did not result in an antileishmanial effect in vivo in either animal model (Table 1).

In summary, no formulation of AMB tested had an effect on cutaneous leishmaniasis in immunocompetent mice (C57BL/6). In contrast, in the immunodeficient BALB/c strain, there was a slight effect of AMB. This effect was not significantly greater at higher than at lower doses or with liposomal as opposed to free drug.

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