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Activity of a New Liposomal Formulation of Amphotericin B against Two Strains of *Leishmania infantum* in a Murine Model

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The efficacy of a new liposomal formulation of amphotericin B was compared to that of amphotericin B deoxycholate (Fungizone) in a murine model of visceral leishmaniasis induced by *Leishmania infantum*. Median effective doses (ED_{50}) were determined with two different strains: strain 1 was obtained from an untreated patient, and strain 2 was obtained from a patient who had received 12.5 g of amphotericin B over 3 years. BALB/c mice were infected intravenously on day 0 with promastigotes and then treated on days 14, 16, and 18 (strain 1) or on days 21, 23, and 25 (strain 2) with the liposomal formulation of amphotericin B (five doses were tested for each strain: 0.05, 0.1, 0.5, 0.8, and 3 mg/kg of body weight) or with conventional amphotericin B (four doses were tested for each strain: 0.05, 0.1, 0.5, and 0.8 mg/kg). Mice in the control group received normal saline solution. The liposomal amphotericin B formulation was about three times more active than the conventional drug against both strains. ED_{50} of the liposomal formulation were 0.054 (strain 1) and 0.194 (strain 2) mg/kg. ED_{50} of conventional amphotericin B were 0.171 (strain 1) and 0.406 (strain 2) mg/kg. Determination of drug tissular levels, 3 days after the last drug administration, showed a drug accumulation in hepatic and splenic tissues much higher after administration of liposomal amphotericin B than after conventional amphotericin B. A lack of toxicity was noted in all groups treated with the liposomal formulation.

Leishmania is a pathogenic protozoan that causes a wide spectrum of infectious diseases in mammalian hosts, ranging from self-healing cutaneous ulceration to progressive and lethal visceral infection. The estimated prevalence in the world is 12 million cases, with 400,000 to 2,000,000 new cases reported per year (11). In the Mediterranean basin, where *Leishmania infantum* is endemic, *Leishmania*-human immunodeficiency virus (HIV) coinfections, occurring in about 2 to 7% of AIDS patients, are regarded as emerging diseases (10, 24, 30). Today, 25 to 70% of adult visceral leishmaniasis (VL) cases are related to HIV infection in southern Europe (2, 19). In *Leishmania*-HIV-coinfected patients, the first course of antimonial pentavalent compounds is successful in 46 to 75% of cases, with apparent clinical recovery (1, 24). This treatment is initially unsuccessful, totally (with 10% of the patients dying during the course) or partly, in 15 to 19% of cases (1, 14). Relapses after apparent clinical recovery occur after a mean of 4.5 months (28). Relapses following antimonial treatments can be the direct cause of mortality. Therefore, the use of amphotericin B (AmB), an antifungal agent which proved to be also active against *Leishmania*, was promoted as an alternative first-line treatment. AmB was first used with the deoxycholate formulation (Fungizone). Acute and chronic side effects have limited the use of AmB deoxycholate as antileishmanial agent (4). In immunocompetent VL patients the use of low doses of AmB deoxycholate gave a high cure rate with limited side effects (23). Lipid formulations of AmB were also developed in order to reduce toxicity and increase activity (20, 25). The lipid formulations of AmB currently marketed for clinical use or undergoing experiments are structurally different and may be divided into three classes: AmB lipid complex (Abelcet; Liposome Company, Princeton, N.J.) is a concentration of ribbon-like structures of a bilayered membrane, AmB colloidal dispersion (Amphocil; Sequus Pharmaceuticals, Menlo Park, Calif.) is composed of disklike structures of cholesteryl sulfate, and AmB liposomes (Ambisome; Vestar, San Dimas, Calif.) are composed of small unilamellar vesicles made up of a bilayer membrane. AmB lipid complex and AmB colloidal dispersion should not be considered equivalent to true liposomes in terms of toxicity and efficacy. Their pharmacokinetics are dissimilar, and in particular, the uptake of AmB by macrophagic cells varies greatly between the different lipid formulations (21).

