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Antileishmanial Activity of Liposome-Encapsulated Amphotericin B in Hamsters and Monkeys

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Visceral leishmaniasis (kala-azar) results from parasitization of the macrophages of the liver, spleen, and the rest of the visceral reticuloendothelial system with *Leishmania donovani*. Pentavalent antimony is the drug of choice for leishmaniasis chemotherapy; amphotericin B (AmB) is active but is rarely used, because of drug toxicity. AmB encapsulated within macrophage-directed carriers (liposomes) has been used to treat humans with systemic mycoses complicating neoplastic diseases; dosages of up to 5 mg of encapsulated AmB per kg per day for >14 days are without apparent kidney or liver toxicity. In the present work, >99% of *L. donovani* parasites were eliminated from the liver and spleen of infected hamsters by one administration of 1.5 to 11 mg of liposome-encapsulated AmB (L-Amb) per kg. A total of 98 to 99% of hepatosplenic parasites were eliminated from squirrel monkeys by three administrations of 4 mg of L-AmB per kg. L-AmB was 170 to 750 times as active as antimony in hamsters, and approximately 60 times as active as antimony in monkeys. The demonstration that apparently nontoxic human dosages of L-AmB eliminate essentially all hepatosplenic parasites in hamster and primate models suggests that this preparation should be considered for clinical trial against kala-azar.

Leishmania parasites are obligate intramacrophage microorganisms in the mammalian host. Clinical manifestations of visceral leishmaniasis result from multiplication of *Leishmania donovani* amastigotes within the macrophages of the liver, spleen, and the rest of the visceral reticuloendothelial system. The remarkably circumscribed environment in which amastigotes are viable has led to a rational approach to the development of new antileishmanial formulations: the encapsulation of antileishmanial agents within macrophage-directed carriers such as liposomes (artificial lipid membranes). Administration of liposome-encapsulated agents theoretically increases the therapeutic index of the agent in two ways. (i) The uptake of the carrier by macrophages should lead to delivery of large quantities of drug to the macrophage-contained organisms, and (ii) the relatively low uptake of carrier by the organs to which the drug is toxic should lessen toxicity.

Treatment for visceral leishmaniasis is pentavalent antimony (Sb) in the form of sodium stibogluconate (Pentostam) or meglumine antimonate (Glucantime) at a dosage of 20 mg of Sb per kg per day for at least 20 days. Antimony treatment failures can be successfully treated with amphotericin B (AmB), but the nephrotoxicity of this agent has generally led to its rejection in favor of repeated courses of Sb. The spectacular success of liposome-encapsulated Sb—the preparation was up to 1,000 times more active than free Sb against visceral leishmaniasis in rodents (1, 3, 13)—suggested that liposomal Sb should be considered as primary or secondary therapy for kala-azar.

AmB is primarily known as the major agent for the treatment of systemic mycoses. The reason for the activity of AmB against such apparently disparate organisms as fungi and *Leishmania* parasites is that on the basis of sterols the organisms are in fact similar. The major demethylated sterol in mammalian cell membranes is cholesterol, in fungi it is ergosterol; in *Leishmania* organisms it is the ergosterol precursors episterol and 5-dehydroepisterol (4). AmB preferentially intercalates with fungal ergosterol, and presumably *Leishmania* episterols, compared to cholesterol, and thereby disrupts microorganism membranes (6). Several investigators have formulated liposomal preparations of AmB (L-AmB) to decrease the toxicity associated with treatment of systemic mycoses (10, 15). In mice, the anticandidal activity of L-AmB was approximately equal to that of free AmB when used at similar concentrations (8, 10). However, there was no evident drug toxicity in animals administered 12 mg of L-AmB per kg, whereas the 50% lethal dose of free AmB was 1.2 mg/kg. The ability to administer higher dosages of L-AmB made this formulation more active than free AmB against disseminated candidiasis in neutropenic mice (8, 10). New et al. investigated the efficacy of AmB encapsulated within liposomes of various compositions against visceral leishmaniasis in mice (12), and Panosian et al. found that their L-AmB preparation was at best slightly effective against murine cutaneous leishmaniasis (14).

