Antileishmanial Activity of Chlorpromazine

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The antiprotozoal activity of chlorpromazine against the pathogenic protozoan *Leishmania donovani*, in both its amastigote and promastigote stages, was characterized. Chlorpromazine at concentrations ≥ 3.12 μg/ml (9.8×10^{-6} M) produced a significant reduction in viable promastigotes. The minimal protozoacidal concentration for promastigotes, defined as that concentration which produced $\geq 90\%$ reduction in viable parasites after 18 h, was 13.8 μg/ml. The results were similar when promastigote viability was assessed by flagellar motility or by the ability of drug-exposed or control promastigotes to incorporate [^{3}H]uridine and [^{3}H]leucine. Exposure of promastigotes to 50 μg of chlorpromazine per ml reduced O_2 consumption by 87% within 30 min and immobilized 97% of parasites. Morphological disruption of promastigotes was observed by electron microscopy. The mean minimal protozoacidal concentration of chlorpromazine for amastigotes was 13.2 μg/ml. Chlorpromazine given orally (20 mg/kg per day for 14 days) reduced the parasite burden in *L. donovani*-infected hamsters by 64.2% ($P < 0.01$) as measured by the number of amastigotes in touch preparations of livers and by 67.9% ($P = 0.03$) as measured by the number of promastigotes derived from homogenates of spleens. This dose is ca. 10-fold greater than that tolerated by patients being treated for psychiatric illness. Although chlorpromazine will probably not be useful in the treatment of human visceral leishmaniasis, the data suggest that less-toxic phenothiazines might prove to be effective.

Leishmania donovani, the causative agent of visceral leishmaniasis, produces morbidity and mortality in many areas throughout the world. Pentavalent antimonial compounds are widely used for the treatment of visceral leishmaniasis, but they are variably effective, must be given parenterally, and have associated toxicities at high dosages. More-effective and less-toxic compounds, which can be given orally, are needed for the treatment of visceral leishmaniasis.

Phenothiazine drugs, of which chlorpromazine is the prototype, are widely used for their antipsychotic, antianxiety, and antiemetic effects. We have recently found that they also possess protozoacidal activity against *L. donovani* (4). *L. donovani* exists in its arthropod vector, the sandfly, in its flagellated, extracellular promastigote stage and in mammals as an aflagellar amastigote solely within mononuclear phagocytes. The purpose of this study was to further characterize the antileishmanial effects of chlorpromazine against *L. donovani* in its amastigote and promastigote stages in vitro as well as in an in vivo experimental model of visceral leishmaniasis.

MATERIALS AND METHODS

Preparation of *L. donovani*. A Sudanese strain of *L. donovani* (S3) was maintained by serial intracardiac inoculation of amastigotes into Syrian hamsters. At 4 to 6 weeks after inoculation, amastigotes were obtained from the spleens of heavily infected hamsters by methods previously described (5). Amastigotes were used for study as described below or were allowed to convert to promastigotes in a modified form of minimal essential medium (1) to which 10% heat-inactivated (56°C, 30 min) fetal calf serum, penicillin, and gentamicin were added. Incubation was carried out at

26°C in 5% CO_2–95% room air. Promastigotes were harvested on days 4 through 8 of growth and used after being washed twice in phosphate-buffered saline, or they were passaged into fresh medium and used before day 14.

Effect on promastigotes. The effect of phenothiazines on promastigotes was assessed by incubating promastigotes (3 $\times 10^6$/ml) at 26°C in control medium or with serial twofold dilutions of drug (0.78 to 50 μg/ml) in medium with 10% heat-inactivated (56°C, 30 min) fetal calf serum and antibiotics. The phenothiazines used in this study were obtained from the Neurosciences Research Branch of the National Institute of Mental Health, Rockville, Md. Each individual experiment was done in duplicate. The minimal protozoacidal concentration was defined as the lowest concentration of drug that reduced the number of viable parasites by $\geq 90\%$ in comparison to control cultures. Viability was assessed after 18 h by morphological criteria: presence of flagellar motility; uptake of the supravital stain, neutral red dye (5); or reculture after drug exposure. In selected studies, viability was determined by uptake of [^{3}H]uridine and [^{3}H]leucine by a modification of the method of Reiner and Kazura (6). In these experiments promastigotes were incubated for 2 h with serial twofold dilutions of drug or medium alone, and then 2.5 μCi each of [^{3}H]uridine and [^{3}H]leucine per ml were added to each well. After 18 h of incubation, promastigotes were harvested on filter papers, extensively washed, and counted in a scintillation counter (model LS-250; Beckman Instruments, Inc., Irvine, Calif.).

