Lack of Efficacy of Liposomal Amphotericin B Against Acute and Chronic *Trypanosoma cruzi* Infection in Mice

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Abstract. Acute and chronic infection with *Trypanosoma cruzi* affects millions of people. The current therapeutic options are highly toxic and often not effective. Liposomal amphotericin B (LAMB) has been demonstrated previously to have some activity in murine models. In our studies, higher dosages given multiple times were tested for activity against acute or chronic disease, exploring whether intermittent and brief regimens could be effective, as might then prove useful in human, particularly outpatient, therapy. For acute infection, LAMB 25 mg/kg intravenously (i.v.) given one to three times prolonged survival and caused a rapid disappearance of Y strain trypomastigotes from the blood. However, even four or six doses of LAMB 30 mg/kg i.v., did not result in the cure of Y strain infection, with all mice relapsing after being immunosuppressed with cyclophosphamide. Similarly, chronic infection due to the CL strain was found to be unaltered by 1–3 treatments with LAMB 25 mg/kg. All surviving mice had histopathological evidence of infection in one or more tissues and equivalent antibody titers regardless of treatment regimen. Overall, LAMB at doses up to 30 mg/kg i.v. prolonged survival, but these doses were not curative in the regimens studied.

INTRODUCTION

Trypanosoma cruzi, the etiologic agent of Chagas disease, is endemic to the Americas and of particular importance to Latin America where it is a serious cause of morbidity and mortality in Central and South America.^{1–5} Chagas disease was estimated to affect approximately 12–20 million people in 1992.⁵ However, more recent estimates based on 2010 data indicate a reduction in the burden of disease, with an estimate of 5.7 million people with infection.⁶ This reduction is very likely due to efforts in eradication of the vectors, as well as screening of blood supplies. It is increasingly important in the United States and other nonendemic countries because of emigration.⁷ For example, it has been estimated that about 300,000 individuals in the United States have Chagas disease, with up to 45,000 having cardiomyopathies.^{1,2} Although few naturally occurring cases have been reported in the United States, a variety of potential animal reservoirs and insect vectors have been reported, particularly across the southern tier of states.⁸

The current therapies (e.g., benznidazole or nitrofurans) are highly toxic and have limited efficacy.⁹ In addition, infected individuals must take the drugs over a long period. Additional therapies with antifungals, posaconazole, a prodrug of ravuconazole, and other azole derivatives, as well as compounds related to pentamidine, arylimidamides, and derivatives of nitrofurantoin and others have been studied experimentally with encouraging results^{9–12} but have not achieved acceptance as standard clinical practice. Clinical trials with posaconazole and ravuconazole showed that patients treated with either drug as monotherapy had a significantly higher rate of treatment failure than did those given benznidazole alone.^{13–15} Similarly, the use of a combination therapy with posaconazole and benznidazole showed no benefit over monotherapy with benznidazole.¹⁶ Few studies have been performed using amphotericin B. In one study, conventional

amphotericin B was reported to have good efficacy for three treated patients.¹⁷ In two experimental studies, liposomal amphotericin B (AmBisome, Gilead Sciences, Foster City, CA) was tested and showed efficacy.^{18,19} Because cure is elusive, particularly in chronic Chagas disease cases, alternative treatment modalities are needed that are less toxic, can be administered for a shorter period of time, and are curative. Our goal was to determine whether a high-dose regimen of AmBisome (liposomal amphotericin B) given only one or a few times would show efficacy against infection with *T. cruzi*.

METHODS

Murine studies. All murine studies described herein were conducted with the approval of the Institutional Animal Care and Use Committee of the California Institute for Medical Research. All infected mice were housed in sterile micro-isolator cages and provided sterilized food and water ad libitum.

Organism. *Trypanosoma cruzi*, Y and CL strains, were obtained from Dr. Juan Engel at University of California San Francisco. Trypomastigotes were mouse-passed to enhance virulence and increase the number of organisms before the therapy experiment. Trypomastigote stocks were thawed and injected intraperitoneally into five 6-week-old female CD-1 mice for passage. After 15 days, infected mice were bled and trypanosomes counted on a hemacytometer and quantified as trypomastigotes per mL. One additional mouse passage was done and blood was stored in liquid N₂.

