ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2009, p. 5197–5203 Vol. 53, No. 12
0066-4804/09/$12.00 doi:10.1128/AAC.00866-09
Copyright © 2009, American Society for Microbiology. All Rights Reserved.

In Vitro Sensitivity Testing of *Leishmania* Clinical Field Isolates: Preconditioning of Promastigotes Enhances Infectivity for Macrophage Host Cells[▽]

Raquel Inocêncio da Luz,[1] Marieke Vermeersch,[1] Jean-Claude Dujardin,[2]
Paul Cos,[1] and Louis Maes[1]*

Laboratory of Microbiology, Parasitology and Hygiene, Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, University of Antwerp, Groenenborgerlaan 171, Antwerp B-2020, Belgium,[1] and Laboratory of Molecular Parasitology, Institute of Tropical Medicine, Nationalestraat 155, Antwerp B-2000, Belgium[2]

Received 25 June 2009/Returned for modification 6 August 2009/Accepted 3 September 2009

Diagnostic material from patients with leishmaniasis is generally available as promastigotes, and proper testing for susceptibility to first-line drugs by the intracellular amastigote assay is frequently hampered by the poor infectivity of the promastigotes for the macrophage host cell. Several conditions for optimization of the in vitro metacyclogenesis and cell infectivity of *Leishmania donovani*, *L. guyanensis*, and *L. braziliensis* field strains obtained from patients receiving standard antimony medication were investigated. Triggering log-phase promastigotes to become amastigote-like by increasing the temperature or acidifying the culture medium was not successful. Adequate metacyclogenesis and the highest levels of macrophage infection were obtained after 5-day-old late-log-phase promastigote cultures were preconditioned at 25°C to pH 5.4 for 24 h in Schneider's medium prior to infection. The susceptibility assay with primary peritoneal mouse macrophages included pentavalent antimony (Sb^V; sodium stibogluconate), trivalent antimony (Sb^{III}; potassium antimonyl tartrate), miltefosine, and the experimental drug PX-6518. All strains were sensitive to miltefosine (50% inhibitory concentration [IC_{50}] < 10 μM) and PX-6518 (IC_{50} < 2 μg/ml) but showed distinct susceptibility to Sb^V and/or Sb^{III}, depending on whether they were derived from cured, relapse, or nonresponder patients. Within the available set of *Leishmania* species and strains, simultaneous Sb^V-Sb^{III} resistance was clearly associated with treatment failure; however, a larger set of isolates is still needed to judge the predictive value of Sb^V-Sb^{III} susceptibility profiling on treatment outcome. In conclusion, the proposed conditioning protocol further contributes toward a more standardized laboratory model for evaluation of the drug sensitivities of field isolates.

As the failure of first-line treatment for all clinical forms of leishmaniasis is a growing problem, it is pivotal to monitor the efficacy of standard drugs and map the prevalence of drug resistance in areas where the disease is endemic (7, 9, 24). Diagnostic field isolates are mostly provided to the laboratory as promastigotes, but it remains an experimental challenge to appropriately adapt them to the amastigote-macrophage model, still considered the "gold standard" for susceptibility evaluation (23, 31). Infection of the macrophage is generally achieved with metacyclic promastigotes, but unfortunately, infection is subject to a high degree of variability, among several other factors affecting the outcome of the sensitivity test (8, 10). These factors strongly suggest the need for the further standardization of susceptibility testing of clinical field isolates.