Lopez-Berestein et al. (9) used their L-Amb preparation in human trials. ^{99M}TC-labeled liposomes were localized primarily in the liver (45%) and the spleen (26%) (9). In initial studies in which 0.8 to 1.0 mg of L-AmB per kg per day was administered, L-AmB was without demonstrable kidney or liver toxicity and was therefore less toxic than neat AmB, of which the dosage of 1 mg/kg per day characteristically results in nephrotoxicity (7). Ongoing studies have shown that patients receiving up to 5 mg of L-AmB per kg per day for >14 days similarly tolerate treatment without abnormalities of kidney or liver function (G. Lopez-Berestein, submitted for publication).

We evaluated the activity of this clinical preparation of L-AmB against visceral leishmaniasis in rodents and nonhuman primates.

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MATERIALS AND METHODS

Hamster experiments. The activity of antileishmanial drugs against *L. donovani* infections in hamsters was determined as previously described (5). In brief, 60- to 80-g hamsters (*Mesocricetus auratus*) were administered intracardiac injections of approximately 10^7 *L. donovani* amastigotes (Khartoum strain, WR 378). Three or ten days later, the six animals in each group were administered saline (negative controls), meglumine antimonate, liposomal meglumine antimonate, free AmB, L-Amb, or saline liposomes. All preparations were administered via intracardiac injection in less than 1 min, except for meglumine antimonate, which was given intramuscularly. The animals were killed 4 days after this one administration of drug, and the ratio of parasites per host cell nucleus in the liver and spleen was determined from Giemsa-stained impression smears of those organs. This ratio was multiplied by 200,000 (5; the approximate number of host cell nuclei per milligram of organ) and the weight of the organ in milligrams to determine the number of parasites per liver or spleen.

Monkey experiments. The activity of agents against *L. donovani* infections in squirrel monkeys (*Saimiri sciureus*) was determined as recently described (11). Sixteen animals were used. Because only 14 males were available, 2 females were used; these two animals received saline-containing liposomes. Approximately 700-g animals were injected intravenously with 5×10^8 *L. donovani* amastigotes obtained from infected hamsters. Infected monkeys were administered saline, meglumine antimonate, AmB, L-AmB, or saline liposomes intravenously over 1 min or meglumine antimonate intramuscularly daily for 6 days (i.e., on days 17 to 23) and were killed on day 27 (day 11 after the initiation of treatment), at which time the number of parasites per host cell nucleus in the liver and spleen was determined. Blood was drawn for complete blood counts before infection, before therapy, and after therapy on the day of death. The monkeys were necropsied, and all major organs were examined. Selected tissues (heart, liver, spleen, kidneys, lung, and brain) were examined by light microscopy of hematoxylin and eosin staining.

Computations. For each experimental group, the number of parasites per host cell nucleus in the liver or spleen was determined and expressed as a percentage of the number of parasites per host cell nucleus in saline-treated controls. The percent suppression for an experimental group equaled: 100 − percent control parasites in an experimental group. The ED_{50} or ED_{90} (dosage of drug expected to suppress 50 or 90% of amastigotes in an organ when compared with controls) was calculated from a plot of percent control amastigotes versus drug dosage. Dosage of meglumine antimonate refers to the amount of antimony in the form of meglumine antimonate administered to an animal. Sb constitutes approximately 30% of meglumine antimonate by weight. The G value is the ratio of the ED_{50} (or ED_{90}) of the positive control drug meglumine antimonate to the ED_{50} (or ED_{90}) of the test drug and represents the fold increase in activity of the drug relative to meglumine antimonate.

Drug. L-AmB was prepared by incorporation of AmB (E. R. Squibb & Sons, Princeton, N.J.) into the lipid bilayer of dimyristoylphosphatidylglycerol-dimyristoylphosphatidylcholine liposomes (lipid ratio, 3:7) as previously described (7). In brief, multilamellar vesicles were prepared by adding a methanolic solution of the drug to the lipids in chloroform, evaporating the organic solvents in a rotary evaporator, suspending the dried residue in 0.9% NaCl, and washing by centrifugation. The AmB content of the washed preparation was determined by high-performance liquid chromatography. The efficiency of trapping AmB in such a preparation is typically >90% (7), and there is typically 1 mg of AmB per 10 mg of liposomal lipids. L-AmB was used within 2 months of preparation; leakage of AmB from the liposomes is less than 5% over this time (G.L.-B., unpublished observations). Liposomal meglumine antimonate was prepared as previously described (2). In brief, dipalmitoylphosphatidylcholine, cholesterol, dicetylphosphate, and β-tocopherol in chloroform were mixed in a ratio of 1.0:0.75:0.1:0.01 in a rotary evaporator, and the solvent was evaporated. The dried lipids were then swollen with sufficient 0.308 M meglumine antimonate such that the phospholipid was 10 mM, and the untrapped drug was washed away by centrifugation. Approximately 5 to 7% of the drug is entrapped in the liposomes by these procedures (2). The liposomal formulation was used within 2 weeks of preparation, during which time little leakage of meglumine antimonate occurs (2). There is approximately 2 mg of Sb in the form of meglumine antimonate per 7 mg of liposomal phospholipid in such preparations. The antimony content was determined by atomic absorption. To provide dilutions of encapsulated drugs, the liposomal preparations were diluted with saline.