The activity of liposomal AmB (Ambisome) was demonstrated against *L. donovani* in a murine model. The reported median effective dose (ED_{50}) was 0.15 to 0.25 mg/kg of body weight (6). The activity of Ambisome against *L. infantum* was studied in only a few models (16, 18), and no ED_{50} data are available. Immunocompetent and immunocompromised VL patients have received liposomal AmB in some clinical trials (7–9, 33). Most of treated patients were initially considered cured, but immunocompromised patients relapsed clinically and parasitologically after 3 to 22 months (8). Secondary Ambisome cures were similarly followed by relapses. These relapses, related to the immune status of patients, probably also reflect ineffective drug levels in unusual localizations of *Leishmania* in HIV patients (22, 31).

A new liposomal formulation of AmB (AmB liposomes) was developed and is available at a reduced cost compared to Ambisome (1/10 of the current price of Ambisome) (13). The aim of the present work was to compare, by determination of ED_{50}, the efficacies of AmB deoxycholate and AmB liposomes against two different strains of *L. infantum* in a murine model of VL. One strain was obtained from a patient who had received 12.5 g of amphotericin B over 3 years. The other strain was obtained from an untreated patient.

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MATERIALS AND METHODS

Mice and strains of *Leishmania*. Experiments were conducted with BALB/c male mice (5 weeks old, 20 ± 2 g) purchased from IFFA CREDO (L'arbresle, France) and two strains of *L. infantum*. The first strain (strain 1) of *L. infantum* MON1 (MHOM/PT/93/CRE2g) was isolated from an AIDS patient who had not been treated with AmB. The second strain (strain 2) of *L. infantum* MON1 (MHOM/DZ/92/CRE25) was isolated from an AIDS patient who had received a 12.5-g total amphotericin B dose over 3 years. *Leishmania* promastigotes were injected into a hamster by the intraperitoneal route and maintained in McNeal-Novy-Nicolle medium at 27°C for 8 days. Bulk culture of infectious promastigotes was initiated and propagated in supplemented RPMI 1640 medium as previously described (15). Preliminary assays showed that strain 2 needed a period of incubation longer and a number of infectious promastigotes higher than those of strain 1 to obtain a similar *Leishmania* burden in liver. On day 0, mice were inoculated intravenously (tail vein) with 10^7 infective *L. infantum* promastigotes of strain 1 or with 4×10^7 infective *L. infantum* promastigotes of strain 2, each in a 0.1-ml volume. This procedure induced a heavy *Leishmania* liver burden after 12 days for strain 1 and after 19 days for strain 2.

Therapeutic protocols. Mice were randomly assigned to two groups. In group A, mice were infected with strain 1 and were treated on days 14, 16, and 18. In group B, mice were infected with strain 2 and were treated on days 21, 23, and 25. After the inoculation phase, mice were randomly assigned to one of three different regimens: normal saline solution (control group) (12 mice per strain), AmB deoxycholate (24 mice per strain), and AmB liposomes (30 mice per strain). Formulations were purchased as follows: normal saline solution and AmB liposomes were from Pharmacie centrale des Hôpitaux, Paris, France, and AmB deoxycholate was from Squibb (Neuilly-sur-Seine, France). AmB deoxycholate was administered at 0.05, 0.1, 0.5, and 0.8 mg/kg and injected by the intravenous route in a 0.1-ml volume. AmB liposomes were given at 0.05, 0.1, 0.5, 0.8, and 3 mg/kg. Liposomal AmB was prepared according to Good Manufacturing Practices, on a pilot scale (1,000 vials per batch), by the method previously described by Fessi et al. (13). Briefly, an organic phase containing phospholipids and the drug was introduced under magnetic stirring in an aqueous phase. The organic solvent was evaporated, and the liposomes obtained were filtered and lyophilized. Prior to administration, 50 mg of lyophilized AmB liposomes was resuspended in sterile distilled water (20 ml), shaken for 3 min, and then diluted in 5% dextrose. The liposomes obtained were oligomultilamellar with a 300-nm mean diameter and were stable over 8 days at 4°C.

Sacrifice. The animals were killed by cervical dislocation 21 and 28 days after the initial infection in groups A and B, respectively. The Guiding Principles for Biomedical Research involving animals, published by the Council for International Organizations of Medical Sciences (5a) were followed during all procedures.

Assessment of efficacy. The following parameters were used to assess treatment efficacy: parasite burden, ED, and levels of AmB in tissue.