Inoculum preparation. The inoculum was prepared by mouse passage. Stored trypanosome stocks were thawed and injected intraperitoneally into fifteen 6-week-old female CD-1 mice at 2×10^5 trypanosomes/mouse. Mice were bled (exsanguinated) on day 8 postinfection and the number of trypomastigotes/mL determined as described above. The inoculum was prepared from the collected blood by dilution with RPMI+10% FCS to 5×10^5 trypomastigotes/mL. Excess blood was stored in liquid N₂.

Therapy of acute disease. Forty 6-week-old female BALB/c mice from Charles River Laboratories were used in the

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FIGURE 1. Kaplan-Meier plot of the cumulative mortality of mice infected with *Trypanosoma cruzi* and treated with AmBi at 25 mg/kg given one, two, or three times, or mice treated with D5W. Statistical comparison of survival by log rank showed that all treatment groups survived significantly longer than did the D5W-treated controls ($P < 0.0001$). There were no differences between treatment with AmBi at 25 mg/kg 1x, 2x, or 3x ($P > 0.05$).

therapy model. Mice were infected intraperitoneally with 1×10^5 trypomastigotes/mouse. On day 5 postinfection, all mice were bled via tail vein phlebotomy and 0.1 mL of blood collected and heparinized. Blood was diluted in the ratio of 1:2 in RPMI 1640 with L-glutamine and NaHCO_3 with 10% fetal calf serum and the number of trypanosomes/mL of blood from each mouse was determined by counting in a hemacytometer.

Mice were assigned to one of four treatment arms for the study. These groups were 5% dextrose water (D5W) control, or AmBisome (AmBi; Gilead Sciences) at 25 mg/kg given one, two, or three times. AmBi was prepared per the manufacturer's instructions. Treatments began on day 5 postinfection subsequent to the blood sampling. Groups of 10 mice received either AmBi at 25 mg/kg or D5W. Treatment was administered via lateral tail vein in 0.25 mL volume. Those given AmBi were dosed on day 5 (1x), days 5 and 7 (2x), or days 5, 7, and 9 (3x). Mice receiving D5W were dosed on days 5, 7, and 9.

Blood sampling. Blood samples were taken from each mouse 7 days after the last dose of AmBi. Mice given AmBi 1x were sampled on day 12, those given 2x sampled on day 14, and those given 3x sampled on day 16. D5W-treated mice were sampled on day 12. All surviving mice were exsanguinated on day 30. Blood was handled as described above and the number of trypomastigotes/mL determined by hemacytometer count.

Experimental parameters of antitrypanosomal activity. Drug activity was based on survival through 30 days of infection and comparative survival assessed by log rank test. Infectious burden in the blood was determined by hemacytometer count and efficacy determined by comparison of trypomastigote counts between groups at each day of sampling. Comparisons were done by Mann-Whitney U test.

Does therapy clear acute disease? Thirty 6-week-old female BALB/c mice from Charles River Laboratories were infected intraperitoneally with 1×10^5 trypomastigotes of the Y strain of *T. cruzi*. These animals were divided into groups, with 10 mice assigned per group. Treatment groups were No treatment (control, D5W) or AmBisome (AmBi) at 30 mg/kg given four or six times. AmBi was prepared per manufacturer's instructions and dilutions made in sterile D5W. Treatments

FIGURE 2. (A) Enumeration of trypomastigotes in blood before treatment on day 5 of infection, 7 days after the last treatment on days 12, 14, or 16 of infection, and at day 30 of infection. All groups started with $N = 10$. Lack of a data point indicates death of a mouse. Bars represent the median. The lower limit of detection was $< 10^5$ trypomastigotes/mL of blood, indicated by symbols assigned a value of 10^4 . (B) As in (A), symbols are the median number of trypomastigotes/mL of blood for the group.

began on day 5 postinfection. Treatment was administered via lateral tail vein in 0.25 mL volume.

Those given AmBi were dosed either on day 5, 7, 9, days 5, 7, 9, and 12 (4x) or days 5, 7, 9, 12, 14, and 16 (6x). Parasitemia was assessed on days 15 and 30 using samples of blood from the tail vein.

