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Nanodisk-Associated Amphotericin B Clears *Leishmania major* Cutaneous Infection in Susceptible BALB/c Mice

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Nanometer-scale, apolipoprotein-stabilized phospholipid bilayer disk complexes (nanodisks [ND]) harboring the toxic and poorly soluble antileishmanial agent amphotericin B (AMB) were examined for efficacy in treatment of *Leishmania major*-infected BALB/c mice (*Mus musculus*). *L. major*-infected mice were intraperitoneally (i.p.) treated with AMB-ND in 0-, 1-, and 5-mg/kg doses at 24 h, 48 h, and 4, 7, 14, and 21 days postinfection in two experiments. *L. major*-infected mice were i.p. treated with phosphate-buffered saline, 5 mg/kg AMB-ND, or 5 mg/kg lipid-associated amphotericin B (liposomal amphotericin B, AmBisome) at 24 h, 48 h, and 10, 20, 30, and 40 days postinfection in one experiment. Parasite numbers, footpad lesion size progression, and development of cytokine responses were assayed at days 7, 15, 30, 50, 140, and 250 or at days 14, 30, 50, 95, and 140 postinfection. Mice administered AMB-ND in 1- or 5-mg/kg doses were significantly protected from *L. major*, displaying decreases in lesion size and parasite burden, particularly at the 5-mg/kg dosage level. In contrast to the i.p. treated AmBisome group, BALB/c mice treated with i.p. AMB-ND completely cleared an *L. major* infection by 140 to 250 days postinfection, with no lesions remaining and no parasites isolated from infected animals. Restimulated mixed lymphocyte culture cytokine responses (interleukin-4 [IL-4], IL-12, IL-10, NO, and gamma interferon) were unchanged by AMB-ND administration compared to controls. The marked clearance of *Leishmania* parasites from a susceptible strain of mice without an appreciable change in the cytokine response suggests that AMB-ND represent a potentially useful formulation for treatment of intrahistiocytic organisms.

Leishmaniasis is a tropical disease caused by a genus of protozoal parasites, *Leishmania* spp. Infection with *Leishmania* spp. will cause disease ranging from single cutaneous ulcers to life-threatening hepatomegaly and splenomegaly. With its emergence as an opportunistic infection in human immunodeficiency virus-positive individuals, effective treatment of leishmaniasis is of increased importance (14). Currently there are no vaccine protocols for leishmaniasis, and many therapies are limited by toxicity, long-term courses of treatment, expense, and development of parasite resistance to chemotherapeutics (22). Currently used drugs include pentavalent antimonials such as sodium stibogluconate (Pentostam), different amphotericin B (AMB) lipid formulations, and other, lesser used drugs, such as pentamidine, allopurinol, miltefosine, and ketoconazole (4, 10, 22). With all of these agents varying degrees of toxicity are seen, and full clearance of parasites is not always achieved in cutaneous leishmaniasis, both in clinical practice and in experimental studies (22, 23). As a method of countering these limitations, efforts have been made to develop both new drugs and improved methods of delivering known antiprotozoal drugs without concomitant toxicity to the human host. Of particular interest is the possibility of targeting therapeutics to the macrophage phagolysosome, the site of *Leishmania* amastigote reproduction (1, 4–6, 12, 18, 22, 25). Such a strategy would conceivably reduce the required treatment dose and thereby the associated toxicity, expense, and treatment duration.

Recently, methods for the generation of AMB-enriched reconstituted high-density lipoprotein particles have been described (20). The particles generated are comprised of a nanometer-scale, disk-shaped phospholipid bilayer, hereafter referred to as nanodisks (ND), whose periphery is circumscribed by amphipathic apolipoprotein molecules. Apolipoproteins are well known plasma components that function in transport of hydrophobic biomolecules, including cholesterol, triacylglycerol, phospholipid, and fat-soluble vitamins. A common property shared by these proteins is an ability to disrupt certain phospholipid bilayer vesicles and transform them into disk-shaped lipid/protein complexes (19, 21). Hydrophobic drugs such as the polyene antibiotic AMB can intercalate between phospholipids in the bilayer component of the complex, effectively solubilizing the active biomolecule. ND are distinguished from conventional liposomes or lipid microvesicles in that they do not possess an aqueous core, they are fully soluble in aqueous media, their diameters range from 8 to 20 nm (rather than 60 to 250 nm for liposomes), and apolipoproteins are an intrinsic structural element of the complex (19).

In the susceptible BALB/c mouse model, there is evidence that conventional drug therapies alone are often insufficient for clearance of cutaneous *Leishmania* infection. Nabors et al. (17) and Li et al. (11) showed that clearance of established cutaneous *L. major* infections in BALB/c mice required not only Pentostam (sodium stibogluconate) but also therapy with

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interleukin-12 (IL-12) or gamma interferon (IFN-γ) to upregulate IL-12 levels and cause a trend towards a Th1 immune response in the animals. A similar necessity for IL-12 has been shown by other researchers (16). In short, in the murine model, successful treatment of cutaneous leishmaniasis with pentavalent antimonials must often be accompanied by an immune response similar to that seen in a resistant animal to achieve full clearance of infection. AMB therapy may also require an immune response, especially tumor necrosis factor (15). Other researchers, however, have claimed that AMB can act independent of an immune response (8, 16). There is recent evidence that AMB derivatives may negatively influence B-cell responses, as well as increase tumor necrosis factor alpha production (7).

