Axenically Cultured Amastigote Forms as an In Vitro Model for Investigation of Antileishmanial Agents

DENIS SERENO* AND JEAN-LOUP LEMESRE
Laboratoire d’Épidémiologie des Maladies à Vecteur, Unité de Biologie Parasitaire, ORSTOM, Montpellier, France

Received 29 October 1996/Returned for modification 13 December 1996/Accepted 20 February 1997

Leishmaniasis is a significant cause of morbidity and mortality in several countries. A vertebrate host is infected with flagellate, extracellular promastigotes of the parasite via the bite of a sand fly. Promastigotes are rapidly transformed into nonflagellated amastigotes within the mononuclear phagocytes of the vertebrate host. The basic treatment consists in the administration of sodium stibogluconate (Pentostam), meglumine (Glucantime), or pentamidine. Treatment failure, especially in kala-azar, mucosal leishmaniasis, and diffuse cutaneous leishmaniasis, is becoming a common problem in many areas where the diseases are endemic. There is now strong indication that treatment failure may be partly due to the drug resistance of the parasite (15). In addition, the low efficacy of pentavalent antimony in the treatment of patients coinfected with AIDS and Leishmania spp. is often noticed (28). These problems prompted the development of additional antileishmanial drugs.

The development of new drugs has been impeded by the lack of a simple and rapid drug evaluation system directly applied to the parasite at the mammalian stage. Leishmania organisms in the amastigote form have been cultured in a wide range of mammalian cells. Some of them, including sticker dog sarcoma cells (fibroblasts) (22, 23), transformed rodent macrophages, primary isolated mouse peritoneal macrophages (27), human monocyte-derived macrophages (6, 7), and human leukemia monocyte THP-1 cells (12), have been used in in vitro drug-screening procedures. Great variations in the activity of pentavalent antimony (Pentostam and Glucantime) were shown, depending on the model used. Pentostam, which was shown to be poorly toxic for amastigote forms of Leishmania donovani and L. mexicana in cell lines (26), was in contrast highly toxic in the human-derived monocyte macrophage or mouse peritoneal macrophage model (6, 27). There is still no indication of the activity of this drug against a dividing population of extracellular amastigotes. The recent development of axenic cultures of amastigote forms of different Leishmania species (3, 4, 20a) provided new opportunities to investigate the leishmanicidal activities of new compounds directly at the mammalian stage of the parasite.

In this report we evaluate the ability of axenically grown amastigotes to be used in a relevant drug-screening procedure. The chemosensitivities of extracellular amastigote forms of three Leishmania species to different drugs, including those currently used for leishmaniasis, are evaluated and compared with those of the promastigote forms. Results are compared with those previously reported by researchers using classical screening procedures. These axenically cultured amastigotes appear to be a useful tool for the early determination of antileishmanial activity.

MATERIALS AND METHODS

Antileishmanial agents. Pentamidine isethionate, amphotericin B, diminazene aceturate, sodium arsenite, and potassium antimonyl tartrate trihydrate were supplied by Sigma (St. Louis, Mo.). Pentostam was a generous gift of H. Amini, University of Teheran, (Teheran, Iran). Our batch contained 0.1% m-chloroacetic acid as a preservative. Primaquine was given by J. Rodriguez (Bel Horizonte, Brazil).

Parasites. L. infantum clone 1 (MHOM/MA/67/ITMAP-263), L. amazonensis clone 1 (MHOM/BR/76/LTB-012), and L. mexicana clone 3 (MNYC/BZ/62/M-379) were cloned by a micromanipulation method.

Cultures. We have successfully established axenic cultures of amastigote forms of different Leishmania species. A general procedure was used to generate large quantities of each parasite stage. Axenically grown amastigote forms of L. amazonensis and L. mexicana were maintained at 32 ± 1°C, and L. infantum was maintained at 36 ± 1°C with 5% CO2 by weekly subpassages in a cell-free medium called MAA/20 (medium for axenically grown amastigotes) in 25-cm2 flasks. From a starting inoculum of 5 × 107 amastigotes/ml, cell densities in the range of 2 × 108 to 7 × 108/ml were obtained on day 7. MAA/20 consisted of modified medium 199 (Gibco BRL) with Hank’s salts, supplemented with 0.5% soybean cryptotanshinonic acid, 3 mM L-cysteine, 15 mM β-glucose, 5 mM

http://aac.asm.org/
TABLE 1. Susceptibilities of axenically grown amastigote and promastigote forms of L. mexicana, L. amazonensis, and L. infantum to drugs currently used to treat leishmaniasis

