Pentostam Induces Resistance to Antimony and the Preservative Chlorocresol in *Leishmania donovani* Promastigotes and Axenically Grown Amastigotes

M. EPHROS,1 E. WALDMAN,2 AND D. ZILBERSTEIN2*

Department of Pediatrics, Carmel Medical Center, and the Faculty of Medicine, Technion-Israel Institute of Technology, Haifa 34362,1 and Department of Biology, Technion-Israel Institute of Technology, Haifa 32000,2 Israel

Received 7 August 1996/Returned for modification 3 January 1997/Accepted 25 February 1997

An axenic amastigote culture system was utilized to directly assess the stage-specific antileishmanial effects of antimony on amastigotes of *Leishmania donovani* devoid of the macrophage host cell. Pentostam, which contains antimony in the form of sodium stibogluconate and the preservative chlorocresol, was used. Cell density was quantified by measuring the activity of the stable enzyme ornithine decarboxylase. Dose-response curve analyses show that *Leishmania* promastigotes are susceptible to Pentostam, with the 50% inhibitory concentration (IC50) being 104 μg/ml, while amastigotes are more susceptible, with the IC50 being 24 μg/ml. Promastigotes and amastigotes are also susceptible to chlorocresol, with IC50s being 1.27 and 1.82 μg/ml, respectively. Given that promastigotes are insensitive to antimony, these results suggest that the increased susceptibility of amastigotes to Pentostam is due to the stage-specific activity of sodium stibogluconate. To further study this phenomenon, spontaneous resistance to Pentostam was induced in *L. donovani* promastigotes by increasing the concentration of Pentostam in the growth medium in a stepwise fashion. Two mutants, Ld1S.04 and Ld1S.20, grew at 0.4 and 2.0 mg of Pentostam per ml, respectively. Promastigotes of these mutants were 11 and 21 times, respectively, more resistant to Pentostam than the wild type. Amastigotes were 40 and 148 times, respectively, more resistant than the wild type. The mutants were also chlorocresol resistant; promastigotes were 6 and 9 times, respectively, more resistant than the wild type, and amastigotes were 14 and 35 times, respectively, more resistant than the wild type. These data show that resistance to Pentostam induced in antimony-insensitive promastigotes is manifested in amastigotes as resistance both to pentavalent antimony and to chlorocresol. The axenic amastigote system is a unique tool which enables direct evaluation of the activity of antileishmanial compounds on the amastigote devoid of its host cell.

*Leishmania donovani* is the major causative agent of visceral leishmaniasis. It exists either as the extracellular promastigote found in the alimentary tracts of sandflies or as the obligatory intracellular amastigote found in the phagolysosomes of mammalian macrophages (5, 6). Historically, promastigotes have been readily cultured in cell-free media, while amastigotes have been maintained either in animals or in macrophage cell lines (5, 15). Thus, most of the in vitro studies on antileishmanial drug action or resistance have been performed on promastigotes. During the last few years, several laboratories have succeeded in culturing *L. donovani* amastigotes axenically (7, 10, 14a, 16). This technique allows for direct evaluation of the antileishmanial activities of drugs on the human form of the parasite, independent of the host macrophage. The treatment of choice for human visceral leishmaniasis is administration of the pentavalent antimony-containing drug sodium stibogluconate or meglumine antimoniate. Pentostam (sodium stibogluconate; Wellcome, Beckenham, United Kingdom) contains the preservative 4-chloro-3-methylphenol (chlorocresol). No information on the effects of chlorocresol on old-world *L. donovani* is available. The only data available show that promastigotes of (new-world) *Leishmania panamensis* are susceptible to chlorocresol and are apparently resistant to sodium stibogluconate (14). Chlorocresol antimastigote activity could not be assessed due to the cytotoxic effects of this compound on macrophage cell lines. This precluded the quantitative evaluation of the role of chlorocresol in the cytotoxicity of Pentostam against amastigotes. Hence, the use of axenic amastigotes should overcome this obstacle. Furthermore, most attempts to date to induce antimony resistance in vitro were carried out with promastigotes by using pentavalent antimony-containing compounds (3, 12). The results obtained were purported to be applicable to amastigotes as well.