In this study, we determined the efficacy of AMB-ND complexes in treatment of *L. major* cutaneous infection. The data demonstrate that six 5-mg/kg doses of AMB-ND delivered at 1- to 10-day intervals over the course of 3 to 5 weeks are capable of clearing an *L. major* infection in the BALB/c mouse. This novel lipid formulation of AMB is significantly more efficacious for treatment of cutaneous leishmaniasis than similar doses of the liposomal AMB formulation AmBisome, and it resulted in parasite clearance without statistically significant changes in immune response compared to controls.

MATERIALS AND METHODS

Mice. Adult female BALB/c mice (6 to 10 week old) were procured from the National Cancer Institute. Mice were kept in colonies at Colorado State University (CSU) under supervision of the CSU Laboratory Animal Resources Department with authorization by the Animal Care and Use Committee. Mice were anesthetized with intraperitoneal (i.p.) Ketamine (75 mg/kg) and Xylazine (15 mg/kg) prior to subcutaneous inoculation with 1×10^6 *L. major* parasites in 50 μl of Dulbecco's modified Eagle medium (DMEM) in the left hind footpad. Mice were treated with AMB-ND or AmBisome (Astellas Pharma US, Deerfield, IL) at a concentration of 1 mg/kg or 5 mg/kg AMB in a 200-μl total volume/mouse. Control mice were injected with an equivalent amount of empty ND, lacking AMB, in the first two experiments and with phosphate-buffered saline (PBS) in the final experiment. Mice were injected in the peritoneal cavity at each treatment time point. Treatment time points for the first experiments (0, 1, and 5 mg/kg AMB-ND) were 24 h, 48 h, 7 days, 14 days, and 21 days postinfection. The final experiment, with PBS (control), AmBisome (5 mg/kg AMB), or AMB-ND (5 mg/kg AMB) groups, had treatments at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days postinfection. Footpad lesion size was measured using a Vernier caliper at 2- to 4-day intervals throughout a given experiment. For measurement of lesion size, the infected left footpad was compared to the normal right footpad. Mice were euthanized in a CO_2 chamber at day 7, day 15, day 30, day 50, and the indicated later time points to assess parasite burden and cytokine responses of restimulated lymph node cells.

Parasites. *L. major* parasites from the LV39 strain were grown on sheep blood agar in parasite growth medium (RPMI-1640 [Sigma-Aldrich, St. Louis, MO] with 5% fetal bovine serum [HyClone, Logan, UT], 10 mM HEPES [Sigma-Aldrich], 100 U/ml penicillin [Gibco, Carlsbad, CA], 100 μg/ml streptomycin [Gibco], 2 mM L-glutamine [Gibco], 1 mM sodium pyruvate [Gibco], 0.2 mM L-asparagine [Calbiochem, San Diego, CA], 0.6 mM L-arginine [Calbiochem], and 2% sterile-filtered normal human urine). Parasites were passaged through mice every 2 months to retain virulence and from flask to flask twice a week. At 5 to 6 days after flask inoculation, parasites were removed from the growth medium, centrifuged to remove dead parasites, washed in DMEM, and counted on a Neubauer hematocytometer prior to resuspension in DMEM.

Nanodisks. AMB-ND or empty ND were produced essentially as described by Oda et al. (20). Briefly, 10 mg of dispersed phospholipid vesicle substrate comprised of 7 mg dimyristoylphosphatidylcholine and 3 mg dimyristoylphosphatidylglycerol plus 2.5 mg AMB was incubated with 4 mg recombinant human apolipoprotein A-I at 24°C. Following nanodisk formation and dialysis against PBS, ND-associated AMB was determined spectrophotometrically at 416 nm by dissolving an aliquot of the ND solution in dimethyl sulfoxide (extinction coefficient, $1.214 \times 10^5\ M^{-1}\ cm^{-1}$). Sterile filtered ND were stored in the dark at 4°C for <40 days. ND preparations were diluted in PBS, pH 7.0, to the respective concentrations for each treatment group.

Parasite quantification. Parasite burden was evaluated using a limiting-dilution assay. Footpads were skinned and removed from euthanized mice. Footpads from multiple mice from the same group were ground in a tissue homogenizer in modified Schneider's insect medium (Sigma-Aldrich) containing 10% fetal bovine serum, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μg/ml gentamicin (Sigma-Aldrich), 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2 mM L-asparagine, 0.6 mM L-arginine, and 2% sterile-filtered normal human urine. Pooled samples were diluted in 5 ml modified Schneider's insect medium per infected footpad. Samples were plated in 100-μl aliquots on 96-well plates, with a 10-fold dilution of each row. Plates were sealed and set in the dark at room temperature for 10 to 14 days before evaluation. Plates were scored and the results evaluated using the ELIDA program (ELIDA software 1985–2005; Carl Taswell) for statistical analysis (24).

