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Therapy of Visceral Leishmaniasis Due to *Leishmania infantum*: Experimental Assessment of Efficacy of AmBisome

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The tolerance and efficacy of amphotericin B (AmB) deoxycholate (Fungizone) were compared with those of liposomal AmB (AmBisome) in a murine model of visceral leishmaniasis induced by *Leishmania infantum*. Control groups consisted of untreated mice and mice treated with a pentavalent antimonial (Glucantime). BALB/c mice were infected intravenously on day 0 with 10^7 promastigotes of *L. infantum* and then treated from day 7 to 17 (early treatment group) or from day 60 to 70 (delayed treatment group). The pentavalent antimonial was administered daily by intraperitoneal injection, whereas AmB formulations were administered intravenously on alternate days. On days 20, 60, and 120 (early treatment group) and on days 72 and 125 (delayed treatment group), parasite burdens in the liver, spleen, and lungs were determined by subculturings using a microtitration method. A dose range study showed that administration of AmBisome at the well-tolerated doses of 5 or 50 mg/kg of body weight completely eradicated the parasites from the tissues. At 0.8 mg/kg, AmBisome proved more efficacious than AmB deoxycholate administered at the same dose. We also compared the levels of AmB deoxycholate and AmBisome in plasma and tissue. Mice treated with AmBisome had levels of AmB in tissue much higher than did AmB deoxycholate-treated mice with persistent detectable levels 14 weeks after treatment. These results seem to account for the remarkable efficacy of the liposomal formulation of AmB in the treatment of visceral leishmaniasis due to *L. infantum*.

Visceral leishmaniasis (VL) is a widespread disease caused by the kinetoplastid protozoa *Leishmania donovani*, *L. chagasi*, and *L. infantum*. In patients infected with human immunodeficiency virus, VL is an important opportunistic infection (21), since 40 to 50% of the cases of VL reported in southern Europe occur in these patients (25).

L. infantum is the causative agent in most of the cases (2, 11), and no consensual treatment protocol has presently been adopted for immunocompromised patients (14). The main difficulties involved in treating VL in AIDS patients are related to the high rates of relapse after initial treatment with pentavalent derivatives of antimony (22). Alternate therapies using amphotericin B (AmB) prepared with deoxycholate (Fungizone; Squibb, Neuilly-sur-Seine, France) have been proposed since AmB has good activities against *Leishmania* spp. in vitro (4), but AmB deoxycholate is potentially nephrotoxic (18). Recently, it has been shown that administration of lower doses or daily administration of the drug was effective, with limited side effects, in the treatment of Indian VL (20, 28). Other alternatives to reduce toxicity and increase efficacy are the use of lipid formulations of AmB.

Liposome-encapsulated AmB for the treatment of experimental leishmaniasis was first used by New (23) and Panosian et al. (24), with fewer side effects and higher tolerable dosages than with AmB prepared with deoxycholate. In vitro studies with the commercially prepared liposomal AmB (AmBisome; Vestar, San Dimas, Calif.) showed good activities against *L. donovani* (7), but only limited data on experimental VL caused by *L. infantum* treated with AmBisome are available. Gradoni et al. (15) have shown that AmBisome clears parasites from the livers of mice infected with *L. infantum* after three consecutive daily doses, while meglumine antimoniate (Glucantime; Specia, Rhône-Poulenc Rorer, Paris, France) requires 21 doses. In this study, parasite loads were evaluated by the direct enumeration of amastigotes in smears prepared from livers during acute stages of infection. Several clinical trials with lipid-associated formulations of AmB, including AmBisome (8, 9, 13, 19, 26, 30), Amphocil (3, 12), and AmB lipid complex (27), reported favorable outcomes either in first-line therapy or in cases of drug-resistant VL. However, relapses were observed, especially in immunocompromised patients (10), and this was probably related to the persistence of parasites and/or to insufficient drugs levels in the infected tissues.

Thus, this study was designed to compare the efficacies of AmB deoxycholate, AmBisome, and meglumine antimoniate as reference therapy in the treatment of murine VL, using long-term follow-up and concurrent monitoring of tissue parasite burdens and drug levels in blood and infected organs. These studies were done in a mouse model of infection due to *L. infantum*, the most common infecting *Leishmania* species in AIDS patients, with treatment administered either at the acute or at the chronic stage of the disease and sequential titration of the parasite burden using a sensitive culture-based titration method.