The current study investigated the toxicities of Pentostam and chlorocresol against axenic *L. donovani* amastigotes and promastigotes. We show that both promastigotes and amastigotes are susceptible to chlorocresol. Furthermore, spontaneous resistance of promastigotes to Pentostam was manifested as resistance to chlorocresol in both promastigotes and amastigotes and as resistance to sodium stibogluconate in amastigotes.

**MATERIALS AND METHODS**

**Materials.** Pentostam (liquid form, containing 100 mg of sodium stibogluconate per ml and 0.1% chlorocresol) was obtained from Wellcome, Ltd. 4-Chloro-3-methylphenol, ornithine, leupeptine, and E64 were obtained from Sigma Chemical Co. (St. Louis, Mo.). Medium 199 and fetal calf serum were obtained from Biological Industries, Inc. (Beit Haemek, Israel). All other chemicals were of analytical grade.

**Parasites.** A cloned line of *L. donovani* 1S was used in all experiments (8). **Cell culture.** Promastigotes were grown in medium 199 supplemented with 10% fetal calf serum at 26°C. In vitro culture of amastigotes was performed as follows. Late-logarithmic-phase culture of *L. donovani* promastigotes was transferred to medium 199 supplemented with 25% fetal calf serum, and the medium...
was incubated at 37°C (5% CO₂) for 16 to 24 h. Subsequently, the cells were centrifuged (1,200 g, at room temperature for 10 minutes) and resuspended in this medium titrated to pH 5.5 with 10 mM succinate–TRIS and incubated as described above. Under these conditions promastigotes differentiate to amastigotes within 96 h (10a, 14a).

Enzymatic assay of ODC activity. Ornithine decarboxylase (ODC) activity was measured according to the method described by Assaraf et al. (1). Briefly, for each measurement, a 1.5-ml aliquot of cell suspension was centrifuged (Eppendorf centrifuge; 3 min at 4°C), and the cells were resuspended to 80 µl of ODC buffer containing 0.5% Triton X-100 and leupeptine and E64 (10 µg/ml each). [3H]ornithine (0.2 mCi of L-[2,3-3H]ornithine; specific activity, 40 Ci/mmol) was added to a final concentration of 25 µM, and the cell extract was incubated at 37°C for 2 h. The resultant [3H]putricine was separated by cation-exchange chromatography (P81 paper; Whatman International, Ltd., Springfield Mill, Medistone, United Kingdom) and quantitatively measured by β-scintillation counting. Relative ODC activity values were plotted against drug concentrations, and 50% inhibitory concentrations (IC₅₀) were determined.

Selection for resistance to Pentostam in promastigotes. Resistance was induced in promastigotes by increasing the concentration of Pentostam in the growth medium in a stepwise fashion. At each step, promastigotes in logarithmic growth phase were cultured continuously in a drug concentration which killed 90 to 95% of the parasites. When each surviving subpopulation of parasites was stabilized (i.e., able to successfully grow in the new Pentostam concentration), it was then exposed to the subsequent increased concentration of Pentostam.

RESULTS

Promastigotes are readily quantified by any conventional counting method, e.g., hemocytometry or Coulter counting. Because axenically cultured amastigotes grow in large aggregates (2, 14a), it is difficult to use these methods to measure parasite growth. An enzymatic assay, therefore, was adapted to measure ODC activity for this purpose. This enzyme is highly stable in L. donovani, with a half-life (t₁/₂) of ≈24 h in both promastigotes and amastigotes. Therefore, ODC activity in cell culture should be proportional to cell number. A linear correlation between cell count and relative ODC activity exists in L. donovani promastigotes (Fig. 1). In control experiments, ODC activity in amastigote and promastigote cell extracts was not affected by Pentostam at concentrations of as much as 5 mg/ml. Therefore, ODC activity can be exploited to quantitatively assay culture density.

