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An Axenic Amastigote System for Drug Screening

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Currently available primary screens for selection of candidate antileishmanial compounds are not ideal. The choices include screens that are designed to closely reflect the situation in vivo but are labor-intensive and expensive (intracellular amastigotes and animal models) and screens that are designed to facilitate rapid testing of a large number of drugs but do not use the clinically relevant parasite stage (promastigote model). The advent of successful in vitro culture of axenic amastigotes permits the development of a primary screen which is quick and easy like the promastigote screen but still representative of the situation in vivo, since it uses the relevant parasite stage. We have established an axenic amastigote drug screening system using a *Leishmania mexicana* strain (strain M379). A comparison of the 50% inhibitory concentration (IC_{50}) drug sensitivity profiles of M379 promastigotes, intracellular amastigotes, and axenic amastigotes for six clinically relevant antileishmanial drugs (sodium stibogluconate, meglumine antimoniate, pentamidine, paromomycin, amphotericin B, WR6026) showed that M379 axenic amastigotes are a good model for a primary drug screen. Promastigote and intracellular amastigote IC_{50}s differed for four of the six drugs tested by threefold or more; axenic amastigote and intracellular amastigote IC_{50}s differed by twofold for only one drug. This shows that the axenic amastigote susceptibility to clinically used reference drugs is comparable to the susceptibility of amastigotes in macrophages. These data also suggest that for the compounds tested, susceptibility is intrinsic to the parasite stage. This contradicts previous hypotheses that suggested that the activities of antimonial agents against intracellular amastigotes were solely a function of the macrophage.

Leishmaniasis, a disease endemic to 80 countries, is a major public health problem worldwide, with approximately 400,000 cases per year (3). The treatment of choice, pentavalent antimony (sodium stibogluconate [Pentostam] or meglumine antimoniate [glucantime]), was developed 50 years ago. Both forms are parenteral drugs which are characteristically moderately toxic. In addition, clinical failures are not uncommon, both because the compounds have low activity against some *Leishmania* strains (40) and because clinical failures following antimony treatment have been increasingly documented, particularly in India and Kenya (23, 24, 31, 45, 49, 69, 72, 73). Antimony-resistant parasites have also been identified in vitro (15, 40, 44, 47, 50, 51). Drugs that are more effective, less toxic, and easier to use are urgently needed.

Antileishmanial primary screening programs are based either on random screening, which relies on a high throughput (approximately 1 compound moves on to advanced development per 10,000 compounds screened), or on directed screening, which relies on informed selection criteria to limit the numbers of compounds screened. The philosophy of the screen (random versus directed) currently has a direct impact on the choice of the *Leishmania* model (promastigote versus intracellular amastigote). The promastigote stage is used in random primary screens that must be able to screen many compounds fast (12, 22, 44, 56). A limitation to this type of screen is that the promastigote is not the clinically relevant stage. The clinically relevant intracellular amastigote form can be used only in directed primary screens that do not rely on the quantity of compounds to obtain results (16, 19, 38, 47, 55), since this model is more difficult technically and more costly. Clearly, the ideal choice combines these two advantages: a rapid primary screen that uses the clinically relevant amastigote.

The concern over the parasite stage is valid. Promastigotes and amastigotes clearly differ morphologically (7, 8, 35, 36, 59). The two stages also differ on the basis of bioenergetics (reviewed by Berman [14]), including the utilization of fatty acids (42), enzymes of fatty acid oxidation, glycolytic enzymes and pathways (27, 32, 48, 52), and glycosomes. Gene expression (29) and protein phosphorylation (4, 34) are developmentally regulated, as are proteinases (46, 57, 61, 67, 68), nucleases (6), and expression of membrane proteins, including gp63, LPG, and a metalloproteinase (5, 70). Not surprisingly, the susceptibilities of amastigotes and promastigotes to antileishmanial compounds are also different (reviewed by Berman [13] and Neal [54]).

The advent of the ability to culture axenic amastigotes in vitro (1, 8, 35, 36, 58, 62, 71) allows the development of a primary drug screen which has the best of both systems: a rapid and easy drug screen which uses the relevant stage of the parasite life cycle. This publication describes, for the first time, the development and validation of an axenic amastigote system by comparing the sensitivity of promastigotes, axenic amastigotes, and intracellular amastigotes to clinically relevant reference antileishmanial agents.