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MATERIALS AND METHODS

Mice and strain of *L. infantum*. Experiments were conducted with adult BALB/c female mice (Iffa Credo, Lyon, France), at an early or a late stage of infection, following challenge with a strain of *L. infantum* (MHOM/FR/91/LEM2259V) isolated from an AIDS patient. This strain was maintained in culture in Schneider's drosophila medium supplemented with 20% heat-inactivated fetal calf serum (Gibco BRL, Eragny, France). Infectious promastigotes were harvested after 7 days of culture and counted with a hemocytometer. On

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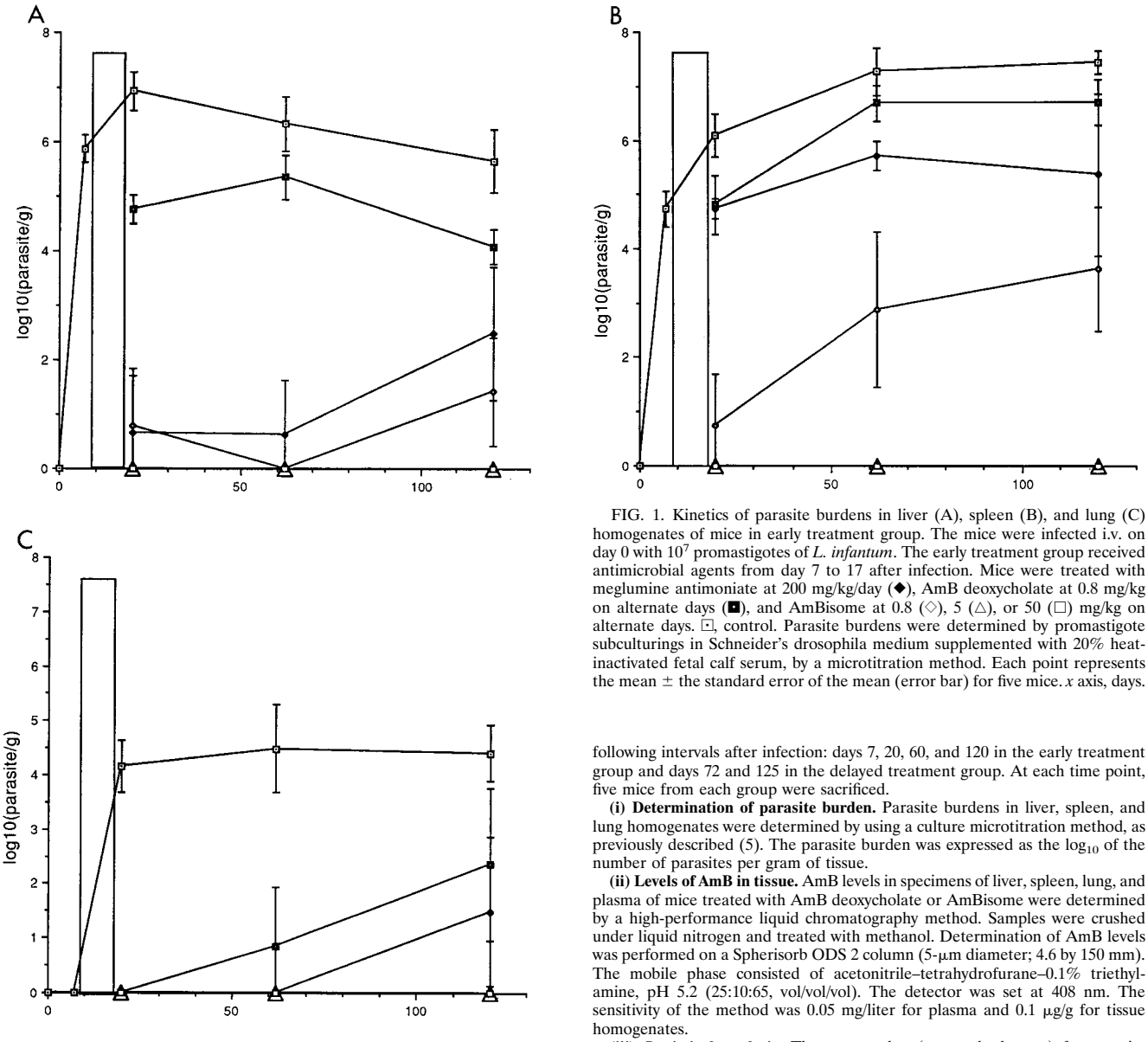