Cytokine assays. Popliteal and inguinal lymph nodes draining the infected footpads were removed from euthanized mice. Lymph nodes from multiple mice in the same treatment groups were pooled. Lymph nodes were placed in DMEM (Gibco) and squashed using sterile metal screens to release cells. Mixed cell populations isolated from the lymph nodes were washed and pelleted three times in DMEM at 1,000 rpm. Resuspended cells were mixed 1:1 with Trypan blue stain (Invitrogen) and counted on a Neubauer hematocytometer. Counted live cells were diluted to 5×10^6 cells/ml in DMEM cell medium (DMEM, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 2 mM L-glutamine, 1 mM sodium pyruvate, 0.2 mM L-asparagine, 0.6 mM L-arginine, and 0.5% normal mouse serum) and added to 24-well plates at 1 ml/well. *L. major* promastigotes were added to the plates at 2×10^6 parasites/well. Lymph node cells were cultured at 37°C in 5% CO_2 for 48 h, at which time supernatants were collected and stored at −20°C. Commercially available enzyme-linked immunosorbent assays (ELISAs) were performed to determine levels of IFN-γ, IL-12, IL-4, and IL-10 (BD PharMingen, San Jose, CA) in supernatants. Samples were assayed for levels of NO by utilizing the Griess reaction (Sigma).

Anti-*Leishmania* antibody assay. *L. major* promastigotes were washed in 1× PBS, resuspended at a concentration of 1×10^8 parasites/ml, and sheared with a 30-gauge needle. LDS loading dye (Invitrogen, Carlsbad, CA) was added and the lysates stored at −20°C. Aliquots of the *L. major* lysate (10 μl) were heated at 95°C for 5 min, cooled, loaded into lanes of a 4 to 12% bis-Tris polyacrylamide gel (Invitrogen), and separated by electrophoresis in 1× MOPS (morpholinepropanesulfonic acid) buffer under reducing conditions. The gel was transferred to nitrocellulose in Towbin buffer (10% methanol, 25 mM Tris-HCl, 192 mM glycine) and blocked in 1% gelatin. The nitrocellulose was tested for completeness of protein transfer with MemCode (Pierce, Rockford, IL). Nitrocellulose strips were incubated for 1 h with a given mouse serum diluted 1:100 in 50 mM Tris-HCl, pH 7.5, 150 mM sodium chloride, 0.1% Tween 20 (TNT). Blood from different *L. major*-infected BALB/c mice was collected by cardiac puncture at euthanasia 50 days following inoculation with 1×10^6 LV39 *L. major* promastigotes and subsequent treatment with PBS (control), AmBisome (3 mg/kg or 5 mg/kg AMB), or AMB-ND (3 mg/kg or 5 mg/kg AMB). Blood from mice in each treatment group was centrifuged to obtain serum, which was stored at −20°C until use. Following incubation with serum, nitrocellulose strips were washed three times with TNT and then reacted with a 1:1,000 dilution of alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G in TNT. After multiple washings, the nitrocellulose strips were developed with the BCIP (5-bromo-4-chloro-3-indolylphosphate)/nitroblue tetrazolium reagent. An identical gel was subjected to silver stain analysis (SilverExpress; Invitrogen) following the supplier's directions.

Statistical analysis. The Student paired *t* test and analysis of variance (ANOVA) were used for statistical analyses of the footpad lesion size data and the cytokine response data (Systat 9.0). The parasite numbers per footpad was analyzed using the ELIDA software (24) for initial analysis and, secondarily, ANOVA on linear plots of the data. Differences were considered significant when the P value was <0.05.

RESULTS

Effect of AMB-ND on survival and lesion size in *L. major*-infected mice. Footpad injection of BALB/c mice with *L. major* promastigotes results in the appearance of measurable lesions. In untreated animals or those treated with empty ND lacking AMB, lesion size continued to increase as a function of time, up to day 50, when lesions were judged to be severe and mice