MATERIALS AND METHODS

Cell culture and drugs. Promastigotes of *Leishmania mexicana* MNYC/BZ/62/M379 (gift of Paul Bates, University of Glasgow) were maintained at 25°C in Schneider's insect medium, pH 7.4 (Gibco BRL), supplemented with 20% heat-inactivated fetal bovine serum (FBS; Intergen) and 1% of a penicillin (50 U/ml)-streptomycin (50 μg/ml) solution (Sigma). Axenic amastigotes (strain M379) were also maintained in Schneider's *Drosophila* medium supplemented with 20% FBS and 1% of a penicillin-streptomycin solution, but the medium was acidified to pH 5.5, and the incubation temperature was increased to 32°C (8). The J774A.1 monocyte-macrophage mouse line (ATCC TIB67; American Type Cul-

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ture Collection, Rockville, Md.) was maintained at 37°C in the presence of 5% CO_2 in RPMI medium (Sigma) supplemented with 10% FBS. The reference antileishmanial agents (amphotericin B, pentamidine, paromomycin, WR6026, solid sodium stibogluconate [lot no. BL06916; Sb analysis = 28 to 30% by weight], and solid *n*-methylglucamine antimoniate [lot no. BL09186; Sb analysis = 27 to 29% by weight]) were obtained from the Chemical Inventory of Walter Reed Army Institute of Research, Division of Experimental Therapeutics.

Axenic amastigote drug susceptibility assay. Axenic amastigote drug susceptibility determinations were made using a modification of the promastigote direct counting growth inhibition assay (25). Amastigotes were seeded at an initial concentration equivalent to early log phase (2×10^5 amastigotes/ml) and allowed to multiply for 90 h either in medium alone or in the presence of serial dilutions of drug until late log phase (5×10^6 cells/ml). As in the promastigote model, axenic amastigote numbers doubled four to five times during the assay. Drug susceptibility experiments were performed in the maintenance media. Drug dilutions encompassed the 10, 50, and 90% inhibitory concentrations (IC_{10}, IC_{50}, and IC_{90}). Amastigotes were counted using a Coulter Counter Multisizer IIe after being passed three times through a 27-gauge needle in order to separate clumps, as needed, for accurate cell determinations. The results correlated with hemocytometer measurements. All experiments were repeated at least three times in triplicate, unless otherwise indicated. The IC_{50} is the concentration of drug which decreases cell numbers by 50% compared to numbers of control cells grown in the absence of drug.

Promastigote drug susceptibility assay. Promastigote drug susceptibility determinations were made using a previously described direct counting assay based on growth inhibition (25). Promastigotes were seeded at an initial concentration equivalent to that of early log phase (2×10^5 promastigotes/ml) and allowed to multiply for 60 h in medium alone or in the presence of serial dilutions of drug until late log phase (10^7 parasites/ml). Drug susceptibility experiments were performed in the maintenance medium except that glucantime assays were performed in modified Medium 199 (Sigma) with 10% FBS to facilitate the dilution of high concentrations of drug. All other parameters were the same as for axenic amastigotes.

Amastigote in macrophage drug susceptibility assay. Drug susceptibilities of M379 amastigotes in the J774A.1 monocyte-macrophage mouse line were determined by following a modification of the method of Chang (28). Briefly, J774A.1 macrophages were seeded at 4×10^5 macrophages/well in RPMI with 10% FBS in chamber slides. Following a 3-day incubation at 37°C to allow attachment, macrophages were infected with axenic amastigotes (4×10^5 amastigotes/well in RPMI with 20% FBS) and then incubated for 4 h at 34°C to allow infection. The chamber slides were washed once with Dulbecco's phosphate-buffered saline (Sigma), drug was added to the appropriate wells diluted in RPMI with 20% FBS, and slides were incubated at 34°C for a further 72 h before staining with Dif-Quik (Baxter). Medium and drug were changed every 24 h to prevent desiccation.

The initial infection was determined by fixing two wells with Dif-Quik immediately following the 4-h incubation. Control wells of infected macrophages were incubated in medium alone to determine the doubling times of amastigotes in macrophages over the length of the experiment (72 h). The viabilities of drug-treated and untreated uninfected macrophages were also assessed at 72 h. Experiments were repeated at least three times in duplicate. The initial percentage of macrophages infected with amastigotes was determined by randomly looking at 50 macrophages in two wells under an inverted microscope (100×), determining the number infected for each duplicate, multiplying by 2, and calculating the average. The number of amastigotes per macrophage was determined by determining the number of amastigotes in 50 randomly chosen macrophages in each duplicate well, dividing by 50, and calculating the average. IC_{50}s were calculated by using the values for the number of amastigotes/macrophage.