<table>
<thead>
<tr>
<th>Parasite type</th>
<th>Parasite</th>
<th>Pentostam [µg of Sb(V)/ml]</th>
<th>Pentamidine [µM]</th>
<th>Amphotericin B [µM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amastigote</td>
<td>L. mexicana</td>
<td>220 ± 30 (4)</td>
<td>7.95 ± 1.63 (5)</td>
<td>0.21 ± 0.03 (5)</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>270 ± 50 (3)</td>
<td>4.52 ± 0.13 (4)</td>
<td>0.26 ± 0.02 (3)</td>
</tr>
<tr>
<td></td>
<td>L. infantum</td>
<td>134 ± 45 (4)</td>
<td>4.55 ± 0.32 (4)</td>
<td>0.79 ± 0.11 (6)</td>
</tr>
<tr>
<td>Promastigote</td>
<td>L. mexicana</td>
<td>470 ± 50 (4)</td>
<td>1.03** ± 0.10 (6)</td>
<td>0.14 ± 0.02 (4)</td>
</tr>
<tr>
<td></td>
<td>L. amazonensis</td>
<td>410 ± 72 (4)</td>
<td>0.48** ± 0.13 (4)</td>
<td>0.16* ± 0.05 (3)</td>
</tr>
<tr>
<td></td>
<td>L. infantum</td>
<td>248 ± 36 (4)</td>
<td>0.45** ± 0.11 (4)</td>
<td>0.24*** ± 0.11 (3)</td>
</tr>
</tbody>
</table>

* n, number of independent experiments, in triplicate; *, P < 0.05; **, P < 0.005; ***; P < 0.001.

The 8-aminoquinoline compound primaquine, which has been described to be highly potent against the intramacrophagic amastigote form of L. donovani (27), was highly toxic to the axenically cultured amastigote forms of the three Leishmania species. L. amazonensis and L. infantum at both parasite stages did not show differences in their chemosensitivities to

1-glutamine, 4 mM NaHCO₃, 0.023 mM bovine hemin, and 25 mM HEPES at a final pH of 6.5 and supplemented to 20% with pretested fetal calf serum. The population of axenically grown amastigotes appeared homogeneous, round to oval, and immotile when examined under a phase-contrast light microscope. Axenically grown amastigotes had morphological, biochemical, and biological characteristics similar to those of in vivo-isolated amastigotes (20h).

Promastigote cultures were derived from axenically cultured parasites at the amastigote stage and were maintained at 25°C by weekly subpassages in RPMI 1640 medium (Gibco BRL) buffered with 25 mM HEPES and 2 mM NaHCO₃ (pH 7.2) and supplemented with 20% heat-inactivated fetal calf serum. Initial parasitic concentrations were 5 × 10⁶ flagellates per ml of medium.

**Viability test.** To estimate 50% inhibitory concentrations (IC₅₀), the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) micromethod previously described (34) was used. Briefly, axenically grown amastigotes or promastigotes, from late log phase of growth, were seeded in 96-well flat-bottom microtins (Nunc) in a volume of 100 µl under adequate culture conditions. Drugs were added at 1/10 of the final concentration in a volume of 10 µl in triplicate (range depended on the drug). After 72 h of incubation, 10 µl of MTT (10 mg/ml) was added to each well and plates were further incubated for 4 h. The enzyme reaction was then stopped by addition of 100 µl of 50% isopropanol-10% sodium dodecyl sulfate. The plates were incubated for an additional 30 min under agitation at room temperature before the optical density at 570 nm was read with a 96-well scanner. Two or more independent experiments in triplicate were performed for the determination of sensitivity to each drug. As a control, the activity of a drug alone in reagent was determined, and no substantial interaction was found.

**Data analysis.** The results of each experiment were analyzed by the method described by Hills et al. (18) and Huber and Koella (19). Briefly, Hills proposed finding two concentrations, x₁ and x₂, such that the parasite density, y₁, at concentration x₁ (and all lower concentrations) was more than half of the density found in the control, y₀, and that the parasite density, y₂, at concentration x₂ (and all higher concentrations) was less than half of y₀. The IC₅₀ was then found by linear extrapolation between x₁ and x₂: log(IC₅₀) = log(y₀) + [(log(y₁) - log(y₂))] [(log(x₂) - log(x₁))].