As shown in Fig. 2A, the dose-response curve of L. donovani promastigotes exposed to Pentostam was evaluated both by cell counting and by determining ODC activity. As shown, promastigotes are susceptible to Pentostam, with the IC₅₀ being about 0.2 mg/ml as determined by both methods. Axenic amastigotes are also susceptible to Pentostam, with the IC₅₀ being 0.029 mg/ml (Fig. 2B). To verify the applicability of this assay to axenic amastigotes, the following experiment was performed: amastigotes (an aliquot) which were tested for ODC activity were retransformed back to promastigotes by transferring them to promastigote growth medium (medium 199 supplemented with 10% fetal calf serum), which was incubated for 48 h at 26°C and either counted or tested for ODC activity. There was a direct correlation between the number of recycled promastigotes and the level of ODC activity measured in drug-treated amastigotes. Since parasites do not proliferate during differentiation (14a), the number of recycled promastigotes counted at 48 h represented the number of viable amastigotes present when ODC activity was measured.

FIG. 1. Comparison of hemocytometry and ODC activity assays. Promastigotes of L. donovani were grown in 24-well plates (0.5 × 10⁶ to 1 × 10⁶ cells per ml in a 2-ml final volume) in medium 199 without carbonate, with 25 mM morpholinepropanesulfonic acid (MOPS). At each time point, a 1.5-ml aliquot from each well was assayed for ODC activity (●) and a 20-µl aliquot was counted by hemocytometry (○). Inset: plot of ODC activity against cell density. The ODC results are expressed as means ± standard deviations (SD) (n = 3).
Resistance was induced in promastigotes by increasing the concentration of Pentostam in a stepwise fashion, as described in Materials and Methods. Two mutants were chosen for further analysis: Ld1S.04 and Ld1S.20. Ld1S.04 was isolated from a population which grew successfully in 0.4 mg of Pentostam per ml, and Ld1S.20 was isolated from a population which grew successfully at 2 mg of Pentostam per ml (Fig. 3A).

Ld1S.04 and Ld1S.20 promastigotes were 11 and 21 times, respectively, more resistant to Pentostam than wild-type promastigotes (Table 1). Mutant promastigotes were readily transformed in vitro to amastigotes and possessed all of the amastigote-specific activities previously ascribed to wild-type parasites. These include the lack of lipopolysaccharide synthesis and proline transport with a pH optimum at pH 5.0 (10a, 14a). Ld1S.04 and Ld1S.20 amastigotes were 40 and 148 times, respectively, more resistant to Pentostam than wild-type amastigotes (Fig. 3B; Table 1).

To ascertain the stability of the mutations in Ld1S.04 and Ld1S.20, promastigotes were grown continuously for 6 months in the absence of Pentostam. Both mutants remained resistant for the entire period, with no decrease in IC50. In addition, when the resistant mutants were cycled between amastigotes and promastigotes in the absence of Pentostam, no changes in resistance were found.

**DISCUSSION**

In vitro evaluation of drug activity against obligatory intracellular parasites such as *Leishmania* is complicated by the organism’s presence within the host cell. To better understand a drug’s mode of action on an intracellular parasite and to identify the possible contribution of the host cell, it is necessary to separate the parasite from its host cell. This study utilized the axenic amastigote culture technique to examine the direct effect of Pentostam on *L. donovani* amastigotes devoid of the host macrophage.

In contrast to the relative ease with which promastigotes can be counted, axenic amastigotes grow in large conglomerates which are separable only by potentially damaging mechanical disruption, making direct counting impossible (2, 7, 14a, 16). Recently, an enzymatic assay of acid phosphatase was used to quantify cell density (4). *L. donovani* possesses two types of
L. donovani promastigotes and amastigotes are susceptible to Pentostam; however, the IC$_{50}$ for amastigotes was approxi-
mately one-fourth of that for promastigotes. Previous studies
indicated that promastigotes of L. panamensis are resistant to
sodium stibogluconate but are susceptible to the preservative
chlorocresol (13, 14). The susceptibility of amastigotes to chlo-
rocresol could not be determined due to the toxicity of this
compound to macrophages. The current study indicates that
promastigotes of L. donovani are also susceptible to chloro-
cresol, with the IC$_{50}$ being similar to that of the chlorocresol
concentration in Pentostam (Table 1 (values shown in pare-
theses)). Furthermore, by using axenic amastigotes, it was pos-

tible to measure the IC$_{50}$ of chlorocresol. L. donovani amasti-
gotes are susceptible to chlorocresol, as are promastigotes
(IC$_{50}$s of 1.8 and 1.3 µg/ml for amastigotes and promastigotes,
respectively). Despite the fact that amastigotes and promastig-
gotes are similarly susceptible to chlorocresol, the calculated
centration of chlorocresol at the IC$_{50}$ of Pentostam for
amastigotes was one-eighth (0.24 versus 1.82 µg/ml) that of the
IC$_{50}$ of chlorocresol as determined directly for axenic amasti-
gotes. Therefore, the increased susceptibility of amastigotes to
Pentostam is probably due to their susceptibility to sodium
stibogluconate. These data support the previously described
stage specificity of leishmanial susceptibility to pentavalent
antimony (13).