The effects of chlorpromazine on promastigotes were further assessed by monitoring the oxygen consumption of 4 $\times 10^7$ parasites with an oxygen-monitoring system (USI model 53 Biological Oxygen Monitor; Yellow Springs Instrument Co., Yellow Springs, Ohio). Oxygen consumption was quantified before and after the addition of chlorpromazine (50 μg/ml). Finally, promastigotes which had been exposed to chlorpromazine (50 μg/ml) for 20 min and control parasites were examined by transmission electron microscopy to

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assess the effects of chlorpromazine on parasite morphology.

Effects on phagocyte-free amastigotes. The effects of chlorpromazine on amastigotes were determined by incubating amastigotes (10^6/ml), released from the spleens of infected hamsters, with serial twofold dilutions of the drug (0.78 to 50 μg/ml) or medium alone at 37°C for 2 h. All studies were done in duplicate. The amastigotes were then washed twice in phosphate-buffered saline and allowed to convert to promastigotes at 26°C in 2 ml of medium which contained 10% heat-inactivated fetal calf serum and antibiotics. The number of motile promastigotes derived from amastigotes was determined at 48 h in control and treated tubes.

Effect of chlorpromazine on _L. donovani_ in experimentally infected Syrian hamsters. Weanling Syrian hamsters were infected with amastigotes in 0.2 ml of phosphate-buffered saline by intracardiac inoculation. Two weeks after inoculation of amastigotes, chlorpromazine treatment was initiated. Animals received a single daily dose for 14 days by gavage after they were lightly anesthetized with ether. Control animals received saline. On day 15, all hamsters were killed. The parasite burden in the liver was determined by a modification of the method of Stauber et al. (9) in which duplicate liver touch preparations were fixed with methanol and stained with a Wright-Giemsa preparation (Diff-Quik; Dade Diagnostics, Inc., Aguada, P.R.). The number of parasites per host cell nucleus was then determined by counting ≥200 nuclei on coded monolayers. The number of parasites in the spleen was determined concurrently, by weighing the spleen and culturing homogenized samples of known weights (the mean weight of splenic material cultured was 334 ± 32 mg) in 10 ml of growth medium at 26°C. The number of motile promastigotes derived from splenic amastigotes was determined after 48 h of incubation, and the number of promastigotes per total spleen was then calculated.

Statistical analysis. Comparisons between groups were evaluated by paired _t_ test, where appropriate, and Student's two-tailed _t_ test for independent means.

RESULTS

The dose-dependent antileishmanial effect of chlorpromazine against promastigotes is demonstrated in Fig. 1. A significant reduction in viable parasites compared with untreated controls was seen at chlorpromazine concentrations ≥9.8 × 10^{-6} M (3.12 μg/ml) ($P < 0.01$). The results were similar when promastigote viability was assessed by morphological criteria and by the ability of drug-exposed and control parasites to incorporate [³H]uridine and [³H]leucine. No difference was noted between the number of motile promastigotes under control and drug-exposed conditions and the number which stained with the supravital dye, neutral red. In addition, when promastigotes were exposed to chlorpromazine for 2 h, washed twice in phosphate-buffered saline, and recultured in modified minimal essential medium under optimal promastigote growth conditions, there was no increase in the number of motile promastigotes after 48 h compared with the number of motile parasites at 2 h, further indicating that drug-exposed, immobilized promastigotes were dead. The geometric mean minimal protozoacidal concentration of chlorpromazine for promastigotes was 13.8 ± 0.4 (standard error of the mean) μg/ml ($n = 14$).

In studies in which promastigote viability was assessed by oxygen consumption, chlorpromazine (50 μg/ml) added to 4 × 10^7 promastigotes reduced oxygen consumption by 87% within 30 min ($n = 2$) while reducing the number of motile
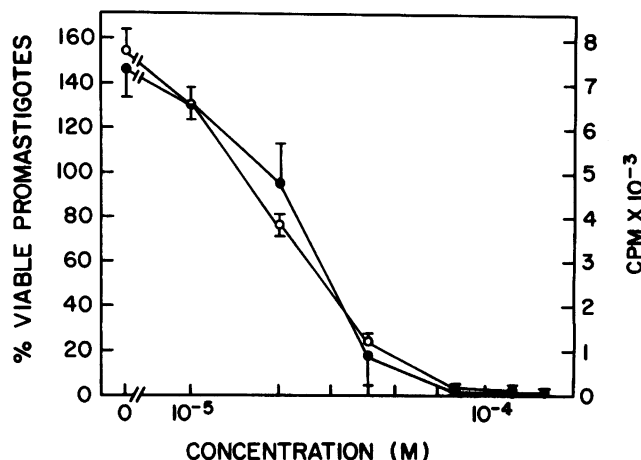