(i) Parasite burden. The liver parasite burden was evaluated after Giemsa staining of the smears. The number of amastigotes per 500 hepatocytes was calculated and related to liver weight (in milligrams) by the formula of Stauber et al. (32). The percentage of parasite suppression was calculated as [1 − (mean Stauber count of the treated group/mean Stauber count of the control group)] × 100.

(ii) ED. ED_{25}, ED_{50}, and ED_{90} (doses of the drug calculated to eliminate 25, 50, and 90% of parasites compared to controls) were determined by using a Michaelis-Menten model.

(iii) Levels of AmB in tissue. After sacrifice, samples of liver and spleen were immersed into liquid nitrogen. AmB levels in liver and spleen in mice treated with AmB deoxycholate and AmB liposomes were determined by a modified high-performance liquid chromatography method (34). Briefly, after thawing, samples were homogenized in 0.5 ml of phosphate-buffered saline, and AmB was extracted by methanol (4 ml). Methanolic extract was introduced on a solid-phase column (Bond-Elut C_{18}, 1 ml) under pressure. AmB was eluted with a solution composed of 50% acetonitrile in 2.5 mM Na_2EDTA (1 ml). The eluate was injected into a reverse-phase C_{18} column (Shandon; Hypersil, 5 μm; internal diameter, 250 by 4.6 mm) with a mobile phase composed of 40% acetonitrile in 2.5 mM Na_2EDTA. The flow rate was 1 ml/min. AmB was detected by UV absorption at 410 nm. The limit of detection was 25 ng/g.

Statistical analysis. Results were expressed as means ± standard errors of the means. A one-way analysis of variance or a U test was performed to compare the influences of the various parameters. A P value lower than 0.05 was considered statistically significant.

RESULTS

Control experiments. In group A, mice treated with normal saline solution showed a mean of 0.51 *L. infantum* amastigotes per liver cell nucleus at the end of the 21-day period of experimentation (range, 0.12 to 0.9; $n = 12$). In group B, mice treated with normal saline solution had a mean of 0.49 *L. infantum* amastigotes per liver cell nucleus at the end of the 29-day period of experimentation (range, 0.2 to 1.0; $n = 12$). In groups A and B, the average liver parasite burdens reached 1.4×10^8 and 1.6×10^8 amastigotes (Stauber count), respectively, with 1×10^7 and 4×10^7 promastigotes (Tables 1 and 2). The *Leishmania* burdens were similar in the two cases ($P = 0.718$).

TABLE 1. Suppression of experimental leishmaniasis in mice treated with AmB deoxycholate or with AmB liposomes in group A[a]

Treatment (mg/kg)	No. of amastigotes/ 500 hepatocytes	% Suppression (range)[b]
Control	257.5 ± 61	
AmB deoxycholate		
0.05	243.5 ± 68	18.1 ± 11.1 (0–47.8)
0.1	113.5 ± 38	46.3 ± 13.6 (14.3–84.1)
0.5	67.7 ± 20	64.8 ± 8.3 (38.3–88.1)[c]
0.8	26.2 ± 13	93.9 ± 1.9 (90–98)
AmB liposomes		
0.05	140.0 ± 13	44.3 ± 4.8 (34.8–49.3)
0.1	45.2 ± 26	69.7 ± 13.6 (33.0–93.3)
0.5	13.2 ± 4	95.1 ± 2.0 (89.8–99.4)[c]
0.8	3.0 ± 2	98.8 ± 1.9 (95.9–100.0)
3	1.5 ± 1	99.6 ± 0.2 (97.5–100.0)

[a] Mice were infected with strain 1 (native AmB).
[b] Calculated by using the Stauber count versus untreated mice. Data are expressed as means ± standard errors for six mice per group except the control group ($n = 12$). The U test was used to compare the groups.
[c] Significantly different from the value for the same dose of AmB liposomes ($P < 0.01$).

Treatment groups. (i) Group A. Maximal parasite suppression obtained with AmB deoxycholate was 93.9% ± 1.9% (Table 1). This result was not significantly different from the one observed with AmB liposomes at the same dose (0.8 mg/kg) (98.8% ± 1.9%).