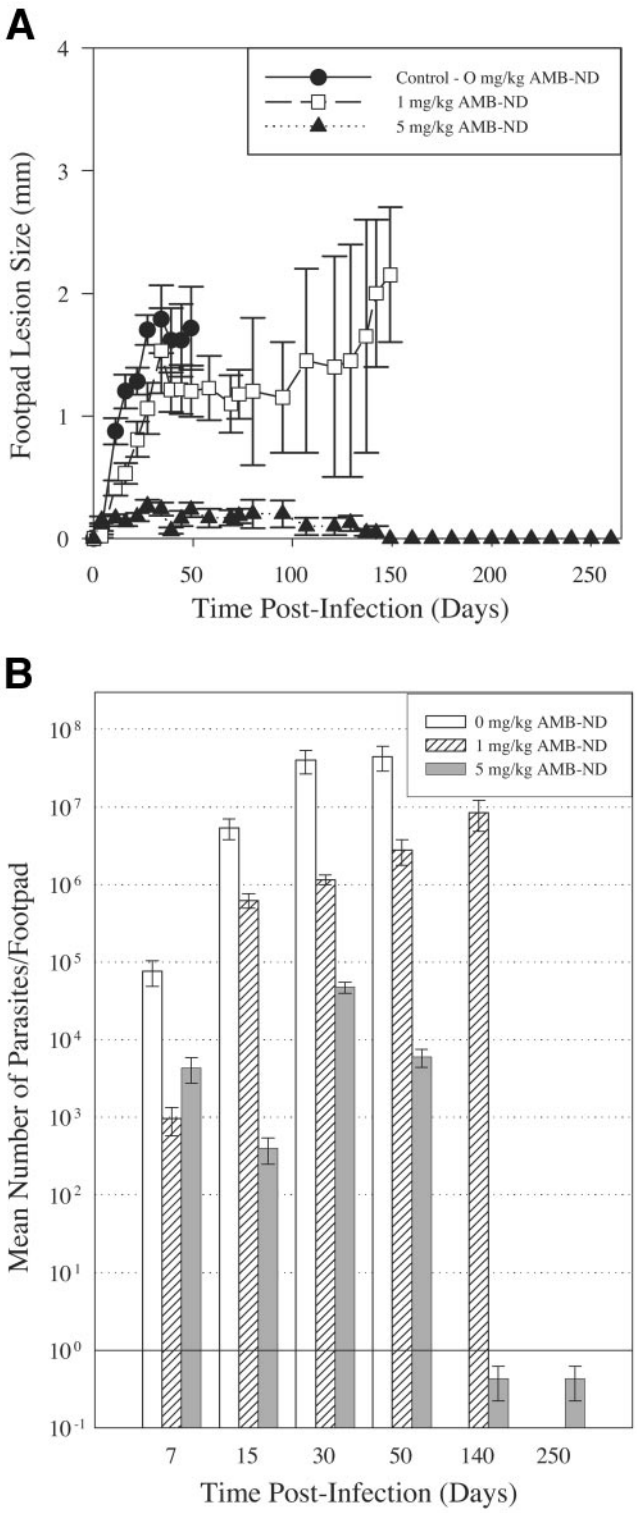

FIG. 1. Effects of AMB-ND treatment on disease progression in *L. major*-infected BALB/c mice. Mice were infected with 1×10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 7 days, 14 days, and 21 days postinfection with empty ND or AMB-ND (0, 1, or 5 mg/kg AMB per treatment). Values are averaged from multiple ($n = 2$) experiments utilizing identical parameters. (A) Effect of AMB-ND treatment on footpad lesion size in *L. major*-infected BALB/c mice. Footpad lesion size was measured at the indicated time points. Values plotted are the means ± standard deviations of 4 to 20 measurements. (B) Effect of AMB-ND treatment on parasite burden in *L. major*-infected BALB/c mice. Parasite numbers per footpad were determined as described in Materials and Methods from samples obtained at the indicated time points. Values reported are the means ± 95% confidence limits of parasite numbers based on limiting-dilution analysis as described in Material and Methods.

were euthanatized (Fig. 1A). By contrast, *L. major*-infected mice treated with AMB-ND at 1 mg/kg or 5 mg/kg survived a minimum of 100 days longer than controls treated with empty ND ($P < 0.0001$) before developing comparable lesions and being euthanatized. Animals in all groups were euthanatized if they reached a predetermined level of lesion severity and/or displayed clinical signs of distress or pain. A dose-response relationship was observed between lesion size and AMB-ND treatment, with decreased lesion size associated with increasing AMB-ND dose ($P < 0.0001$). Remarkably, animals treated with 5 mg/kg AMB-ND had no measurable footpad lesions by day 150 postinfection.

Effect of AMB-ND treatment on *L. major* parasite number. *L. major*-infected mice treated with AMB-ND at a concentration of 1 mg/kg or 5 mg/kg had significantly ($P < 0.05$ by ELIDA [24]) lower parasite numbers/footpad than untreated controls (Fig. 1B). Following cessation of therapy, mice in the 1-mg/kg AMB-ND treatment group eventually developed parasite levels equivalent to those seen in control animals at the time of euthanasia, although this took over 100 days longer. On the other hand, mice treated with 5 mg/kg AMB-ND had parasite numbers/footpad that were progressively lower than those from both the control and 1-mg/kg AMB-ND treatment groups ($P < 0.0001$ by ANOVA). Most significantly, at the time of the final two determinations of parasite numbers (days 150 and 250), those mice treated with 5 mg/kg AMB-ND had a mean parasite number/footpad of 0.4, with a 95% confidence interval of 0 to 1, indicating effective eradication of the parasite from the infection site.

Cytokine responses in *L. major*-infected mice. ELISAs were performed in triplicate on samples obtained from two separate experiments. Cytokine assays of *L. major*-infected mice showed no significant differences between the AMB-ND and control treatment groups (Table 1). Both groups manifested similar cytokine responses, with low IFN-γ, IL-12, and NO production and moderate to large amounts of IL-4 and IL-10. The only changes evident in the AMB-ND treatment groups were a mild increase in IFN-γ and a slight decrease in IL-4 and IL-10 at later time points (days 50, 140, and 250). However, because no animals in the control, empty ND treatment group survived past day 50, this may be a nonsignificant change for the later time points, although we cannot assess that.