The induction of spontaneous resistance to Pentostam in
promastigotes resulted in as much as 21-fold resistance. We
chose two resistant strains for further study, Ld1S.04 and
Ld1S.20. Both strains were stably resistant for more than 6
months, suggesting that point mutation rather than gene am-
plification caused resistance. The IC$_{50}$ of chlorocresol for
both mutants relative to that for the wild type support the
hypothesis that the susceptibility of promastigotes to Pentos-

tam is primarily due to chlorocresol.

Mutant amastigotes were resistant to chlorocresol, with a
degree of resistance resembling the degree of mutant-proma-
stigote resistance to Pentostam (14 and 11 times and 35 and 21
times, respectively, for Ld1S.04 and Ld1S.20). However, mu-

tant-amastigote resistance to Pentostam (relative to that of

wild-type amastigotes) was much greater than that to chloro-
cresol (40 and 14 times and 148 and 35 times, respectively, for
Ld1S.04 and Ld1S.20). Therefore, it can be assumed that mu-
tant amastigotes are also resistant to sodium stibogluconate,
despite the fact that resistance was induced solely in promas-
tigotes, which are purportedly resistant to sodium stiboglu-
conate.

Dual resistance of L. donovani to both the antimony and the
preservative fractions of Pentostam was induced in promasti-
gotes at a sodium stibogluconate concentration 1/20 the IC$_{50}$
previously measured for intracellular L. panamensis promas-
tigotes (13). That this antimony concentration could induce
resistance in promastigotes is rather surprising. The induction
of dual resistance may be explained by the following: either the
single mutation conferring chlorocresol resistance is pleiotro-
pic, or a double mutation has occurred. These issues are cur-
rently under investigation.

In conclusion, although chlorocresol is present in Pentostam
as a preservative, it is toxic to Leishmania and therefore may
contribute to the overall antileishmanial activity of Pentostam
in vivo. This suggests that previous studies evaluating the ac-

tivity of Pentostam (as opposed to that of sodium stiboglu-
conate) against Leishmania probably measured, at least in part,
the effect of chlorocresol, as confirmed for L. panamensis (13).
Although the axenic amastigote model is removed from the
actual host-parasite environment, it is an easy tool for studying
the direct effects of various antileishmanial compounds on the
human form of the parasite. The axenic system also allows
identification of macrophage-dependent drug mechanisms.

ACKNOWLEDGMENTS

This work was supported by the Faculty of Medicine, Technion-
Israel Institute of Technology, and by the Basic Research Foundation
administered by the Israel Academy of Sciences (grant 508/94).

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TABLE 1. IC$_{50}$s of Pentostam and chlorocresol against both the wild type and Pentostam-resistant mutants of L. donovani$^a$

<table>
<thead>
<tr>
<th>L. donovani strain</th>
<th>IC$_{50}$ of pentostam (mg/ml)$^b$</th>
<th>IC$_{50}$ of chlorocresol (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promastigotes</td>
<td>Amastigotes</td>
<td>Promastigotes</td>
</tr>
<tr>
<td>Ld1 (wild type)</td>
<td>0.104 ± 0.03 (1.04)</td>
<td>0.024 ± 0.002 (0.24)</td>
</tr>
<tr>
<td>Ld1S.04</td>
<td>1.14 ± 0.14 (11.4)</td>
<td>0.95 ± 0.1 (9.5)</td>
</tr>
<tr>
<td>Ld1S.20</td>
<td>2.2 ± 0.9 (22)</td>
<td>3.55 ± 0.5 (35.5)</td>
</tr>
</tbody>
</table>

$^a$ IC$_{50}$ are means ± SD, where n = 3 to 7.
$^b$ Chlorocresol concentrations (in micrograms per milliliter) are given in parentheses.