RESULTS

Standardization of M379 assays. Promastigote, axenic amastigote, and intracellular amastigote drug sensitivity assays are based on inhibition of parasite growth. The growth curves of M379 promastigotes and of axenic and intracellular amastigotes are shown in Fig. 1. Determinations of growth inhibition from drug were made when the parasites were still in log phase, prior to entering stationary phase. M379 promastigotes reach this stage by 60 h; axenic amastigotes require 90 h (Fig. 1a and b). M379 intracellular amastigotes (in J774A.1 macrophages) multiply more slowly than axenic amastigotes, but they consistently double in number over 72 h when inoculated with a low amastigote-to-macrophage ratio (1:1). Higher infection ratios do not show doubling, although growth is still seen (Fig. 1c and data not shown). The percentage of infected macrophages at the time that the antileishmanial agents were added was high and did not change over the length of the experiment

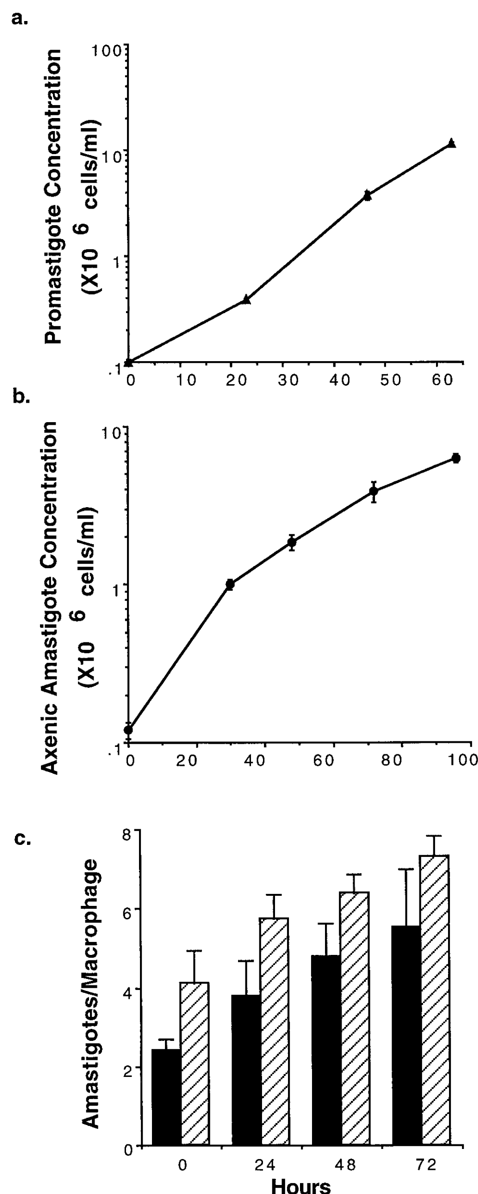

FIG. 1. Representative growth curves of promastigotes, axenic amastigotes, and intracellular amastigotes. (a) Promastigotes of strain M379 were seeded at approximately 10^5 cells/ml. (b) Axenic amastigotes were seeded at approximately 10^5 cells/ml. (c) Macrophages were infected with amastigotes at ratios of approximately 1:1 (solid bar) and 3:1 (hatched bar). Promastigote and amastigote concentrations were determined by using a Coulter Counter Multisizer IIe. Each point represents the mean of two replicates ± the standard deviation. The number of amastigotes/macrophages was determined daily by counting under a light microscope. Each time point represents the mean of three experiments ± the standard error of the mean.

whether a 1:1 or 3:1 ratio (amastigotes:macrophages) was used (90% ± 1% at 4 h and 89% ± 4% at 72 h; 94% ± 2% at 4 h and 95% ± 2% at 72 h, respectively).

Comparison of M379 promastigotes and amastigotes. The sensitivities of M379 promastigotes, axenic amastigotes, and intracellular amastigotes to reference antileishmanial agents (sodium stibogluconate, meglumine antimoniate, pentamidine, paromomycin, amphotericin B, WR6026) were determined (Fig. 2, Table 1). Overall, axenic amastigotes are significantly more similar to intracellular amastigotes in their sensitivity to