Effect of lipid formulation on AMB efficacy in *L. major*-infected mice. To evaluate the extent to which features of the ND particle structure or composition are responsible for the observed efficacy against *L. major* infection in mice, AMB-ND were compared directly with the liposomal AMB formulation AmBisome in a single experiment. Using an identical AMB dose and treatment regimen, AMB-ND-treated mice displayed decreased lesion size as a function of time and increased survival compared to AmBisome-treated mice, which developed

TABLE 1. Effect of AMB-ND therapy on cytokine production in *L. major*-infected mice[a]

Cytokine and day	Concn[b] in treatment group:		
	Untreated controls	1 mg/kg AMB-ND	5 mg/kg AMB-ND
IFN-γ			
15	85.12 ± 51.29	31.99 ± 13.81	42.32 ± 48.01
30	12.75 ± 7.51	14.51 ± 14.65	2.08 ± 2.03
50	15.29 ± 7.14	26.72 ± 5.46	31.66 ± 10.24
140		14.40 ± 2.34	38.97 ± 0.28
250			114.7 ± 3.6
IL-12			
15	173.33 ± 78.76	41.96 ± 49.75	138.30 ± 18.38
30	0	13.86 ± 27.73	88.72 ± 115.25
50	0	7.50 ± 15.00	0
140		0	0
250			66 ± 3.3
NO			
15	2.40 ± 0.38	0.44 ± 0.45	0.39 ± 0.46
30	0.03 ± 0.06	0.71 ± 0.83	0.094 ± 0.19
50	2.31 ± 2.23	0.71 ± 0.64	0.54 ± 0.29
140		4.69 ± 4.15	1.29 ± 0.39
250			0.4 ± 0.01
IL-4			
15	136.82 ± 84.70	109.87 ± 51.96	54.43 ± 12.29
30	104.55 ± 18.68	80.06 ± 10.41	75.00 ± 51.43
50	95.44 ± 32.63	75.03 ± 9.76	121.45 ± 55.53
140		73.03 ± 4.57	10.43 ± 2.05
250			11.50 ± 0.64
IL-10			
15	166.60 ± 29.96	129.23 ± 51.86	72.20 ± 58.77
30	27.97 ± 32.32	33.72 ± 36.03	45.94 ± 52.12
50	96.05 ± 37.23	57.31 ± 18.67	70.93 ± 21.67
140		28.79 ± 8.66	25.05 ± 2.43
250			28.30 ± 5.20

[a] BALB/c mice were infected with 1×10^6 LV39 *L. major* promastigotes, followed by treatment with PBS or AMB-ND (1 and 5 mg/kg AMB) at 24 h, 48 h, 7 days, 14 days, and 21 days. Lymph node culture supernatants were obtained at the specified time points following infection and assayed for cytokine production.

[b] Values reported are the means ± standard deviations of ELISA measurements ($n = 3$) from two separate experiments. Values for IFN-γ, IL-12, and IL-10 are nanograms per milliliter; those for NO and IL-4 are micromoles per milliliter and picograms per milliliter, respectively.

severe, life-threatening lesions by day 95 postinfection and were euthanatized (Fig. 2A). Lesion size was uniformly statistically significantly different between the AMB-ND and AmBisome treatment groups ($P < 0.0001$). Similarly, the AMB-ND treatment groups had significantly lower ($P < 0.05$) parasite numbers/footpad than the corresponding AmBisome treatment groups, particularly at later time points (days 36, 50, and 95) (Fig. 2B). A 10- to 100-fold decrease in parasite numbers was observed for the AMB-ND treatment group compared to the AmBisome treatment group when they were administered equivalent doses of AMB at similar time points.

Cytokine assays showed no significant differences between cytokine responses for control, AmBisome, or AMB-ND treatment groups (Table 2).

AMB-ND treatment induces an antibody response to *L. major*. To assess the humoral immune response to *L. major* and verify infection, AMB-ND- and AmBisome-treated animals or control animals treated with PBS were assayed for *L. major* antigen reactivity. Although there was recognition of *L. major* antigens by sera from the 5-mg/kg AMB-ND group, the profile of proteins recognized was different from that of other serum samples. Also, there was an apparent decreased production of