(ii) Group B. Maximal parasite suppression obtained with AmB deoxycholate was 78.9% ± 8.5% (Table 2). This result was significantly different from the one observed with AmB liposomes at the same dose (0.8 mg/kg) (98.7% ± 0.8%; $P =$

TABLE 2. Suppression of experimental leishmaniasis in mice treated with AmB deoxycholate or with AmB liposomes in group B[a]

Treatment (mg/kg)	No. of amastigotes/ 500 hepatocytes	% Suppression (range)[b]
Control	248.3 ± 78	
AmB deoxycholate		
0.05	425.0 ± 72	ND
0.1	311.0 ± 90	22.6 ± 9.60 (0–47.8)
0.5	118.8 ± 69	58.4 ± 20.8 (0–89.5)
0.8	50.0 ± 20	78.9 ± 8.5 (78.9–90.6)[c]
AmB liposomes		
0.05	425.0 ± 95	8.3 ± 8.3 (0–41.7)
0.1	153.0 ± 62	46.5 ± 19.1 (0–83.4)
0.5	45.0 ± 21	82.8 ± 8.0 (60.1–100)
0.8	3.0 ± 2	98.7 ± 0.8 (95.8–100)
3	1.6 ± 1	99.2 ± 0.7 (96.2–100)

[a] Mice were infected with strain 2 (AmB, 12.5 g over 3 years).
[b] Calculated by using the Stauber count versus untreated mice. Data are expressed as means ± standard errors for six mice per group except the control group ($n = 12$). The U test was used to compare the groups. ND, not determined.
[c] Significantly different from the value for the corresponding dose of AmB liposomes ($P < 0.05$).

TABLE 3. Efficacy of AmB deoxycholate and AmB liposomes for two strains of *L. infantum*[a]

Treatment and strain	mg/kg[b]		
	ED_{25}	ED_{50}	ED_{90}
AmB deoxycholate			
Strain 1	0.060 ± 0.015	0.171 ± 0.040[c]	1.042 ± 0.220
Strain 2	0.182 ± 0.038	0.406 ± 0.065[d]	0.918 ± 0.039
AmB liposomes			
Strain 1	0.018 ± 0.003	0.054 ± 0.009[e]	0.336 ± 0.064
Strain 2	0.077 ± 0.008	0.194 ± 0.018	0.576 ± 0.042

[a] Strain 1 was naive, and strain 2 was isolated from a treated patient (AmB, 12.5 g over 3 years).
[b] Calculated by using a Michaelis-Menten model. Data are expressed as means ± standard errors. The U test was used to compare the doses.
[c] Significantly different from the values for AmB deoxycholate with strain 2 and AmB liposomes with strain 1 ($P < 0.05$).
[d] Significantly different from the value for AmB liposomes with strain 2 ($P < 0.05$).
[e] Significantly different from the value for AmB liposomes with strain 2 ($P < 0.01$).

0.0428). For each dose tested, the activities were not significantly different between the two strains except for the 0.05-mg/kg dose of AmB liposomes ($P = 0.0056$).

Evaluation of the ED. In group A, AmB liposomes were threefold more active than the free drug (AmB deoxycholate). In group B, AmB liposomes were about twofold more active than the free drug. The liposomal AmB was significantly more efficient than AmB deoxycholate for both strains ($P < 0.05$) (Table 3).

AmB levels. Administration of AmB deoxycholate led to undetectable drug levels (detection limit, 25 ng/g) in the liver and in the spleen for doses below 0.5 mg/kg (Table 4). At 0.8 mg/kg, drug levels were higher in the spleen than in the liver (0.24 versus 0.057 μg/g). Administration of liposomal AmB led to much higher drug levels than that of free drug in these two organs. For the only comparable dose (0.8 mg/kg), the AmB liposomes/AmB deoxycholate ratio was about 100 in the liver and 8 in the spleen. Comparable results were obtained with strain 2 ($P > 0.05$).