FIG. 1. Kinetics of parasite burdens in liver (A), spleen (B), and lung (C) homogenates of mice in early treatment group. The mice were infected i.v. on day 0 with 10^7 promastigotes of *L. infantum*. The early treatment group received antimicrobial agents from day 7 to 17 after infection. Mice were treated with meglumine antimoniate at 200 mg/kg/day (◆), AmB deoxycholate at 0.8 mg/kg on alternate days (■), and AmBisome at 0.8 (◇), 5 (△), or 50 (□) mg/kg on alternate days. ⊡, control. Parasite burdens were determined by promastigote subculturings in Schneider's drosophila medium supplemented with 20% heat-inactivated fetal calf serum, by a microtitration method. Each point represents the mean ± the standard error of the mean (error bar) for five mice. *x* axis, days.

day 0, each mouse was inoculated intravenously (i.v.) with 10^7 promastigotes, i.e., 0.2 ml of inoculum (i.v.) per mouse.

Therapeutic protocols. Mice were randomly assigned to two groups: (i) the early treatment group, which was treated from day 7 to 17, and (ii) the delayed treatment group, which was treated from day 60 to 70. In each group, meglumine antimoniate and AmB deoxycholate were evaluated at high dosages that were previously found to be efficacious and nontoxic in preliminary experiments. Meglumine antimoniate (200 mg/kg of body weight; Specia, Rhône-Poulenc Rorer) was administered daily for 11 days (total dose, 2,200 mg/kg). For each injection, 5-ml ampoules of meglumine antimoniate (85 mg of pentavalent antimony per ml) were diluted with 5% glucose, and 0.2 ml was injected intraperitoneally into each mouse. AmB formulations were administered i.v. on days 7, 9, 11, 13, 15, and 17 in the early treatment group and on days 60, 62, 64, 66, 68, and 70 in the delayed treatment group. AmB deoxycholate (Squibb) was given at 0.8 mg/kg per injection (total dose, 4.8 mg/kg), and AmBisome (Vestar) was given at 0.8, 5, or 50 mg/kg per injection (total dose, 4.8, 30, or 300 mg/kg, respectively). AmB deoxycholate (vials with 50 mg of lyophilized AmB deoxycholate powder) was resuspended in 5% glucose, and AmBisome (vials with 50 mg of lyophilized powder of AmB encapsulated in liposomes) was resuspended in sterile distilled water. Suspensions were shaken for 2 min and then diluted in 5% glucose prior to administration. Drug suspensions were prepared daily, and 0.2 ml was injected into each mouse.

Assessment of efficacy. Mice were studied for 4 months after infection. Sequential examinations of parasite burden and AmB levels were performed at the following intervals after infection: days 7, 20, 60, and 120 in the early treatment group and days 72 and 125 in the delayed treatment group. At each time point, five mice from each group were sacrificed.

(i) Determination of parasite burden. Parasite burdens in liver, spleen, and lung homogenates were determined by using a culture microtitration method, as previously described (5). The parasite burden was expressed as the $\log_{10}$ of the number of parasites per gram of tissue.