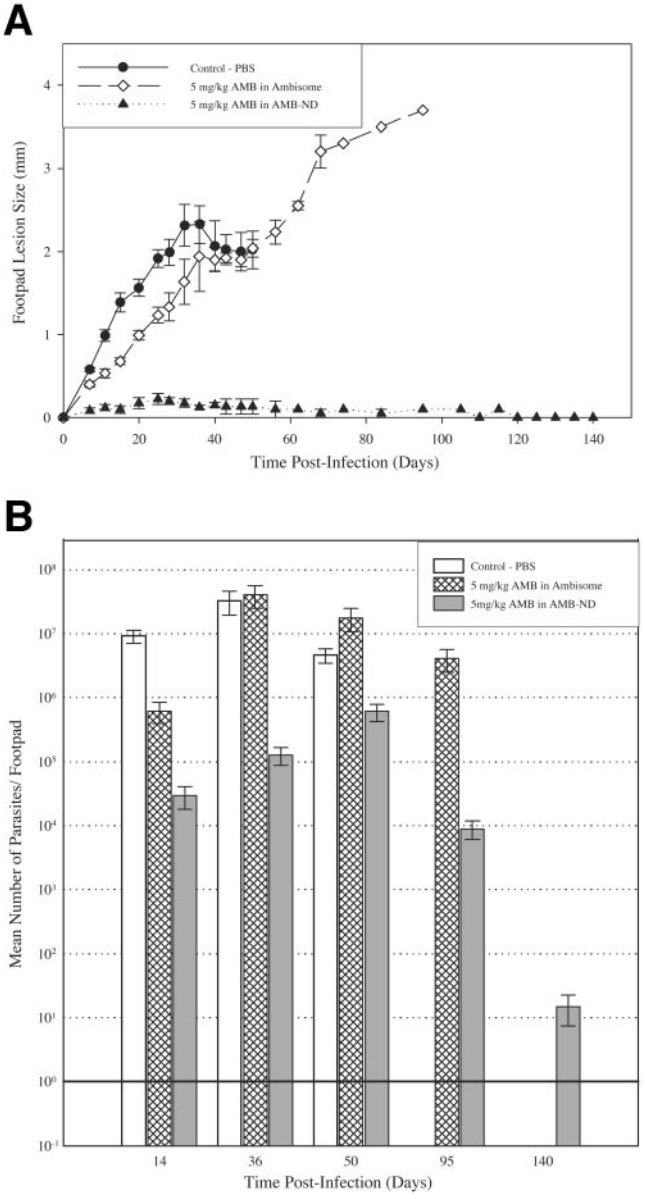

FIG. 2. Effect of AMB formulation on disease progression in *L. major*-infected BALB/c mice. Groups of mice were infected with 1×10^6 LV39 *L. major* promastigotes and treated at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days with PBS (control), 5 mg/kg AMB in AmBisome, or 5 mg/kg AMB in AMB-ND. Data are derived from a single experiment ($n = 1$). (A) Effect of AMB formulation on footpad lesion size in *L. major*-infected BALB/c mice. Footpad lesion size was measured at the indicated time points. Values plotted are the means ± standard deviations of 2 to 14 measurements. (B) Effect of AMB formulation on parasite numbers per footpad in *L. major*-infected BALB/c mice. Parasite numbers per footpad were determined as described in Materials and Methods from samples obtained at the indicated time points. Values reported are the means ± 95% confidence limits of parasite numbers based on limiting-dilution analysis as described in Material and Methods.

TABLE 2. Effect of AMB formulation on cytokine production in *L. major*-infected mice[a]

Cytokine and day	Concn[b] in treatment group:		
	Untreated controls	5 mg/kg AmBisome	5 mg/kg AMB-ND
IFN-γ			
14	24.6 ± 2.9	18.6 ± 0.92	23.4 ± 0.76
36	0.06 ± 0.002	0.62 ± 0.02	1.2 ± 0.05
50	3 ± 0.23	3.4 ± 0.24	0.58 ± 0.08
95		1.6 ± 0.36	1.64 ± 0.35
140			39.3 ± 7.5
IL-12			
14	68 ± 8.7	109 ± 7.1	134 ± 13
36	18.9 ± 7.2	33.6 ± 7.9	14.5 ± 9
50	64.2 ± 5.6	44.7 ± 26.8	56.9 ± 16.2
95		49.4 ± 3.1	38.8 ± 6
140			17.3 ± 4.6
NO			
14	0.17 ± 0.02	0.03 ± 0.01	0.11 ± 0.02
36	0	0.2 ± 0.07	0.03 ± 0.01
50	0.28 ± 0.02	0.34 ± 0.03	0.2 ± 0.01
95		0.08 ± 0.01	0.05 ± 0.01
140			0
IL-4			
14	11.7 ± 0.87	10.5 ± 1.08	3.6 ± 0.61
36	5.2 ± 0.55	8.4 ± 0.9	4.5 ± 0.53
50	6.2 ± 0.43	6.8 ± 0.35	16.4 ± 3.1
95		4.6 ± 0.26	2.8 ± 0.15
140			42.7 ± 2.8
IL-10			
14	160 ± 14	137 ± 17	160 ± 31
36	49.2 ± 4.3	78.8 ± 4.4	51.6 ± 1.7
50	69.3 ± 3.8	71.2 ± 7.4	91 ± 6.6
95		53.5 ± 4.3	47.3 ± 3.6
140			90.6 ± 1.2

[a] BALB/c mice were infected with 1×10^6 LV39 *L. major* promastigotes and treated with PBS, 5 mg/kg AMB in AmBisome, or 5 mg/kg AMB in AMB-ND at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days. Lymph node culture supernatants were obtained at the specified time points and analyzed for cytokine production.