In the normal course of events, infective metacyclic promastigotes are inoculated by the sand fly into the mammalian host, where they rapidly penetrate susceptible cells, undergo intracellular transformation to the amastigote form, and start dividing. The procyclic and metacyclic phases observed during in vitro culture appear to be similar to the stages that occur in the sand fly gut. Moreover, the infectivity for the vertebrate host steadily increases from the log to the stationary phase and is linked to the progressive increase in the numbers of metacyclic promastigotes (5, 27). Metacyclic promastigotes are identified as small, slender promastigotes (≤8 μm by 1.2 to 1.5 μm) with a flagellum measuring at least twice the length of the cell body, and they occur in the greatest numbers in vitro during late stationary phase. However, the level of metacyclogenesis may vary considerably depending on the species, strain, and culture conditions (13). Since the cells in stationary-phase cultures appear to be a heterogeneous population, another relevant limitation is the incomplete transformation into actively dividing amastigotes that occurs after internalization by the macrophage host cell. Not all metacyclic promastigotes appear to have the capability to transform into amastigotes (19), thereby influencing again the outcome of sensitivity testing. The latter is particularly relevant for drugs that specifically act on intracellular amastigotes, such as pentavalent antimony (Sb^V) compounds (4) and the experimental antileishmania compound PX-6518 (22, 31).

The aim of this study was to investigate how promastigotes of different *Leishmania* species can be effectively triggered to differentiate into highly infective metacyclic promastigotes. Metacyclogenesis has been stimulated by culturing promastigotes at acidic pH, which leads to a more homogeneous sta-

* Corresponding author. Mailing address: Laboratory for Microbiology, Parasitology and Hygiene (LMPH), Faculty of Pharmaceutical, Biomedical and Veterinary Sciences, Antwerp University, Groenenborgerlaan 171, Antwerp B-2020, Belgium. Phone: 32 3 265 3354. Fax: 32 3 311 6301. E-mail: louis.maes@ua.ac.be.
▽ Published ahead of print on 14 September 2009.

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

TABLE 1. Susceptibilities of clinical field isolates to Sb^V, Sb^{III}, MIL, and the experimental drug PX-6518

Species	Strain[a]	Sb treatment outcome[b]	IC_{50} for intracellular amastigotes				AI[c]	
			Sb^V (μg/ml eq.)	Sb^{III} (μg/ml eq.)	MIL (μM)	PX-6518 (μg/ml)	Sb^V	Sb^{III}
L. donovani	BPK043	Definitive cure	13.3 ± 4.9	6.0 ± 2.0	1.5 ± 0.8	0.3 ± 0.1	1.0	1.0
	BPK091	Definitive cure	49.7 ± 23.0	24.2 ± 17.4	3.2 ± 1.6	0.3 ± 0.1	3.2	3.9
	BPK178	Definitive cure	49.5 ± 23.7	21.8 ± 8.8	5.5 ± 1.3	0.8 ± 0.2	3.1	3.6
	BPK206	Definitive cure	37.5 ± 19.7	11.0 ± 2.9	4.0 ± 2.8	0.4 ± 0.0	3.6	1.8
	BPK206cl		65.8 ± 7.8	5.0 ± 0.0	2.0 ± 0.0	0.9 ± 0.1	**5.5**	0.8
	BPK274	Definitive cure	51.2 ± 22.3	16.2 ± 10.2	7.1 ± 5.4	0.8 ± 0.7	3.2	2.7
	BPK294	Definitive cure	24.5 ± 2.1	6.0 ± 1.4	3.0 ± 0.0	0.4 ± 0.0	1.5	1.0
	BPK298	Definitive cure	30.3 ± 5.7	12.0 ± 7.0	3.4 ± 3.0	0.4 ± 0.0	1.9	2.0
	BPK177	Relapse	≥77 ± 0.0	32.0 ± 11.9	2.0 ± 0.0	0.4 ± 0.2	**6.6**	**5.3**
	BPK190	Nonresponder	≥77 ± 0.0	27.3 ± 8.7	4.5 ± 0.7	0.1 ± 0.0	**6.6**	**4.5**
	BPK190cl		55.0 ± 25.5	24.0 ± 10.0	3.0 ± 0.0	0.2 ± 0.1	**4.2**	4.0
L. guyanensis	PER106	Definitive cure	13.0 ± 1.4	12.0 ± 5.7	10.0 ± 1.4	2.0 ± 0.0	1.0	1.0
	PER008	Definitive cure	41.5 ± 10.6	18.0 ± 2.8	5.0 ± 1.4	1.5 ± 0.7	3.2	1.6
	PER054	Unknown	50.0 ± 5.7	18.0 ± 2.8	9.5 ± 3.5	0.9 ± 0.1	4.0	1.6
	PER072	Definitive cure	77.0 ± 0.0	19.0 ± 8.5	10.0 ± 1.4	1.0 ± 0.0	**6.0**	1.6
	PER080	Definitive cure	41.5 ± 15.6	14.5 ± 2.1	7.5 ± 2.1	0.9 ± 0.1	2.5	1.4
L. braziliensis	PER002cl	Unresponsive	57.5 ± 16.3	13.5 ± 13.0	10.3 ± 5.6	1.7 ± 1.7	**4.5**	1.3
	PER005cl	Unresponsive	75.0 ± 4.0	25.0 ± 10.2	10.0 ± 5.8	0.5 ± 0.2	**5.8**	2.4