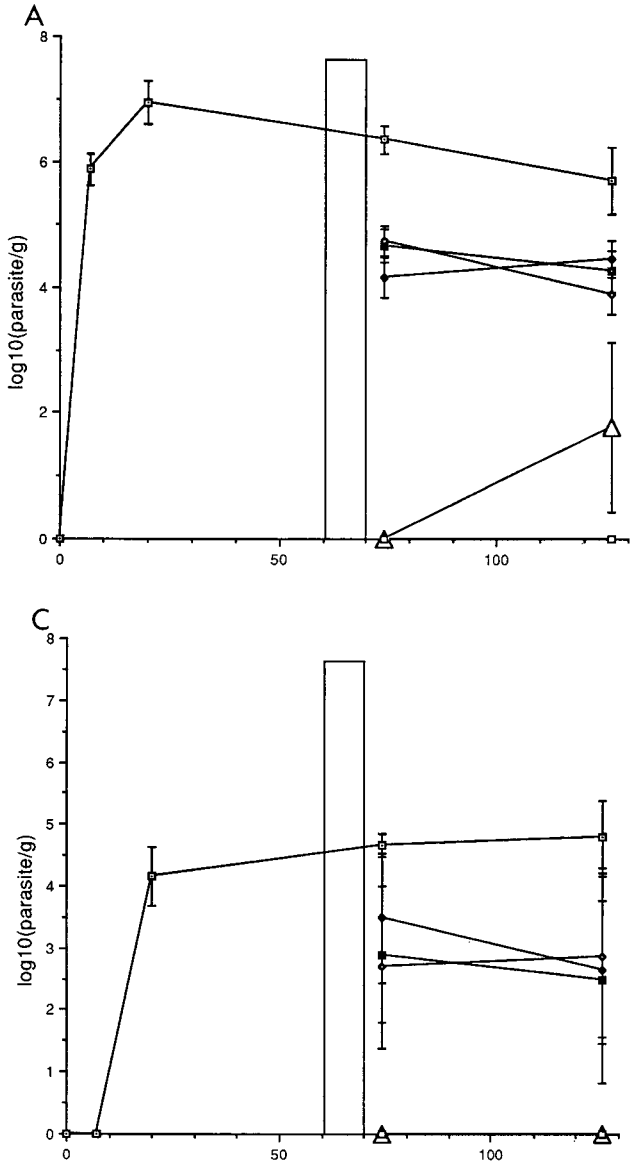
(ii) Levels of AmB in tissue. AmB levels in specimens of liver, spleen, lung, and plasma of mice treated with AmB deoxycholate or AmBisome were determined by a high-performance liquid chromatography method. Samples were crushed under liquid nitrogen and treated with methanol. Determination of AmB levels was performed on a Spherisorb ODS 2 column (5-μm diameter; 4.6 by 150 mm). The mobile phase consisted of acetonitrile–tetrahydrofurane–0.1% triethylamine, pH 5.2 (25:10:65, vol/vol/vol). The detector was set at 408 nm. The sensitivity of the method was 0.05 mg/liter for plasma and 0.1 μg/g for tissue homogenates.

(iii) Statistical analysis. The mean value (± standard error) for parasite burdens of five mice was calculated for each time point.

RESULTS

None of the untreated control mice died before the end of the experiment. Mortality was observed neither in the 25 mice treated with meglumine antimoniate nor in 25 mice treated with AmBisome (50 mg/kg). One of 25 mice died in each group treated with 0.8- and 5-mg/kg doses of AmBisome, and 2 of 25 mice died in the group treated with AmB deoxycholate (0.8 mg/kg).

Analysis of early treatment (day 7 to day 17 postinfection) (Fig. 1). The kinetics of parasite burdens in control mice, examined on days 7, 20, 60, and 120, showed an early and considerable colonization of the liver and spleen (6 and 4 $\log_{10}$ parasites per g, respectively, on day 7). In the lungs, the parasites were detected beginning on day 20, with lower levels detected in the lungs than in the other organs. Meglumine antimoniate treatment resulted in a marked reduction of the parasite burdens in the liver, i.e., 3 to 6 $\log_{10}$ parasites per g