FIG. 1. Dose-dependent effect of chlorpromazine on promastigote viability. Symbols: ●, mean ± the standard error of the mean percentage of promastigotes that were viable as determined by flagellar motility after 18 h of incubation with chlorpromazine in comparison to the initial inoculum ($n = 6$); ○, mean counts per minute ± standard error of the mean of triplicate samples (corrected for background) of [³H]leucine and [³H]uridine incorporated by control and chlorpromazine-exposed promastigotes.

promastigotes by 97%. Loss of nuclear and cytoplasmic detail was documented by electron microscopy (Fig. 2). In some instances, disruption of the plasma membranes of the parasites was observed. In addition, the minimal protozoacidal concentration of chlorpromazine seemed to correlate inversely with the oxygen concentration. Under relatively anaerobic conditions ($PO_2 < 3$%), the minimal protozoacidal concentration of chlorpromazine was 41.7 μg/ml, 3.0-fold higher than in room air.

Chlorpromazine also killed _L. donovani_ amastigotes, which had been released from the spleens of infected hamsters. The geometric mean minimal protozoacidal concentration of chlorpromazine was 13.2 ± 0.5 μg/ml ($n = 6$). To evaluate the effect of chlorpromazine on amastigotes in vivo, we treated _L. donovani_-infected Syrian hamsters by gavage with chlorpromazine (20 mg/kg per day) for 14 days. This resulted in a 64.2% ($P < 0.01$) decrease in the number of amastigotes per host cell nucleus in touch preparations from the livers of chlorpromazine-treated animals compared with the untreated controls (Table 1). There was also a 65.2% decrease in the number of amastigotes in the liver as approximated by multiplying the ratio of amastigotes to host cell nucleus times the liver weight in one experiment ($P < 0.05$; data not shown). Finally, there was a 67.9% ($P = 0.03$) reduction in the number of viable parasites in the spleens of treated animals based on the ability of the parasites to convert from the amastigote to the promastigote stage. At the dosage of chlorpromazine used, treated animals were mildly to moderately lethargic and lost 1.7 ± 1.4 g in body weight over 14 days, whereas untreated _L. donovani_-infected animals gained 4.4 ± 2.7 g ($P < 0.01$). Treatment with chlorpromazine at a lower dosage (5 mg/kg per day) did not significantly reduce the parasite burden. Thus, chlorpromazine at a high dosage level did not cure infected hamsters but did produce a significant reduction in the parasite burden in both their livers and spleens.

DISCUSSION

Chlorpromazine and analogs of chlorpromazine are lethal for the human pathogen _L. donovani_. Our studies indicate
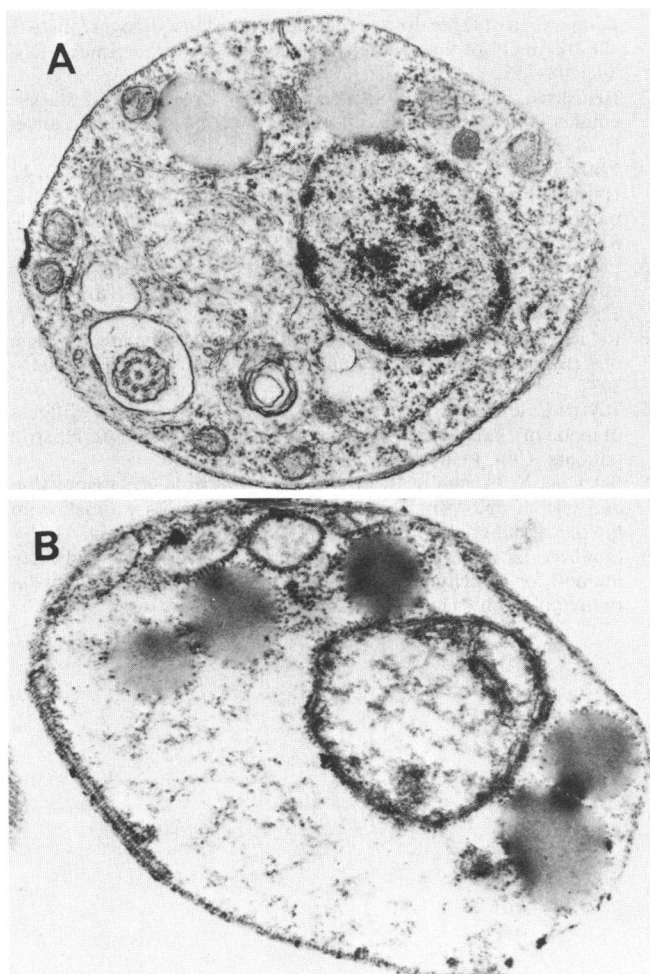
FIG. 2. Effects of chlorpromazine on promastigotes. Electron micrographs of a normal promastigote (in cross-section) (A) and a promastigote treated with chlorpromazine (50 μg/ml) for 25 min (B). Exposure of promastigotes to chlorpromazine was accompanied by loss of nuclear and cytoplasmic detail and membrane defects, indicating that parasites had been killed.