Immunosuppression. Mice that had no observable parasitemia at day 30 were moved into the second phase of the study. For immunosuppression, we used six doses of 100 mg/kg of cyclophosphamide given intraperitoneally, a regimen previously shown to reactivate subclinical experimental Chagas disease. Doses were given beginning on day 35 postinfection on days 35, 37, 46, 50, 54, and 56. Mice were assessed for parasitemia on day 48, 51, 55, and 58 as described previously. Mice showing parasitemia were euthanatized on that day.

Chronic model. Because preliminary attempts to establish a chronic model with the Y strain, studying various inbred strains of mice, resulted in too many deaths, even with low

challenge inoculum, we established a chronic model of infection using the CL strain of *T. cruzi*. Inoculum and handling of the strain was done as described previously. Forty 8-week-old C57BL/6 female mice (Charles River) were infected intraperitoneally with 10^4 trypomastigotes of the CL strain. The mice were preassigned to a therapy group observed for 44 days before the initiation of therapy. At the initiation of therapy, the mice received either D5W (no therapy controls), or one, two, or three doses of AmBi at 25 mg/kg given intravenously (i.v.) once weekly. Mice were followed through 126 days of infection, at which time all surviving mice were exsanguinated while anesthetized with isoflurane gas. After euthanasia, organs were collected into 10% buffered formalin for histopathology study. Organs were embedded in paraffin, sectioned, and stained with hematoxylin and eosin. Serum was separated from the blood samples after clotting and stored frozen at -80°C . Antitrypanosomal antibody titers in the serum were determined using ELISA as described previously.²⁰

RESULTS

Therapy of acute disease. In our initial studies, we examined whether one to three doses of AmBi at 25 mg/kg given i.v. could prolong the survival and reduce the burden of trypomastigotes in the blood. The infection established proved to be highly lethal for nontreated mice, with all D5W mice succumbing to infection by day 14 postinfection (Figure 1). No mice treated with AmBi, regardless of the number of treatments given, died during the 30-day experiment. Comparatively, all AmBi regimens significantly prolonged the survival ($P < 0.0001$), but were not different from one another ($P > 0.05$). These results demonstrate that even a single high dose of AmBi was sufficient to protect mice against lethal *T. cruzi* (Figure 1).

Serial assessment of the burden of trypomastigotes in the blood was carried out. On day 5 postinfection before the initiation of treatment, the median number of trypomastigotes/mL of blood was 1×10^5 (Figure 2). Several mice in the treatment arms had no observable infection at this early time (Figure 2). Statistical analysis on day 5 for trypomastigotes per mL of blood yielded no significant differences among the four treatment groups. By day 12, 14, or 16 postinfection (7 days after stopping therapy), all mice showed infection, with trypomastigotes observed and counted in all samples.

Statistical analysis of the counts obtained 7 days after the cessation of therapy showed that all AmBi regimens were active, with significantly lower counts than for the D5W regimen ($P = 0.001$ – 0.0007). For the majority of these treated animals, with counts below the level of detection, complete clearance of infection may have occurred. There was no significant difference between the counts from AmBi groups receiving treatment 2 $\times$ or 3 $\times$ ($P > 0.05$), and both AmBi 2 $\times$ or 3 $\times$ were better than AmBi 1 $\times$ ($P = 0.002$ or 0.0005 , respectively). On day 30, no mice given D5W remained, whereas all AmBi-treated were still alive. Statistical analysis yielded no significant differences in numbers of trypomastigotes among the AmBi treatment groups ($P > 0.05$).

On a temporal basis, all treatment arms showed a rise in the number of trypomastigotes over that observed at day 5 postinfection (Figure 2). However, the rise in counts for each group showed dose-responsiveness, with those receiving the

highest dosage of AmBi having the lowest counts and those given lesser amounts of AmBi or no treatment had the highest counts. It should be noted that trypomastigote counts showed a significant increase ($P < 0.001$ – 0.03 , dependent on group) in each group from day 5 to day 12, 14, or 16 (Figure 2). However, by day 30, the counts in the AmBi-treated mice had significantly decreased ($P < 0.0001$ – 0.0002 , dependent on group) from those on day 12, 14, or 16.