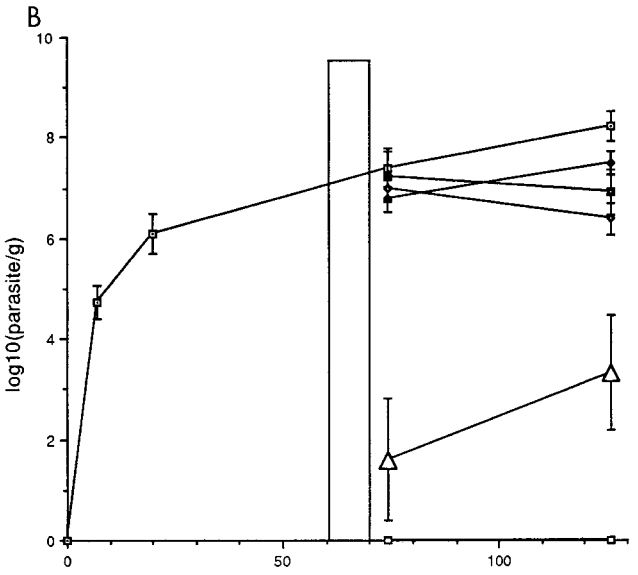


FIG. 2. Kinetics of parasite burdens in liver (A), spleen (B), and lung (C) homogenates of mice in delayed treatment group. The mice were infected i.v. on day 0 with 10^7 promastigotes of *L. infantum*. The delayed treatment group received antimicrobial agents from day 60 to 70 after infection. Mice were treated with meglumine antimoniate at 200 mg/kg/day (◆), AmB deoxycholate at 0.8 mg/kg on alternate days (■), and AmBisome at 0.8 (◇), 5 (△), or 50 (□) mg/kg on alternate days. ⊡, control. Parasite burdens were determined by promastigote subculturings in Schneider's drosophila medium supplemented with 20% heat-inactivated fetal calf serum, by a microtitration method. Each point represents the mean ± the standard error of the mean (error bar) for five mice. *x* axis, days.

fewer parasites than in untreated mice. The therapeutic effect of meglumine antimoniate was less in the spleen, where the parasite load was decreased by 1 to 2 $\log_{10}$, with the persistence of focal infection sites despite treatment. In the lungs of meglumine antimoniate-treated mice, no promastigotes were detected until day 120. Administration of AmB deoxycholate (0.8 mg/kg) decreased the parasite loads in the liver and in the spleen by approximately 1 to 2 $\log_{10}$ and decreased those in the lungs by 2 to 3 $\log_{10}$. At the same dose of AmBisome (0.8 mg/kg), a reduction of 4 to 6 $\log_{10}$ parasites per g in the liver and in the spleen compared with loads for control mice was observed. Preparations from the lung homogenates were parasite negative. This effect in the lungs persisted for 14 weeks after treatment. When we administered higher doses of AmBisome (5 and 50 mg/kg), no parasites were detected in the tissue homogenates from any of the three organs. The absence of culturable *Leishmania* parasites from the liver, spleen, and lung homogenates of these mice persisted throughout the 14-week follow-up period following cessation of therapy.

Analysis of delayed treatment (day 60 to day 70 postinfection) (Fig. 2). When treatments were given 2 months after infection, the mice presented with a chronic VL with high and stable parasite loads (6, 8, and 5 $\log_{10}$ parasites per g in liver, spleen, and lung homogenates, respectively). Treatment efficacies for these mice were lower than those for the mice in the early treatment group. Treatment with meglumine antimoniate (200 mg/kg) produced a reduction of the parasite burdens by approximately 2 $\log_{10}$ in the liver and in the lungs. In the spleen, this regimen caused only a mild effect, with persistence of the parasite load (6 to 7 $\log_{10}$ parasites per g of tissue). The administration of AmB deoxycholate (0.8 mg/kg) did not markedly reduce the parasite burdens in the three sites. On day 125, 4, 6, and 2 $\log_{10}$ parasites per g were present in the liver, spleen, and lung homogenates, respectively. Similar results were obtained with AmBisome administered at the same dose. Treatments with higher doses of AmBisome were more effective. Administration of AmBisome at 5 mg/kg (total dose, 30 mg/kg) resulted in clearance of culturable organisms from the liver and the lungs on day 72; parasite persistence in the spleen was observed, although the levels were lower than those in the other groups (less than 2 $\log_{10}$ parasites per g on day 72 and 3 $\log_{10}$ parasites per g on day 125). When administered at 50 mg/kg, AmBisome produced clearance of parasites from all three organ homogenate subcultures. This effect persisted throughout the 8-week follow-up period following cessation of therapy.