[a] Several strains are the same as those described in references 26 and 32.

[b] Patients were treated with Sb^V at 20 mg/kg of body weight intramuscularly for 1 month. Clinical follow-up was performed at 1, 3, 6, and 12 months after the start of treatment. Nonresponders were cases with a positive parasitology result after the full 30-day treatment course; definitive cure was defined as no signs or symptoms of relapse at 12 months of follow-up; relapse was defined as an initial cure but the reappearance of clinical symptoms and a positive parasitology result during follow-up (26, 32).

[c] An AI of >4 is indicative of resistance (boldface).

tionary-phase population of metacyclic promastigote-like cells (33, 34). However, continuous growth under acidic conditions leads to the early appearance of metacyclic promastigotes and a lower final cell density (1, 6). Therefore, we hypothesized that after growth at neutral pH, promastigotes could be more optimally conditioned by briefly exposing them to a lower pH just prior to infection, leading to a higher final cell density and more reproducible intracellular infections. Different strains could even be synchronized to infect macrophages at the same time, which may be a practical advantage in laboratories that must evaluate the sensitivities of a large number of field isolates in the context of epidemiological studies.

MATERIALS AND METHODS

Culture media, products, reagents, and animals. Adenosine, folic acid, D-biotin, hemin, $NaHCO_3$, potato starch, dimethyl sulfoxide, Giemsa stain, resazurin, and trivalent antimony (Sb^{III}; potassium antimonyl tartrate thrihydrate) were purchased from Sigma, whereas minimal essential medium (MEM), RPMI 1640 medium, Schneider's medium, L-glutamine, and fetal calf serum (FCS) were supplied by Invitrogen. Miltefosine (MIL) and Sb^V (sodium stibogluconate) were kindly provided by TDR, WHO. The experimental antileishmania compound PX-6518 was available from previous work (22). Stock solutions of Sb^{III} and Sb^V were prepared in preheated phosphate-buffered saline at 37°C immediately before use. MIL, amphotericin B, and PX-6518 were dissolved in Milli-Q water and stored at 4°C.

Swiss mice were supplied by Janvier (France). The animal experiments were approved by the Ethical Committee of the University of Antwerp.

Parasite and cell cultures. (i) Parasites. Field isolates from clinical cases of visceral leishmaniasis (VL; *Leishmania donovani*), cutaneous leishmaniasis (CL; *L. guyanensis*), and mucocutaneous leishmaniasis (MCL; *L. braziliensis*) were obtained from the Institute of Tropical Medicine, Antwerp, Belgium (26, 32) as promastigote cryostabilates after primary isolation on NNN blood slopes with a saline-antibiotic overlay. After the strains were thawed, they were subcultured twice a week on 3 N blood slopes for 2 to 3 weeks, and adaptation to MEM consisted of at least two additional passages. In total, about 10 passages were generally performed before in vitro susceptibility testing was performed. Culture of the promastigotes was performed in MEM supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, 10% heat-inactivated FCS (FCS_i), 40 mg/liter adenine, 3 mg/liter folic acid, 2 mg/liter D-biotin, and 2.5 mg/liter hemin. The culture had an initial pH of 7.5 and was kept at 25°C under normal atmospheric conditions. All strains were derived from patients (patients with VL from Nepal, patients with CL and MCL from Peru) who had received standard antimony treatment and whose treatment outcomes were adequately documented. For some strains, clones were also available (Table 1).