**Statistical analysis.** The Student t test, with significance at P values of <0.05, <0.005, and <0.0001, was used to compare drug susceptibilities of parasites at both stages.

**RESULTS**

**Activities of drugs currently used to treat leishmaniasis against axenically grown amastigotes.** The presence in culture of Pentostam at a concentration to give about 134 µg of Sb(V) per ml for 3 days resulted in the elimination of 50% of the amastigotes of L. infantum. Concentrations of Pentostam yielding approximately 250 µg of Sb(V) per ml had the same effect on the viability of L. amazonensis and L. mexicana (Table 1). These concentrations, which were about 6- to 13-fold higher than the peak level of Pentostam in human serum (14, 30), could not explain the high in vivo activity of this drug.

Concentrations of pentamidine in the range of 4.5 to 10.0 µM killed approximately 50% of the parasites at the mammalian stage for the three species studied. Even if slight differences in pentamidine susceptibility were noticed among Leishmania species (Table 1), pentamidine was only poorly potent against the amastigote forms at the concentration achieved in the sera of humans (about 1 µM) given intramuscular injections of 4 mg/kg of body weight (35).

As indicated in Table 1, amphotericin B showed a high toxicity for all the amastigote forms studied, at concentrations close to those found in the plasma of humans receiving intravenously 70 mg of amphotericin B (about 1 µM) (1). However, L. infantum was found to be the least susceptible species.

**Differences in the chemosusceptibilities of parasites at the two stages.** Promastigote forms of three species were fairly susceptible to Pentostam. L. mexicana and L. amazonensis, with IC₅₀ of 470 ± 50 and 410 ± 72 µg/ml, respectively, were the less susceptible species (Table 1). Promastigotes were significantly less susceptible to this drug than amastigotes (Table 1).

IC₅₀ for pentamidine were in the range of the micromolar concentrations of the drug for the promastigote forms of L. infantum, L. amazonensis, and L. mexicana (Table 1). Amastigote forms of the three species studied showed an eightfold reduction in susceptibility to pentamidine when they were compared with the respective promastigote forms (P < 0.005) (Table 1).

Amphotericin B was highly toxic to parasites at the two stages. IC₅₀ ranged between 0.1 and 0.2 µM for promastigotes and varied from about 0.2 to about 0.8 µM for organisms at the intracellular stage (Table 1). Amastigotes of L. infantum (P < 0.001) and L. amazonensis (P < 0.05) were significantly less susceptible than their corresponding promastigotes (Table 1), whereas L. mexicana showed virtually the same amphotericin B susceptibility at both parasite stages.

**Activities of other leishmanicidal compounds.** As shown in Table 2, potassium antimony tartrate trihydrate [Sb(III)], a trivalent antimonial, was more toxic than pentavalent antimony (Pentostam) to the three species of parasites at the two developmental stages studied. L. infantum organisms in the promastigote form were less susceptible to this drug than the two other species, in particular, L. amazonensis [IC₅₀, 1.58 ± 0.65 µg of Sb(III)/ml], L. mexicana expressing an intermediate susceptibility. The two cutaneous species (L. amazonensis and L. mexicana) at the mammalian stage were significantly less sensitive to Sb(III) than their corresponding promastigotes (P < 0.005 and P < 0.001, respectively), whereas amastigotes of the visceral species (L. infantum) were about 10-fold more susceptible (P < 0.005) than the promastigotes (Table 2).

The 8-aminoquinoline compound, primaquine, which has been described to be highly potent against the intramacrophagic amastigote form of L. donovani (27), was highly toxic to the axenically cultured amastigote forms of the three Leishmania species. L. amazonensis and L. infantum at both parasite stages did not show differences in their chemosensitivities to...
promastigote, unlike *L. mexicana* in its amastigote form, which was about twofold more susceptible than its promastigote form (*P* < 0.001) (Table 2).

As shown in Table 2, *L. mexicana* organisms at both parasite stages were equally sensitive to sodium arsenite, a metalloid. By contrast, major differences were noticed between the two stages of the other two species. Axenically grown amastigotes of *L. amazonensis* and *L. infantum* were about twofold and fourfold more susceptible than their corresponding promastigotes, respectively (*P* < 0.005 and *P* < 0.001, respectively) (Table 2).

Diminazene aceturate showed differences in toxicity not only among species (with IC$_{50}$s in the range of 1 to 10 µM) but also between both parasite stages of *L. infantum* (*P* < 0.001) (Table 2).