Liposomes containing saline rather than drug were prepared by the same procedures as those used to prepare drug-containing liposomes. Saline liposomes comparable to meglumine antimonate liposomes were used in the hamster experiments in which both meglumine antimonate liposomes and AmB liposomes were administered. Saline liposomes comparable to AmB liposomes were used in the monkey experiments in which only AmB liposomes were administered. Saline liposomes were administered at a lipid dosage equal to the dosage in the undiluted drug-liposome preparation.

Unencapsulated AmB in the form of Fungizone was purchased from Squibb. Unencapsulated meglamine antimonate (Glucantime) was obtained from Rhone Poulenc, Paris, France. The AmB content of Fungizone and the Sb content of unencapsulated meglamine antimonate were calculated from the information on the package labels.

RESULTS

Hamster experiments. The antileishmanial activity of L-AmB was first tested in our standard model in which hamsters are infected with *L. donovani* for 3 days before drug administration (5). Pentavalent antimony in the form of meglumine antimonate was active in this model, and liposomal meglumine antimonate was 832 to 933 times as active as the unencapsulated drug (Table 1), in agreement with previous reports (1). The activity of unencapsulated AmB in this model has not been reported. Unencapsulated Amb was active against both hepatic and splenic parasites and was approximately 150 times as active as unencapsulated meglumine antimonate against parasites in both organs. L-AmB was 331 to 750 as active as meglumine antimonate and was therefore two to five times more active than unencapsulated AmB.

The activity of L-AmB was next ascertained in hamsters infected for 10 days before drug administration. Because drugs were expected to be less active in hamsters with a higher parasite burden, this experiment was designed to ascertain whether the formulation is active under more stringent conditions. The higher ED_{50} and the lack of an

TABLE 1. Activity of liposomal preparations against *L. donovani* in hamsters infected for 3 days[a]

Drug	Dosage (mg/kg)	Liver amastigotes			Spleen amastigotes		
		% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}/G_{90}	% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{90}
Sb[b]	416	99 ± 0.4	75/232	1/1	91 ± 7	—[c]/≅416	1
	104	77 ± 3			61 ± 10		
	52	17 ± 7			72 ± 9		
	13	3 ± 11			0		
Liposomal Sb[b]	50	99 ± 0	—/0.3	—/933	99 ± 0.5	—/0.5	832
	12.5	99 ± 0			99 ± 0.3		
	3.2	99 ± 0.3			98 ± 1		
	0.8	99 ± 0.9			98 ± 1		
	0.2	86 ± 3			83 ± 10		
AmB	6.0	99 ± 0	≅0.4/≅1.5	187/155	99 ± 0.2	0.3/3.2	130
	1.5	92 ± 2			84 ± 9		
	0.4	54 ± 5			67 ± 9		
	0.1	21 ± 12			0		
L-AmB	6.0	99 ± 0	≅0.1/0.7	750/331	99 ± 0.5	—/1.0	416
	1.5	99 ± 0.5			99 ± 0.1		
	0.4	70 ± 5			80 ± 9		
	0.1	48 ± 2			72 ± 7		
Liposomes		0			3 ± 16	—/—	—

[a] In control animals, there was a mean ± standard error of 743 × 10^6 ± 48 × 10^6 parasites per liver and 6.5 × 10^6 ± 1.3 × 10^6 parasites per spleen.
[b] In the form of Glucantime.
[c] —, Not determined.