that these drugs can kill promastigotes, as well as amastigotes released from the spleens of infected hamsters. More importantly, in this preliminary appraisal, chlorpromazine produced a significant reduction in the number of parasites in the spleens and livers of hamsters infected with L.

donovani, as assessed by the ratio of amastigotes to host cell nuclei and by the product of that ratio and the weight of the liver, a parameter reported by Stauber et al. (9) to be directly proportional to the total number of parasites in the liver.

Our in vivo method differed from that used by Stauber et al. (9) in that we allowed L. *donovani* infection to become established for 14 days before the initiation of drug therapy. The parasite burdens in the livers and spleens of our hamsters were consequently much greater than those in the studies made by Stauber et al. and allowed for assessment of the number of viable amastigotes in the spleen as determined by the ability of the parasites to convert to the promastigote stage. The reduction in parasite burden in the livers and spleens of treated animals may have been due to a direct effect of chlorpromazine on the parasite or to an effect(s) on infected macrophages or lymphocytes. Hamsters have little or no natural immunity against L. *donovani*, and although our studies did not allow us to identify the mechanism of drug action, we believe that chlorpromazine probably acted directly on the parasite since it killed intracellular (4) and extracellular amastigotes in vitro at equivalent drug concentrations.

Although the full clinical utility of our findings remains to be determined, Henriksen and Lende (3) have recently reported that topically applied chlorpromazine ointment (2%) produced healing of cutaneous lesions of L. *aethiopica* in three patients with diffuse cutaneous leishmaniasis (3), a condition usually resistant to parenteral therapy with pentavalent antimonials. It is unlikely that chlorpromazine itself will be useful in human visceral leishmaniasis for three reasons: (i) the dosage of chlorpromazine which we found to be effective in reducing the parasite burden in experimentally infected hamsters was ca. 10-fold higher on a milligram-per-kilogram basis than the dosage routinely used for psychiatric illnesses; (ii) the concentration of chlorpromazine which killed parasites in vitro was >0.75 μg/ml, a concentration that, when achieved in plasma, is associated with unacceptable side effects in humans (7); and (iii) the reduction in parasite burden in vivo was <1 logarithm. Nevertheless, phenothiazines are lipophilic compounds and are concentrated in tissues including the reticuloendothelial system, the site of leishmanial infection. In canine studies, for example, the tissue-to-plasma ratio for chlorpromazine was found to be 68:1 for brain, 26:1 for spleen, and 13:1 for liver (8). In addition, viable parasites can be grown from splenic aspirates after clinically successful treatment of a subset of humans with pentavalent antimonials (2), leading some investigators to postulate that chemotherapy in humans need only reduce the parasite burden to a critical level. Further

TABLE 1. Effect of chlorpromazine on L. *donovani* in livers and spleens of Syrian hamsters[a]

Expt	Mean ± SEM no. of amastigotes per host cell nucleus in liver			Mean ± SEM no. of promastigotes in spleen ($\times 10^6$)		
	Control animals	CPZ-treated animals	% Reduction	Control animals	CPZ-treated animals	% Reduction
A[b]	4.7 ± 1.4 (4)[c]	1.3 ± 0.5 (5)	72.3	16.8 ± 7.0	5.0 ± 1.1	70.2
B	3.3 ± 1.6 (4)	0.6 ± 0.3 (5)	81.2	1.3 ± 0.3	0.4 ± 0.2	69.2
C	3.0 ± 1.4 (4)	2.0 ± 0.4 (4)	33.3	16.9 ± 9.1	5.4 ± 1.5	68.
x̄ ± SEM	3.6 ± 0.8	1.3 ± 0.8	64.2[d]	11.2 ± 4.0	3.6 ± 0.8	67.9[e]

[a] Methods are discussed in the text. CPZ, Chlorpromazine.
[b] Average weight of animals: experiment A, 100 ± 2 g (mean ± standard error of the mean); experiment B, 121 ± 2 g; experiment C, 78 ± 2 g.
[c] Number of animals is shown in parentheses.
[d] $P < 0.01$.
[e] $P < 0.03$.

studies are warranted to determine whether related but less-toxic phenothiazines might be effective in the treatment of this serious systemic parasitic infection.

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