DISCUSSION

Results of this study showed that liposomal AmB was more active than AmB deoxycholate against the two strains of *L. infantum* in our murine model. The determination of ED_{50} for the two strains demonstrated that *Leishmania* strains may require different drug levels in situ to be cleared. These results corroborated observations by Gangneux et al. (16), who have studied the activity of another liposomal formulation of AmB (Ambisome) versus AmB deoxycholate against a strain of *L. infantum* in a BALB/c mouse model. The same trends were observed by Croft et al. (6) with *L. donovani*-infected BALB/c mice. For single doses of Ambisome versus single doses of AmB deoxycholate, ED_{50} reported by these authors were, respectively, 0.15 to 0.25 and 0.95 to 4.9 mg/kg.

TABLE 4. AmB levels in liver and spleen for strain 1[a]

Dose (mg/kg)	μg/g			
	AmB deoxycholate		AmB liposomes	
	Liver	Spleen	Liver	Spleen
0.05	ND[b]	ND	0.028 ± 0.002	0.025 ± 0.015
0.1	ND	ND	0.170 ± 0.030	0.098 ± 0.027
0.5	ND	0.055 ± 0.018	2.640 ± 0.220	0.455 ± 0.017
0.8	0.057 ± 0.017	0.237 ± 0.052	6.460 ± 0.800	2.048 ± 0.507
3			28.64 ± 3.760	12.50 ± 1.200

[a] Mice were sacrificed 3 days after cessation of treatment. Each mouse received three doses of AmB deoxycholate or AmB liposomes on days 14, 16, and 18. The data are expressed as means ± standard errors for six mice per group.
[b] ND, not detectable (<25 ng/g).

Determination of drug levels 3 days after the last injection showed a higher drug accumulation in hepatic and splenic tissues following liposomal AmB administration compared to that of AmB deoxycholate. Very high concentrations of AmB were found in the two organs evaluated after three doses of 3 mg of liposomal AmB per kg without immediate apparent toxicity. Mice were not treated with 3 mg of AmB deoxycholate per kg because doses >1 to 2 mg/kg were reported to be very toxic or lethal to normal mice (17, 27): in C57BL/6 mice, the median lethal dose reported after one injection of AmB deoxycholate was 2.3 mg/kg (29). The liposomal formulation of AmB evaluated in the present work led to drug levels similar to those obtained with Ambisome (3 mg/kg × 3) in BALB/c mice infected with *L. infantum* (18). Higher drug levels were obtained by Gangneux et al. in the liver and, especially, in the spleen in *L. infantum*-infected mice treated with six doses of Ambisome (0.8 mg/kg × 6) (16). The access of liposomal formulations to the spleen and probably to other organs following hepatic saturation has already been described: it increases with the dose and the number of administrations. Multiple doses of Ambisome led to similar drug levels in the spleen and in the liver after 28 administrations of 1 or 5 mg/kg in rats (29). A more rapid uptake of liposomes by hepatic tissue, involving an early saturation of the liver, has been reported for liposomes >150 nm (5). The access of liposomal formulations to organs rich in macrophagic cells such as bone marrow, gastrointestinal tract, or skin (22, 26, 31), where *Leishmania* may form cryptic foci, is essential to prevent relapses especially in immunosuppressed patients showing atypical dissemination of parasites in mucosal and/or dermal sites and in unusual cells such as epithelial cells or polynuclear neutrophils. Further studies are required to assess the internalization of the liposomal formulation of AmB by these nonmacrophagic cells.

The difference between the activities of the AmB liposomes and the free drug was not so dramatic, as AmB deoxycholate already has one of the highest intrinsic activities known against *L. infantum* (3). In comparison, pentamidine bound on methacrylate nanoparticles was six times more active than free drug in a similar model (12). The major advantage of liposomal formulations may be related to reduced toxicity and persistent high drug levels in tissues more than increased activity. The liposomal formulation of AmB tested in this work was well tolerated by mice. The median lethal dose, previously determined in BALB/c mice, was 22 mg/kg (13). In the same way, good tolerance of the liposomal formulation of AmB (Ambisome) has been reported following either single-dose or multidose treatment in rodents (29) and in humans (9). The prohibitive cost of Ambisome had limited its use (9). Therefore, the evaluation of new and less expensive liposomal AmB formulations is of particular interest, as the number of *Leishmania*-HIV-coinfected patients is still increasing. In vivo data suggest that the AmB liposomal formulation tested in this work warrants further investigation in the treatment of visceral leishmaniasis.

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