(ii) Cell culture. Primary peritoneal mouse macrophages were collected from Swiss mice 2 days after peritoneal stimulation with a 2% potato starch suspension. Cells were collected and grown in RPMI 1640 medium supplemented with 200 mM L-glutamine, 16.5 mM $NaHCO_3$, and 5% FCS_i at 37°C under 5% CO_2.

Preconditioning of promastigotes. Temperature, differentiation time, and acidification of the medium were used as variables for preconditioning of the promastigote cultures. The influence of temperature was evaluated by incubating the promastigotes from patients with MCL, CL, and VL at 25°C, 34°C, or 37°C. The conditioning time for the promastigotes varied from 24 h to 72 h. The following protocols were adopted for acidification: (i) standard growth in MEM with spontaneous acidification and metacyclogenesis, with the latter largely being dependent on the inoculum size and the speed of growth (for slowly growing strains, the starting inoculum was adaptively increased); (ii) artificial acidification of MEM with 1 N HCl to pH 5.4 during late log phase, i.e., 5-day old cultures; and (ii) transfer of 5-day-old promastigote cultures into Schneider's medium at pH 5.4 supplemented with 20% FCS_i (11).

Intracellular drug sensitivity assays. Primary peritoneal mouse macrophages were seeded in 96-well microtiter plates at 3×10^5 cells/well and were left for adhesion and differentiation for 48 h before infection with metacyclic promastigotes at an infection ratio of 10:1. After 24 h, noninternalized promastigotes were removed and the medium was replaced with medium containing twofold drug dilutions starting from 77 μg Sb^V/ml, 88 μg Sb^{III}/ml, 40 μM MIL, and 2 μg/ml PX-6518. After 5 days of incubation in 5% CO_2 at 37°C for VL and 34°C for CL and MCL, parasite burdens [calculated as the percentage of infected macrophages × (mean number of amastigotes/macrophage)] were microscopically assessed on Giemsa-stained preparations and compared to the burdens for the untreated infected controls. At least 100 macrophages were counted, and infection was judged to be adequate if 70 to 80% of the macrophages were infected. The results are expressed as the percent reduction of the total parasite

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

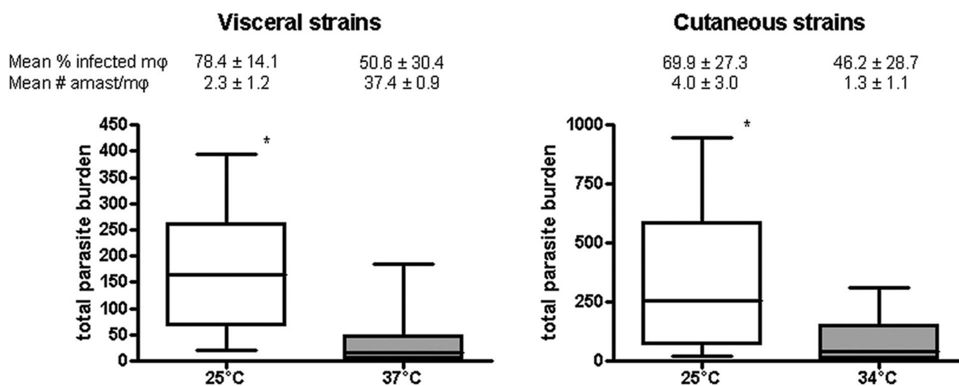

FIG. 1. Total macrophage amastigote burdens after transfer of log-phase promastigotes to acidic conditions at either 37°C (VL) or 34°C (CL, MCL) and 25°C (VL, CL, MCL). The conditioned promastigotes were subsequently used to infect macrophages, and the parasite burdens were determined on Giemsa-stained slides. *, $P < 0.05$.

burdens, and 50% inhibitory concentrations (IC_{50}) were calculated (Statview). At least six independent replicate tests were performed for each observation. To more easily compare the results obtained with Sb^V and Sb^{III} in the different experimental series, an activity index (AI), which represented the ratio of the IC_{50} for the field strain/IC_{50} for the sensitive reference strain, was calculated. Strains BPK043 and PER106 were chosen as Sb-sensitive reference strains (Table 1). An AI of ≥4 was defined as resistance (32).