Clearing of acute disease by AmBi. The results of the initial study were encouraging and suggestive that clearance may have been attained by only two or three doses of AmBi at 25 mg/kg. To determine whether mice could in fact be cured by high-dose AmBi, we performed another study of similar design but increased the dosage to 30 mg/kg and examined the activity of AmBi given 4 $\times$ or 6 $\times$. Through the first 30 days of infection, the survival results are very similar to previous studies. All control mice died of acute disease by day 15 of infection. In contrast, no mice given AmBi 4 $\times$ or AmBi 6 $\times$ died before day 30 (Figure 3). Both treatment regimens were equivalent and significantly prolonged survival over that of the controls ($P < 0.0001$).

Assessment of the blood trypomastigote counts was carried out at day 15 and 30. On day 15, only three mice given AmBi 4 $\times$ had detectable infection and no mice given AmBi 6 $\times$ had detectable infection. After 30 days of infection, none of the treated mice had detectable infection.

The second phase of the study was to determine whether cure had been obtained by these regimens. This was carried out by immunosuppression with cyclophosphamide (100 mg/kg) of mice that had no detectable counts of trypomastigotes at day 30 of infection. The immunosuppression was carried out to determine whether the animal had been cured from infection; those not cured would have relapse of disease with countable trypomastigotes in the blood. All mice in the AmBi 4 $\times$ or AmBi 6 $\times$ treatment groups relapsed at some point

FIGURE 3. Survival of mice infected with *Trypanosoma cruzi* Y strain and given no treatment (Control), four doses of AmBi at 30 mg/kg (days 5, 7, 9, and 11) or six doses of AmBi at 30 mg/kg given intravenously (days 5, 7, 9, 11, 13, and 15). All mice were free of trypomastigotes in the blood on day 30 and were immunosuppressed with cyclophosphamide on the days indicated in the figure (days 35, 37, 46, 50, 54, and 56). Blood samples were examined for the presence of trypomastigotes; mice were euthanized when trypomastigotes were observed.

TABLE 1
Histological evaluation, survival, and blood parasitemia of surviving mice with chronic trypanosomiasis treated with AmBi at 25 mg/kg

Group	Brain		Heart		Intestine		Muscle		Parasitemia*		Died†	
	NI	Abn	NI	Myocarditis	NI	Abn	NI	Myositis	No	Yes	No	Yes‡
D5W	9	0	2	7	9	0	2	7	9	0	9	1
AmBi 1x	5	3	0	8	8	0	0	8	8	0	8	1
2x	8	1	1	8	9	0	0	9	9	0	9	1
3x	9	1	5	5	10	0	2	8	10	0	10	0

NI = normal; Abn = abnormal.

* All mice were positive before the initiation of therapy on day 40.

† After the cessation of therapy.

‡ Only deaths after cessation of therapy were tallied.

after the initiation of immunosuppression, with trypomastigotes visible in the blood of these animals. Figure 3 shows the days of cyclophosphamide dosing and the results of the mice with trypomastigotes in the blood being euthanatized. Thus, cure was not obtained with either AmBi regimen.

Chronic infection studies. These studies were conducted to assess the potential activity of AmBi therapy initiated after the establishment of disease and development of the chronic phase of infection, where no or few trypomastigotes are

found in the blood. Thus, therapy began on day 40 of infection. At day 126 of infection, serum was collected and organs were assessed by histology. As shown in Table 1, few mice died and none had parasitemia the day of euthanasia. However, there was evidence of ongoing infection. Histologically, the majority of the animals had myocarditis or myositis, with some treated mice showing involvement of the brain; none had visible abnormalities of the intestine. No mouse was clear of infection in all organs. Examples of the lesions noted in the brain, heart,

FIGURE 4. (A) Meningitis with amyloid-like degeneration of arterial wall (asterisk). (B) Individual organisms (arrows) are seen at higher power. (C–E) Skeletal myositis with amyloid-like degeneration of arterial wall (asterisks in C and D). Individual organisms (arrows, D) and encysted amastigotes (arrows, E) are seen at higher power. (F) Example of myocarditis. Original magnifications: (A) 80×; (B) 240×; (C) 40×; (D and F) 160×; (E) 300×.

and muscle are shown in Figure 4. The evidence of tissue disease, despite apparent clearing of parasitemia, is consistent with the prior studies demonstrating reactivation of latent tissue infection at a time when there was evident clearing of parasitemia.