[b] Values reported are the means ± standard deviations of ELISA measurements ($n = 3$) from one experiment. Values for IFN-γ, IL-12, and IL-10 are nanograms per milliliter; those for NO and IL-4 are micromoles per milliliter and picograms per milliliter, respectively.

antibody against *L. major* antigens by mice in this treatment group, as evidenced by decreased immunostaining (Fig. 3). In this treatment group, bands seen in other treatment groups at 55, 30, and 17 to 20 kDa were not present. However, there was a reaction against a unique band at ~32 kDa. Treatment with 3 mg/kg AMB-ND, 3 mg/kg AmBisome, or 5 mg/kg AmBisome did not produce any significant differences in antibody responses to *L. major* antigens compared to PBS-treated controls, as judged by Western blot analysis.

DISCUSSION

In this study, we evaluated a novel lipid formulation of AMB in treatment of *L. major* infection in susceptible BALB/c mice. The data reveal that nonlethal doses of AMB-ND eradicate an experimental cutaneous *L. major* infection. Numerous studies have confirmed the efficacy of AMB lipid formulations in treatment of visceral *Leishmania* infection, achieving clearance rates of up to 96% of organisms with high-dose treatment and long-term recrudescence rates of as low as 10 to 20% (7, 8, 15). Similar studies have shown increased efficacy and decreased toxicity of liposome-complexed AMB in treatment of experimental *L. infantum* infection (9). Treatment of cutaneous *L. major* infection with AMB lipid formulations (specifically AmBisome) has resulted in temporary reductions in lesion size without complete clearance of the parasites (26, 27). To assess the efficacy of AMB-ND, we compared i.p. injected AMB-ND, empty ND, and AmBisome with respect to their abilities to decrease pathology (e.g., cutaneous lesion size) and parasite burden in *L. major*-infected BALB/c mice. The results show that at 5 mg/kg AMB, under the treatment regimen employed, AMB-ND elicit a marked improvement over AmBisome in terms of pathology, parasite burden, and survival. At the same time, empty ND were ineffective. AmBisome has been reported to be effective for temporary lesion resolution at an intravenous (i.v.) 25- to 50-mg/kg dose for a 6- to 12-day, six-dose treatment regimen, with lesions dramatically reduced over the following 4 weeks, although lesions recurred within 7 to 8 weeks posttreatment (26, 27). Based on the fact that parasite inoculum, treatment frequency, and dosage of AMB

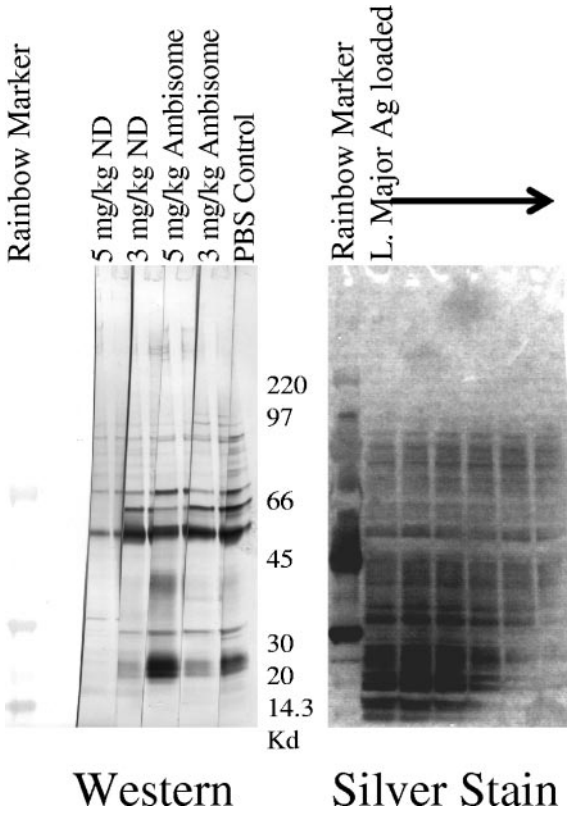

FIG. 3. Immunoblot of *L. major* antigens. *L. major* promastigote lysate proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. The membrane was probed with anti-*L. major* BALB/c mouse serum collected 50 days following inoculation with 1×10^6 LV39 *Lm* promastigotes and subsequent treatment with PBS (control), AmBisome (3 mg/kg or 5 mg/kg AMB), or AMB-ND (3 mg/kg or 5 mg/kg AMB) at 24 h, 48 h, 10 days, 20 days, 30 days, and 40 days. Images collected with Adobe Photoshop 6.0 and Windows Powerpoint 2003.

were identical in the ND and AmBisome treatment groups, the data suggest that some aspect of the ND particle structure may be enhancing the therapeutic potential of the antibiotic. A potential confounding factor may be the administration of AmBisome and AMB-ND via the i.p. route rather than i.v., as it is known that AmBisome has reduced effectiveness when administered subcutaneously near lesions and may be less effective when administered i.p. (26). However, the same might well be true for AMB-ND, and future studies using i.v. administration of AMB-ND will address this question. Mechanisms for increasing the effectiveness of the AMB-ND formulation may include increased time in circulation, bioavailability, or increased delivery of AMB-ND complexes to the infected macrophages.