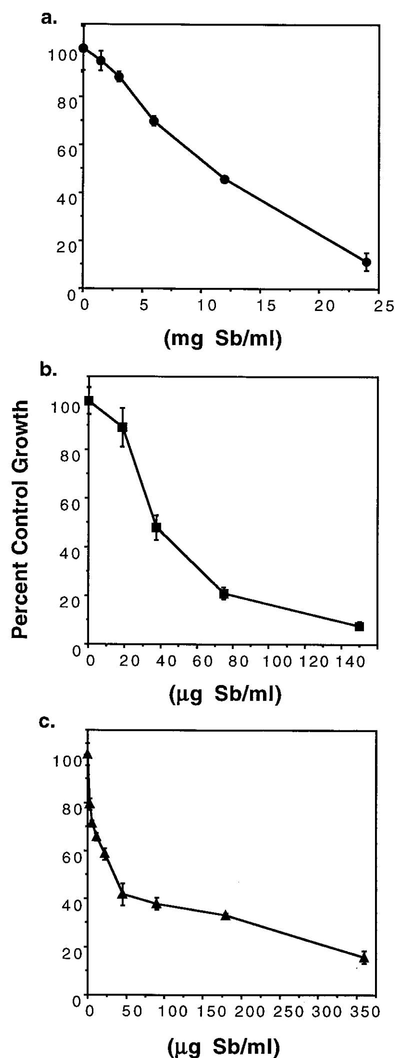

FIG. 2. Inhibition of multiplication of strain M379 promastigotes, axenic amastigotes, and intracellular amastigotes by sodium stibogluconate. The results of a representative experiment are shown for promastigotes (direct counting method) (a), axenic amastigotes (b), and intracellular amastigotes (c). Promastigotes were incubated in the presence of milligram quantities of sodium stibogluconate for 60 h, and axenic and intracellular amastigotes were incubated with microgram quantities for 90 and 72 h, respectively. The percent control values were calculated by dividing parasite numbers (or amastigotes/macrophage) in the presence of drug (minus initial numbers) with numbers in the absence of drug (minus initial numbers) and multiplying by 100.

known antileishmanial agents than are promastigotes. Only one of the six reference antileishmanial agents tested (paromomycin) showed a difference between the axenic amastigote and intracellular amastigote assays, and this difference was only twofold. This result shows that the axenic amastigote susceptibility to clinically used reference drugs is comparable to the susceptibility of amastigotes in macrophages. Thus, M379 axenic amastigotes are a good system for a primary drug screen.

In contrast, four of the six reference antileishmanial agents (including paromomycin) tested showed a significant difference (>2-fold) between the promastigote and intracellular amastigote assays (Fig. 2a and c; Table 1). The reference antileishmanial agents glucantime, Pentostam, and paromomycin were less active for promastigotes than for intracellular amastigotes (380-, 330-, and 3-fold, respectively), while pentamidine was more active (5-fold). These results show that the susceptibility of promastigotes to clinically used reference antileishmanial agents is not representative of the drug sensitivities of intracellular amastigotes in our model.

DISCUSSION

We have developed and validated an axenic amastigote drug screen that uses *L. mexicana* M379. We found that the sensitivity of axenic amastigotes to reference antileishmanial agents closely parallels the sensitivity of the same strain tested intracellularly in J774A.1 macrophages. In contrast, the sensitivity of the promastigote stage to reference antileishmanial agents is significantly different from the sensitivities of intracellular and axenic amastigotes.

L. mexicana M379 has been extensively studied and characterized (6, 8–10, 21, 43, 60, 61, 68, 71), and the complete developmental cycle has been replicated in axenic culture (7, 10). Axenic amastigotes have been characterized ultrastructurally by transmission electron microscopy (8, 61) and shown to have amastigote-specific features, including an ovoid shape, a short nonemergent flagellum, no paraxial rod, and megasomes. Biochemical analysis also showed an amastigote-like profile (6, 8, 61). Both cysteine proteinase and 3′-nucleotidase/nuclease activities were higher in amastigotes (lesion derived and axenically cultured) than in promastigotes, and the gelatin sodium dodecyl sulfate-polyacrylamide gel electrophoresis banding patterns of the axenic amastigote proteinase and nucleotidase/nuclease were similar to those seen in lesion amastigotes and different from those seen in promastigotes. Infectivity comparisons in CBA mice also showed a similar pattern for axenic amastigotes and lesion amastigotes and a significantly different pattern for stationary-phase promastigotes (8).

The drug susceptibility assays for the promastigote, axenic amastigote, and intracellular amastigote forms were designed to be as similar as possible. All of the assays were based on direct counting of parasites, either under the light microscope (intracellular amastigotes) or by Coulter Counter (promastigotes and axenic amastigotes). However, the IC_{50}s of M379 promastigotes and axenic amastigotes, generated using the direct counting method, showed significant differences.