To further confirm whether cure had been attained, we determined the antibody titers for all surviving mice. These results, shown in Figure 5, corroborate the histological results and indicate that all mice remained infected, with significant levels of antibody, which were not different between the treatment groups. Thus, AmBi treatment of chronic infection did not appear to be beneficial in either reducing tissue damage or in causing a reduction in the antibody titer against *T. cruzi*.

DISCUSSION

Chagas disease is primarily a New World disease that affects many millions of people. Current therapies are highly toxic and have limited efficacy. The possibility of the use of amphotericin B as a therapy has been addressed previously. The desirable toxic and pharmacological properties of the liposomal amphotericin B formulation, AmBisome, make it a potential drug for use in treatment, where high nontoxic dosages can be administered. A rationale for these regimens is that AmBisome given i.v. to humans has a prolonged terminal half-life, measured in weeks, after the acute high-peak blood levels achieved, and both an acute and a persistent antitypanosomal effect might occur. As demonstrated in our

FIGURE 5. Determination of antitypanosomal antibody (IgG1) by ELISA on the serum of mice chronically infected with the CL strain of *Trypanosoma cruzi*. Each data point represents an individual mouse that had survived through 126 days of infection, at which time all mice were bled and serum was collected. Negative control represents uninfected mice. D5W = 5% dextrose water; AmBi 1x = AmBisome at 25 mg/kg given one time intravenously (i.v.); AmBi 2x = AmBisome at 25 mg/kg given two times i.v.; AmBi 3x = AmBisome at 25 mg/kg given three times i.v.

initial studies on the therapy of acute infection, our results indicate that even a single treatment with a high dosage of AmBi (i.e., 25 mg/kg) is sufficient to provide some lasting protection against acute infection with *T. cruzi*. These results are similar to previous studies using AmBi for the treatment of murine Chagas disease.^{18,19} Although in the Cencig et al.¹⁸ study, mice were given six doses of 25 mg/kg intraperitoneally, there was no cure. Because we thought that the route of AmBi administration may be important, and to mimic potential human therapy, we used i.v. administration, which also prolonged survival but was not curative. Our results are also consistent with those of Yardley et al.¹⁹ who administered only a single 25 mg/kg dose of AmBi i.v., improving survival but not cure.

We further assessed whether even higher dosages of AmBi given i.v. (30 mg/kg versus 25 mg/kg in the prior study) were curative against acute Chagas infection in another set of studies. Although no AmBi-treated mice died in the first 30 days of infection and no trypomastigotes could be found in the blood on day 30, subsequent immunosuppression with cyclophosphamide caused relapse of active infection with trypomastigotes present in blood samples of all treated mice. These results corroborated our earlier studies showing that high-dose AmBi given i.v. could significantly prolong the survival of *T. cruzi*-infected mice, but clearly show that high-dose AmBi did not result in the cure of the treated animals. All animals relapsed after immunosuppression.

Similar to our studies with the Y strain and acute disease, we established a chronic model of infection with the CL strain of *T. cruzi* to determine the activity of high-dose AmBi treatment after trypomastigotes could be found in the blood. Although mice appeared healthy at the end of the study, histopathology showed that amastigotes could be found in tissues of all mice, particularly in the heart and brain. Furthermore, had the AmBi treatments been curative, one would have expected low or no antibody titer. Our results demonstrated that infected mice regardless of treatment regimen had equivalent antibody to the infected, but not treated mice. Thus, cure was not attained in the treatment of chronic infection and did not appear to influence the progression of the chronic infection and damage to the tissues, particularly the heart.

Overall, our studies showed that high-dose AmBisome treatment has activity against acute Chagas disease, causing clearing of the trypomastigotes from the blood and prolonging survival, but is not curative in the repeated dose regimens studied and did not prevent relapse of infection after immunosuppression. Whether even higher dosages of AmBi could be administered short term, or other treatment schedules would be more useful, remain to be determined. Additional studies are needed to find a curative antitypanosomal regimen.

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