ED_{90} for meglumine antimonate in this experiment (Table 2) compared with those in the 3-day experiment (Table 1) confirmed the lower activity of drugs in hamsters under these conditions. Although all the drugs were less active in 10-day-infected hamsters than in 3-day-infected animals, the decrease in activity was generally in proportion to the decrease in activity of the control drug meglumine antimonate, so that the G value changed little. Specifically, liposomal meglumine antimonate was 940 to 1,040 times as active as unencapsulated antimony, and L-AmB had a G value of 170 to 208. The highest administered dosage of L-AmB eliminated >99% of parasites.

TABLE 2. Activity of liposomal preparations against *L. donovani* in hamsters infected for 10 days[a]

Drug	Dosage (mg/kg)	Liver amastigotes			Spleen amastigotes		
		% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}	% Suppression (mean ± SE)	ED_{50}/ED_{90}	G_{50}
Sb[b]	416	68 ± 5	208/>416	1	75 ± 5	188/>416	1
	208	46 ± 10			57 ± 13		
	104	21 ± 7			20 ± 12		
	52	0			25 ± 8		
Liposomal Sb[b]	28	100	0.20/0.8	1,040	100	0.20/0.07	940
	7	99 ± 0			99 ± 0.1		
	1.4	99 ± 0.1			99 ± 0.3		
	0.28	83 ± 3			85 ± 5		
	0.07	4 ± 9			7 ± 11		
AmB	6	78 ± 2	≅1.5/—[c]	139	60 ± 4	4.4/—	43
	1.5	48 ± 3			31 ± 10		
	0.4	5 ± 8			26 ± 10		
L-AmB	11	99 ± 0.05	1.0/2.7	208	99 ± 0	1.1/≅2.8	170
	2.8	93 ± 1.6			91 ± 2		
	0.7	42 ± 4			40 ± 10		
	0.17	21 ± 9			11 ± 19		
	0.04	5 ± 5			0		
Liposomes		0			29 ± 14		

[a] In control animals, there was a mean ± standard error of 1,790 × 10^6 ± 128 × 10^6 amastigotes per liver and 46 × 10^6 ± 4.8 × 10^6 amastigotes per spleen.
[b] In the form of Glucantime.
[c] —, Not determined.

TABLE 3. Activity of L-AmB against *L. donovani* infection in squirrel monkeys[a]

Drug	Dosage	No. of monkeys dead/total (day[s] of death)	% Suppression [mean (range)]	
			Liver amastigotes	Spleen amastigotes
Sb[b]	104 mg/kg per day × 7 days (728 mg/kg)	1/3 (9)	98 (98–100)	99 (99–100)
AmB	2 mg/kg on days 1, 4, and 7 (6 mg/kg)	2/2 (2, 9)	96 (95–97)	98 (96–100)
L-AmB	2 mg/kg on days 1, 4, and 7 (6 mg/kg)	0/3	95 (88–100)	90 (89–92)
	4 mg/kg on day 1 (4 mg/kg)	0/3	90 (85–100)	71 (67–76)
	4 mg/kg on days 1, 4, and 7 (12 mg/kg)	1/3 (10)	99 (98–100)	98 (99–100)
Liposomes		0/2[c]	73 (42–89)	57 (44–61)

[a] In the three control animals, there was a mean (range) of 1.1 (1.04 to 1.12) × 10^9 organisms per liver and 27 (18 to 38) × 10^6 organisms per spleen.
[b] In the form of Glucantime.
[c] This group comprised female monkeys.

Monkey experiments. The activity of L-AmB was next examined in *L. donovani*-infected squirrel monkeys (Table 3). Because of the limited number of animals, meglumine antimonate was administered at one dosage (104 mg/kg per day for 7 days). This dosage eliminated essentially all parasites but also caused the premature death of one of three monkeys. AmB at a dosage of 2 mg/kg per day on days 1, 4, and 7 was approximately as effective as the 728 total mg of meglumine antimonate per kg but caused both of the monkeys in the group to die prematurely. L-AmB was slightly less active and considerably less toxic than unencapsulated AmB in this model. When 2 mg of encapsulated AmB per kg was administered on days 1, 4, and 7, 90 to 95% of parasites were eliminated, and no monkeys died. When the dosage of L-AmB was doubled to 4 mg/kg on days 1, 4, and 7, >98% of parasites were eliminated, but one of three monkeys died. Monkeys given one administration of this higher dosage did not die but evidenced only 71 to 90% parasite elimination. Non-drug-containing liposomes were substantially active in this model. This apparent activity may be spurious and may have been owing to the use of the two female monkeys; *L. donovani* frequently multiplies more slowly in females than in males (W. Hanson, unpublished observation).