AmB levels in plasma, liver, spleen, and lungs (Table 1). (i) Early treatment group. For mice treated with AmB deoxycholate (0.8 mg/kg), AmB levels in plasma and in tissues were consistently low or at undetectable levels when examined 3, 43, and 103 days after the withdrawal of drugs. In mice treated

TABLE 1. AmB levels in plasma, liver, spleen, and lungs[a]

Treatment group	Day	AmB level (mean ± SD) in:			
		Plasma (mg/liter)	Liver (μg/g)	Spleen (μg/g)	Lungs (μg/g)
Early AmB deoxycholate (0.8 mg/kg)	3	0.07 ± 0.01	0.43 ± 0.09	0.88 ± 0.23	ND[b]
	43	ND	ND	0.27 ± 0.09	ND
	103	ND	ND	ND	ND
Delayed AmB deoxycholate (0.8 mg/kg)	2	0.1 ± 0.02	10.3 ± 1.62	4.53 ± 1.11	0.15 ± 0.08
	55	ND	ND	0.11 ± 0.07	ND
Early AmBisome (0.8 mg/kg)	3	0.09 ± 0.01	33.94 ± 5.14	23.84 ± 4.26	ND
	43	ND	3.05 ± 1.69	5.48 ± 2.13	ND
	103	ND	ND	0.53 ± 0.07	ND
Delayed AmBisome (0.8 mg/kg)	2	0.09 ± 0.01	28.14 ± 2.69	5.92 ± 1.67	0.06 ± 0.07
	55	ND	ND	0.29 ± 0.05	ND
Early AmBisome (5 mg/kg)	3	0.17 ± 0.01	209.7 ± 47.4	98.80 ± 19.01	1.64 ± 1.29
	43	0.05 ± 0.01	55.94 ± 2.95	28.72 ± 11.92	ND
	103	ND	2.9 ± 0.70	4.3 ± 1.86	ND
Delayed AmBisome (5 mg/kg)	2	0.14 ± 0.01	189.01 ± 25.88	65.1 ± 33.57	1.27 ± 0.29
	55	0.07 ± 0.01	25.48 ± 4.29	16.6 ± 6.64	ND
Early AmBisome (50 mg/kg)	3	0.36 ± 0.03	2,575.4 ± 860.2	929.22 ± 187.7	35.88 ± 5.70
	43	0.13 ± 0.02	808.4 ± 115.1	124.4 ± 15.89	5.05 ± 2.30
	103	ND	214.9 ± 48.99	100.68 ± 34.85	1.62 ± 0.72
Delayed AmBisome (50 mg/kg)	2	0.28 ± 0.04	1,614.1 ± 194.9	493.7 ± 152.4	26.52 ± 4.01
	55	0.13 ± 0.01	465.6 ± 53.3	322.7 ± 81.99	2.92 ± 1.01

[a] Levels of AmB in plasma, liver, spleen, and lungs were detected by high-performance liquid chromatography analysis 3, 43, and 103 days after cessation of early treatment (day 7 to 17 postinfection) and 2 and 55 days after cessation of delayed treatment (day 60 to 70 postinfection) with AmB deoxycholate and AmBisome. The values presented are means ± standard deviations for five mice per group.

[b] ND, not detectable (level in plasma, <0.05 mg/liter; level in tissue, <0.1 μg/g).

with AmBisome (0.8 mg/kg), the levels of AmB in the plasma were also low to undetectable. In the tissues examined, however, marked drug accumulation was observed, with levels in the liver and in the spleen on day 3 being 33.94 and 23.84 μg/g, respectively, and those on day 43 being 3.05 and 5.48 μg/g, respectively. Furthermore, AmB remained detectable in the spleen until 14 weeks after the cessation of AmBisome therapy. When AmBisome was administered at 5 or 50 mg/kg, drug concentrations in the liver reached 209.7 and 2,575.4 μg/g, respectively, and those in the spleen reached 98.8 and 929.2 μg/g, respectively. Drug levels in these organs decreased very slowly, with persistent levels of drug detectable 14 weeks after treatment. AmB was detectable at much lower levels in the lungs of mice given AmBisome (5 or 50 mg/kg) and remained at low to undetectable levels in the plasma.