**DISCUSSION**

The in vivo efficiencies of drugs have been reported to be under the control of different parameters, such as pharmacokinetic parameters. One of the most important parameters was a drug’s direct activity against a parasite at the mammalian stage. Axenically grown amastigotes may thus become a powerful tool in the isolation of new compounds with high activities against active and dividing populations of amastigote organisms. We and other investigators have previously shown that extracellular amastigotes clearly resembled intracellular amastigotes in their ultrastructural, biological, biochemical, and immunological properties (3, 4, 20a). Moreover, characterized amastigotes, such as intracellular ones, differed from promastigotes in having a variety of biochemical characteristics, including proteinase, ribonuclease, adenine deaminase, peroxidase, and dehydrogenase activities, glucose catabolism, nucleic acid synthesis, and nitric oxide activity (11, 17, 21, 29, 34). In this report, a relevant viability test (34) was used to investigate the ability of axenically cultured amastigotes of different *Leishmania* species to be used in an in vitro drug-screening procedure.

We have found that Pentostam was poorly toxic to axenically grown amastigotes at the concentration achieved in human serum (14, 30). Active and dividing populations of axenically cultured amastigotes were generally more susceptible to Pentostam than their corresponding promastigotes. It is known that sodium stibogluconate is a complex mixture of components (5). A fractionation experiment using Pentostam has shown that the preservative *m*-chlorocresol accounts for virtually all the leishmanicidal activity of the fractionated material (32). So it will be of interest to determine the activities of these compounds against leishmanias in the amastigote forms. The mode of action of the pentavalent antimonials is poorly understood (5, 8, 10). The possibility of metabolic conversion of pentavalent to trivalent antimony in vivo was suggested more than 50 years ago (13, 14). Previous in vitro experiments with *L. mexicana* promastigotes demonstrated that antimony sodium gluconate (Triostam), a trivalent analog of sodium stibogluconate, had a 50% lethal dose of 20 µg of Sb(III)/ml, while sodium stibogluconate at 100 µg of Sb(V)/ml has no effect (25). We and other investigators have shown that trivalent antimonial compounds were highly toxic to different *Leishmania* species in the promastigote form at concentrations ranging from 1.58 to 35.00 µg of Sb(III)/ml (25, 31–33). Potassium antimony (III) tartrate was shown to be substantially more potent than sodium stibogluconate against both promastigotes and amastigotes (31–33). The generally higher toxicity of potassium antimony tartrate, when compared with that of pentavalent antimony, to organisms at both parasite stages, strongly supports the hypothesis of an in vivo reduction of sodium stibogluconate to an active trivalent antimony species. It has been reported that potassium antimony tartrate, which is highly toxic to promastigotes of *L. pifanoi*, which belongs to the *L. mexicana* family, at concentrations of 1 to 2 µg of Sb(III)/ml is less toxic to axenic amastigotes of *L. pifanoi* (20 µg of Sb(III)/ml) (31, 32). In agreement with these findings, we show that extracellular amastigotes of *L. mexicana* and *L. amazonensis* were less susceptible to potassium antimony tartrate than their corresponding promastigotes. More interesting is the finding that amastigotes of *L. infantum* were more susceptible to potassium antimony tartrate than their corresponding promastigotes.

Pentamidine was significantly less active against axenically grown amastigotes than promastigotes of the three *Leishmania* species studied. Using our experimental model of axenic cultures, researchers have shown that axenically grown amastigote organisms, like promastigote organisms, possess an active carrier involved in pentamidine uptake (2). In addition, the same authors have demonstrated that axenically cultured amastigotes have a reduced number of transport sites per cell when compared with promastigotes. These observations may explain the lower susceptibilities of extracellular amastigotes to this drug. At concentrations achieved in serum, 0.5 to 0.8 µM (35), pentamidine has little or no effect on amastigotes. These results suggest a major role for macrophages in the cytotoxicity of pentamidine, either in metabolizing or in concentrating the drug, as was previously described (9).