Pathologic examination of the monkeys did not reveal a cause of death in the animals that died prematurely, nor did it show evidence of toxicity in those that survived the treatment period. One monkey administered unencapsulated AmB had mild kidney tubular necrosis, but the other animal in this group, which also died prematurely, did not. Complete blood counts were within normal limits in all the monkeys after treatment. Our inability to find lesions in the monkeys that died prematurely was similar to previous experience with mice that died of AmB toxicity (10).

DISCUSSION

Pentavalent antimony is the primary chemotherapeutic agent for leishmaniasis, a tropical disease most prevalent in developing countries. Liposomal Sb is considered a potential clinical agent for visceral leishmaniasis because it incorporates the primary agent into a rational carrier and because the complete formulation is spectacularly successful in rodents. Nevertheless, the practical considerations of drug development afford L-AmB an advantage not yet shared by liposomal Sb. AmB is the primary agent for systemic mycoses, and the importance of a less toxic treatment for such diseases, which are prevalent in both the developed and developing nations, has led to the development and testing of an L-AmB formulation in humans. Clinical experience with L-AmB at dosages including 5 mg of AmB per kg per day has shown that the formulation is neither toxic to the kidneys nor detrimental to the several other organs examined by standard clinical and chemical means.

The purpose of these experiments was to determine the in vivo antileishmanial activity of this clinical preparation of L-AmB. Antileishmanial activity was expressed as the ED_{50}, the ED_{90}, or the dose of drug that eliminated >98 to 99% of parasites when the animals were sacrificed. No attempt was made to maintain animals for extended periods to determine relapse. Against *L. donovani* in hamsters, L-AmB was approximately twice as active as unencapsulated AmB, 200 to 400 times as active as unencapsulated Sb, and one-third to one-fifth as active as liposomal Sb on the basis of comparative ED_{50}s. The dose-response curves were sufficiently steep such that there was a dosage of L-AmB that eliminated >99% of parasites even in hamsters with a high parasite burden. Complete dose-response curves could not be performed in monkey experiments, but data from experiments with high dosages of each drug suggest that L-AmB is slightly less active and considerably less toxic than unencapsulated AmB and about 60 times more active than unencapsulated Sb. The highest dosage tested (4 mg of L-AmB per kg per day for three treatments) eliminated >98% of hepatosplenic parasites. A dose-response curve of sorts was demonstrated in that lesser dosages of the preparation were less leishmaniacidal. Although 4 mg of L-AmB per kg per day administered every third day was toxic to one of three monkeys, both monkeys given 2 mg of unencapsulated AmB per kg per day every third day died, and one of three given the control drug (neat meglumine antimonate) died prematurely. This model apparently is one in which maximally effective dosages of any antileishmanial drug can be toxic, as evidenced by increased mortality, and it may not be the optimal model. Nevertheless, the *S. sciureus* model is the only available nonhuman primate model to test agents against visceral leishmaniasis, and it was used for that reason in these experiments.

L-AmB is an attractive formulation for the treatment of visceral leishmaniasis. Both of its components represent rational therapy: AmB interacts with parasite-specific biochemicals, and liposomes direct the drug specifically to infected tissue. The monkey model may be inappropriate for evaluation of toxicity, and at any rate the chronic toxicity of AmB, which limits its clinical use, could not be well evaluated in this relatively short-term study of L-AmB activity. L-Amb is, however, without toxicity as judged by analysis of

liver function, kidney function, and complete blood counts in the ongoing human trial against mycotic disease. The important features of the present work are that L-AmB was much more active than pentavalent Sb and eliminated >98% of hepatosplenic *L. donovani* in all experiments and that the present human dosage (4 mg/kg per day) administered only three times over 1 week eliminated >98% of parasites from monkeys. The toxicity of this preparation is difficult to evaluate based on the present work. The demonstration of high rodent and monkey efficacy with this clinical formulation suggests that L-AmB has strong potential for trial against kala-azar in humans.

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