(ii) Delayed treatment group. Similar results were noted when treatment was administered from day 60 to 70. Only undetectable or low levels of AmB were detected after treatment with AmB deoxycholate (0.8 mg/kg) or AmBisome (0.8 mg/kg). In mice treated with AmBisome (5 or 50 mg/kg), high drug levels were detected in the tissues examined 8 weeks after cessation of therapy, whereas low levels were detected in the plasma at the same time point.

DISCUSSION

The results of this study show that AmBisome has a pronounced efficacy and tolerance for treatment of VL due to *L. infantum* compared with meglumine antimoniate and AmB deoxycholate. The liposomal formulation of AmB (i.e., AmBisome) was well tolerated and could be administered at doses 50-fold higher than those for conventional AmB. This treatment was also efficacious when administered either at the acute or at the chronic stage of infection.

The results of this study confirmed and extended observations by Gradoni et al. (15), since our method of investigation evaluated the efficacies of the drugs in several organs. Moreover, the sensitivity of the microtitration method that we used for the evaluation of parasite burdens allowed us to determine the presence of viable parasites, even at levels which cannot be detected by the conventional technique of direct enumeration of amastigotes in tissue.

By this method, we clearly showed that the antileishmanial effect of AmB was highly dependent on its formulation. In mice treated with AmB deoxycholate (0.8 mg/kg), parasite burdens decreased moderately in the three organs tested, leading to the persistence of leishmanial foci after treatment. In comparison, AmBisome at the same dose (0.8 mg/kg) yielded a marked reduction of the parasite loads in the early treatment group. Moreover, the markedly reduced toxicity of the liposomal formulation enabled us to use higher doses of AmB, resulting in increased and longer-lasting efficacy. In mice who received AmBisome at 50 mg/kg (total dose, 300 mg/kg), complete elimination of the parasite loads was observed in the early treatment group throughout the 14-week follow-up period and in the delayed treatment group throughout the 8-week follow-up period.

In comparison, meglumine antimoniate treatment was much less effective in reducing the parasite burden in the liver and was ineffective in inhibiting parasite growth in the spleen, which could act as a source for relapses after treatment. A similar lack of efficacy of antimonials was previously described for BALB/c mice infected with *L. donovani* (6).

The results of the pharmacokinetic studies of free and encapsulated AmB are in agreement with previous reports and help to explain the favorable outcome of the investigation (1, 16, 17, 29). Both free and liposomal AmB are widely distributed in the body. However, despite similar trough-level concentrations in plasma, peak concentrations of AmB for mice in

the early treatment group treated with AmBisome were >25-fold higher in the spleen and >75-fold higher in the liver than those for mice treated with AmB deoxycholate at the same dose. The higher drug concentration obtained in tissue after AmBisome treatment slowly decreased, yielding significant sustained levels of AmB in the tissues examined after cessation of therapy. In comparison, AmB levels in the tissues examined decreased quickly after treatment with AmB deoxycholate. At higher doses of AmBisome, we detected proportionally higher peak drug concentrations in the tissues examined. The drug accumulation in tissue and the increase in drug delivery to the parasitophorous vacuole, probably due to direct drug targeting in the infected macrophages (7), combined to produce the markedly superior antileishmanial activity of the liposomal AmB formulation (i.e., AmBisome), particularly at the acute stage of the infection. When treatment was administered at the chronic stage, high doses of AmBisome (50 mg/kg) were necessary to induce and maintain the clearance of infection in tissues; this correlated with sustained high levels of AmB in the liver and spleen and, to a lesser degree, in the lungs. At a lower dose of AmBisome, relapses in the liver occurred several weeks after cessation of therapy, indicating that parasites had not been eradicated during the treatment period and were probably the cause of reactivation when drug levels decreased.

These results suggest that the maintenance of high levels of AmB in tissue is a determinant for preventing relapses. This is especially important for AIDS patients who present with severe immune dysfunctions and for whom relapses of VL are common (10). AIDS patients require maintenance therapy to minimize relapses. The persistence of high levels of AmB in tissues after administration of AmBisome favors the use of this drug for such secondary prophylaxis. Furthermore, the sensitivity of the present experimental model for screening recurrent infections makes it useful for further defining optimal drug prophylaxis regimens.

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