Our values for the antileishmanial susceptibilities of both intracellular amastigotes and promastigotes are comparable to values previously reported. The Pentostam (4.1 and 21 μg/ml) and paromomycin (24 μg/ml) susceptibilities of amastigotes of

TABLE 1. Sensitivities of strain M379 promastigotes, axenic amastigotes, and intracellular amastigotes to known antileishmanial agents in vitro

Antileishmanial agent[a]	IC_{50} ± SEM (μg/ml)[b]		
	Promastigotes	Axenic amastigotes	Amastigotes/ macrophage
Amphotericin B	0.14 ± 0.02	0.28 ± 0.02	0.25 ± 0.01
Pentamidine	0.67 ± 0.1	5.0 ± 0.8	3.4 ± 0.4
Paromomycin	54 ± 10	41 ± 10	17 ± 1
WR6026	9.7 ± 3	12 ± 1	7.2 ± 3
Glucantime[c]	11,000 ± 2,000	30 ± 6	29 ± 3
Pentostam[c]	10,000 ± 3,000	48 ± 10	30 ± 6

[a] All compounds are from the Chemical Inventory of Walter Reed Army Institute of Research.

[b] Assays are described in Materials and Methods.

[c] Values for antimonial agents are in micrograms of Sb (rather than of drug) per ml.

the M379 strain of *L. mexicana* were previously reported based on a mouse peritoneal macrophage model (2, 53). Pentostam values for other *L. mexicana* strains in mouse peritoneal macrophages are also similar to our values (17 and 35 μg/ml [18]). No IC_{50}s of the other drugs appear to be available for *L. mexicana* amastigotes or for amastigotes of other New World species. However, values of glucantime (3.8 μg/ml), pentamidine (>1.0 and 6.1 μg/ml), WR6026 (1.2 μg/ml), and amphotericin B (0.04 μg/ml) for *Leishmania tropica* and *Leishmania donovani* amastigotes (Old World cutaneous and visceral strains, respectively) in mouse peritoneal macrophages are similar to our values (18, 55). Although no IC_{50}s for *L. mexicana* promastigotes appear to be available, the sensitivity of promastigotes of *Leishmania panamensis* and *Leishmania amazonensis* (other New World cutaneous strains) to Pentostam (8.7 mg/ml), glucantime (11 mg/ml), and Pentamidine (0.28 and 0.48 μg/ml) are similar to our values (11, 39, 64, 74), as are the IC_{50}s for the Old World cutaneous strains *Leishmania major* and *L. tropica* for paromomycin (12 to 18 and 30 μg/ml, respectively [37, 41]).

The literature shows significant differences between the IC_{50}s of four of the six antileishmanial agents tested in this study (Pentostam, glucantime, paromomycin, and pentamidine) for promastigotes and intracellular amastigotes. The same pattern of higher IC_{50}s of Pentostam, glucantime, and paromomycin and lower IC_{50}s of pentamidine for promastigotes than for intracellular amastigotes was seen in our experiments with promastigotes and intracellular amastigotes of M379. In contrast, IC_{50}s for axenic amastigotes are comparable to those for intracellular amastigotes with all six of these drugs.

Hypotheses for the differences in the susceptibilities of promastigotes and intracellular amastigotes emphasize the possible role of the macrophage in either antimony accumulation or conversion of Sb(V) to Sb(III) to explain the inactivity of antimony for promastigotes in vitro (15, 26, 33, 64, 65). Previously, macrophages were shown to accumulate antimony in vitro (17, 64), and trivalent antimony has been shown to be more active than pentavalent antimony for *Leishmania* spp. in vitro (33, 64–66). However, differences in the drug susceptibilities of the two stages have not been addressed directly. We show, for the first time, that amastigotes are intrinsically more sensitive to Pentostam and glucantime than are promastigotes.

This finding has practical value, since axenic amastigotes can be studied biochemically and the differences between the two developmental stages can be determined. Since antimony is still the drug with the best clinical therapeutic index, understanding the biochemical basis of antimony efficacy is critical for the design of new compounds that kill *Leishmania* spp. and not host cells. We have shown that axenic amastigotes, not promastigotes, contain this information. This discovery should allow the determination of the biochemical basis for the efficacy of antimony.

In summary, the axenic amastigote assay is technically easier, less expensive, and significantly faster, both in terms of personnel-hours and the total length of the assay, than the intracellular amastigote assay. Unlike the promastigote assay, the axenic amastigote assay uses the clinically relevant stage of the parasite. Despite the removal of the macrophage, drugs are still active against axenic amastigotes at levels attainable in serum (Pentostam, 9 to 15 μg/ml; glucantime, 10 to 28 μg/ml; pentamidine, 0.5 to 3.2 μg/ml [20, 30, 63]). In addition, we should be able to automate the axenic amastigote assay by using established promastigote methods. When the conditions for axenic culture of amastigotes have been standardized for all strains and species, this technology will also have a significant impact on in vitro testing of the sensitivities of clinical isolates to antileishmanial agents.

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