Lipid complexes have been used for many modalities of drug delivery and have proven to be efficacious in both reducing toxicity and increasing the circulation residence time of associated drugs (see references 1 and 12 [liposomes and phospholipid microspheres], 9 [AmBisome], and 27 [Ambisome, Amphocil, and Abelcet]). The drug clearance time for AmBisome, in particular, can be dramatically decreased in various tissue compartments, compared to in the blood (9). ND are lipid complexes and, as such, they may have an altered clearance time, potentially producing enhanced effects at a lower dosage frequency. Experiments to assess this were not specifically performed, but circumstantial evidence, consisting of the observed long-term effects of AMB-ND treatment on parasite numbers, wherein parasite burden continued to decline for >100 days following the final AMB-ND treatment at day 21 or 40 postinfection (Fig. 1B and 2B), suggests that the ND lipid milieu may protect associated AMB from degradation or clearance for weeks to months following administration, thus producing the long-term posttreatment effects seen.

Another possibility is that ND are recognized by the class A scavenger receptor (SR-A) on macrophages, the tissue site of *L. major* replication. It is known that apolipoproteins, modified by acetylation, oxidization, or other modifications, serve as ligands for SR-A. Although the apolipoprotein component of AMB-ND (recombinant human apolipoprotein A-I) has not been deliberately modified, recognition of nonself lipoproteins by the murine SR-A cannot be excluded at present. It has been shown that activated macrophages increase expression of scavenger receptors on their surfaces (25) and that macrophages infected with *L. chagasi* have increased scavenger receptors on their surface (3), suggesting that *Leishmania* phagocytosis results in up-regulation of SR-A. Others have observed colocalization of phagolysosome compartments and scavenger receptor-targeted molecules (25), demonstrating delivery of SR-A ligands to the phagolysosome. Increased delivery of AMB-ND to the parasite-containing macrophage population or direct targeting to the phagolysosome compartment would be expected to result in increased efficacy of AMB-ND in *Leishmania*-infected mice. This hypothesis needs further investigation, including assessment of the interaction between macrophage scavenger receptors and ND, as well as investigation of colocalization of ND and phagolysosomes.

With many of the experimental and clinical anticutaneous leishmaniasis therapeutics, a degree of immune response is necessary, as in the case of the pentavalent antimonials (11, 16, 17), where Th2-to-Th1 switching via IL-12 or IFN-γ is necessary for complete clearance. For this reason, we assessed several Th1 (IL-12, IFN-γ, and NO) and Th2 (IL-4 and IL-10) cytokines to investigate the possible role of the cytokine immune response in AMB-ND treatment of cutaneous leishmaniasis. In the present study, we observed no significant differences in any of the cytokines assayed between AMB-ND treatment groups and those treated with PBS or empty AMB-ND alone. These data indicate that there is no requirement for a change in the cytokine response for full and lasting clearance of *L. major* from BALB/c mice with AMB-ND treatment. Furthermore, these results suggest that AMB-ND treatment may function equally well in immunocompromised patients, thereby increasing its potential utility in human immunodeficiency virus-leishmaniasis coinfection therapy. Although there is some evidence that AMB clearance of *Leishmania* infection is nonimmune status dependent, no studies to date have shown definitive clearance of cutaneous *Leishmania* infection without immunotherapy intervention.

As in the control treatment group, mice treated with AMB-ND developed antibodies to *L. major* (Fig. 3). Differences between the antigen banding patterns in the 5-mg/kg AMB-ND group versus all other groups, however, suggest there is some fundamental difference in antibody production associated with early clearance and failure to establish a full *L. major* infection in this treatment group. Decreased antibody binding suggests that antibody production was reduced in this group. A similar decrease in antibody reactivity has been observed in humans and dogs with spontaneous or chemotherapeutic-associated healing of *Leishmania* infections (2, 13). The decreased antibody reactivity in the 5-mg/kg AMB-ND treatment group is possibly associated with early clearance of the majority of the inoculated *L. major*, leaving fewer parasites to produce antigens and thus reduced antigen exposure for the host over the course of the infection, as well as parasite destruction leading to a different antigen profile, including cytoskeletal, organelle, and other antigens from dead or dying *L. major*.

In conclusion, AMB-ND represent a remarkably effective therapy for experimental cutaneous *L. major* infection in BALB/c mice. At an equivalent dosage and treatment frequency it far surpasses commercially available liposomal AMB and results in sterile clearance of *L. major* infection, using a limited and widely spaced therapeutic regimen. The absence of statistically discernible changes in the cytokine response indicates that this therapy is not dependent on host cytokine-based immunity for sterile clearance. Future investigations will explore the interaction between ND and macrophage scavenger receptors. In addition, investigations of the tissue and the plasma pharmacokinetics of ND-associated AMB and toxicity studies of organs affected by AMB, specifically including the renal and hepatic systems, are planned. Given the versatility of the ND formulation with respect to its intrinsic protein component, protein engineering may be used to specifically target ND to macrophages.

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