All the leishmanias at both parasite stages were found to be highly susceptible to amphotericin B, with the amastigote forms being in general less sensitive. These results are in sub-

<table>
<thead>
<tr>
<th>Parasite type</th>
<th>Parasite</th>
<th>Potassium antimony tartrate (µg/ml)</th>
<th>Primaquine (µM)</th>
<th>Sodium arsenite (µM)</th>
<th>Diminazene aceturate (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amastigote</td>
<td><em>L. mexicana</em></td>
<td>100.24 ± 9.51 (3)</td>
<td>22.24 ± 1.94 (5)</td>
<td>6.15 ± 1.36 (4)</td>
<td>6.88 ± 0.53 (3)</td>
</tr>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td>32.22 ± 8.35 (3)</td>
<td>53.09 ± 1.13 (3)</td>
<td>2.68 ± 0.47 (4)</td>
<td>1.49 ± 0.55 (3)</td>
</tr>
<tr>
<td></td>
<td><em>L. infantum</em></td>
<td>3.80 ± 2.50 (6)</td>
<td>62.14 ± 6.78 (4)</td>
<td>4.93 ± 2.72 (3)</td>
<td>7.01 ± 1.03 (3)</td>
</tr>
<tr>
<td>Promastigote</td>
<td><em>L. mexicana</em></td>
<td>21.82*** ± 2.84 (3)</td>
<td>54.80*** ± 3.69 (5)</td>
<td>7.02 ± 1.33 (3)</td>
<td>10.28 ± 3.89 (3)</td>
</tr>
<tr>
<td></td>
<td><em>L. amazonensis</em></td>
<td>1.58** ± 0.65 (3)</td>
<td>42.27 ± 1.13 (3)</td>
<td>1.88** ± 0.15 (5)</td>
<td>1.01 ± 0.53 (5)</td>
</tr>
<tr>
<td></td>
<td><em>L. infantum</em></td>
<td>35.08*** ± 5.20 (3)</td>
<td>59.84 ± 3.33 (3)</td>
<td>16.23*** ± 2.03 (3)</td>
<td>2.47*** ± 0.48 (3)</td>
</tr>
</tbody>
</table>

*a, number of independent experiments, in triplicate; *, *P* < 0.05; **, *P* < 0.005; ***; *P* < 0.001.
stantial agreement with those obtained with a macrophage model (6, 20). Interestingly, this drug, which was used to treat unresponsiveness to Pentostam, was highly potent against extracellular amastigotes at concentrations below the maximal concentration achieved in serum (1). These data showed that amphotericin B was directly toxic to the parasite at the amastigote stage and did not seem to depend upon macrophage activation for its antileishmanial activity (16).

The additional differences in drug susceptibility between axenically grown amastigote and promastigote forms observed for primaquine, dimazene acetate, and sodium arsenite, together with those observed for drugs in use to treat leishmaniasis, indicate the potential of this in vitro model for the assessment of direct drug activity against the parasite stage in contact with drugs.

Collectively, all these data show that most well-known leishmanicidal agents, whose activities have been demonstrated in vitro with macrophage models, significantly inhibit growth of axenic amastigotes. However, some limitations of this method should be enumerated: (i) a drug active in vitro in a macrophage model (Pentostam) does not significantly inhibit growth at concentrations achieved in serum, (ii) drugs which have to be metabolized by the host before affecting the parasite are likely to be missed, (iii) drugs which have to be concentrated by macrophages may show only slight activity in our in vitro model, and (iv) the MTT test may not be accurate, due to the interaction of the drug with tetrazolium. For example, we were not able to determine the activity of Glucantime on promastigote and amastigote forms because of this kind of problem. In fact Glucantime (and/or its preservative) alone in reagent seems to interact with the medium, thus showing inaccurate optical densities (unpublished results). This interaction may have some effect on the drug activity and may explain the different activities observed for this drug under variable growth conditions (24).

However, this in vitro model presents numerous technical advantages over the traditional early drug-screening procedure: (i) reliable experiments tolerating higher drug concentrations and requiring shorter experimental periods can be carried out easily, (ii) interpretation of the results is easier and it is possible to examine variation in drug susceptibility among strains and species, (iii) sensitivities of new isolates of Leishmania can also be ascertained rapidly in order to evaluate their strains and species, (iii) sensitivities of new isolates of Leishmania can also be ascertained rapidly in order to evaluate their drug resistance profiles, and (iv) in vitro models using macrophages, the influence of macrophages on drug activity can be analyzed.

It is now possible to directly evaluate the toxicities of new compounds to a parasite at the mammalian stage. This may help in isolating new leishmanicidal compounds with high specific activities against the parasite at the mammalian stage. This model represents a general and useful tool in the field of pharmacology and parasitology.

ACKNOWLEDGMENTS

This work was supported by grants from ORSTOM institute. We thank J. L. Chevrrolier and G. Nicaise for revising the language of the manuscript.

REFERENCES

31. Roberts, W. L., and P. M. Rainey. 1993. Antimony quantification in Leish-