Morphological studies. (i) Light microscopy. The promastigotes were monitored for morphological changes during preconditioning by preparing thin smears from promastigotes that were air dried, fixed in methanol, and stained with Giemsa. The slides were examined at ×1,000 magnification under immersion (with a Zeiss Axiophot microscope equipped with an Olympus DP70 camera). The length of the flagella and the cell dimensions of 100 parasites per preparation were measured with the CellP program (soft imaging system; Olympus). Promastigotes with a cell body of <8 μm and a flagellum/cell body ratio of ≥2 are considered metacyclic (2, 33).

(ii) Flow cytometry. In view of the morphological differences between the procyclic and metacyclic forms, flow cytometric analysis can be applied for the discrimination of both forms (28). Briefly, nonfluorimetric measurements were performed (Cell Lab Quanta; Beckman Coulter) by suspending 10 μl of promastigote culture in 1 ml phosphate-buffered saline and analyzing the light scatter of the mixture. Dot plots of the electronic volume and the side scatter of 10,000 events were analyzed.

RESULTS

Conditioning factors for promastigotes. Standard growth curves were established for each strain (data not shown). The VL strains entered into log phase on day 2, and early stationary phase was reached within 4 to 5 days, after which spontaneous acidification of the medium became very prominent, leading to a decrease in viable cell numbers by day 7. The CL and MCL strains reached early stationary phase after about 4 to 5 days, but at lower cell densities than the VL strains and with minimal acidification of the medium. One strain (strain PER106) showed a particularly slow initial growth, with parasite numbers still increasing until day 6. Since all cultures reached early stationary phase after about 4 to 6 days, day 5 was retained for artificial preconditioning.

To check the influence of elevated temperatures and the duration of conditioning, 5-day-old promastigote cultures were adjusted to 37°C (VL) or 34°C (CL) for 24, 48, or 72 h. The promastigote cell bodies became more rounded to oval, but with the flagellum still being clearly present. These rounded stages were not able to adequately infect macrophages. The use of elevated temperatures at pH 5.4 produced similar results. However, when the promastigotes were maintained at 25°C under acidic conditions for 24 h, significantly higher levels (>70%) of infection were obtained both for VL strains and for CL and MCL strains (Fig. 1). The VL strains showed declining levels of infection after longer incubation times. The CL and MCL strains produced more variable results: *L. brasiliensis* produced adequate intracellular amastigote burdens after 24 h and 48 h of conditioning, while the *L. guyanensis* strains showed an overall low level of infectivity (data not shown).

Highly variable infection rates were observed with spontaneous late-stationary-phase VL promastigotes (range, 65 to 93%, with variation across the different test replicates). The transfer of 5-day-old cultures to either acidified MEM or Schneider's medium (pH 5.4) resulted in enhanced infection of macrophages, with infection rates of about >80% being achieved, irrespective of the conditioning medium used (Table 2). Surprisingly, the clone of BPK206 (strain BPK206cl) showed a lower level of infectivity after it was conditioned in MEM. The use of spontaneous late-stationary-phase promastigotes of CL and MCL strains was very problematic since almost no infection of macrophages was obtained (infection

TABLE 2. Effects of different conditioning protocols for enhanced metacyclogenesis on promastigote infectivity for primary peritoneal mouse macrophages[a]

Strain	% Infected macrophages[a]					
	MEM, spontaneous		MEM, pH 5.4		Schneider's medium, pH 5.4	
	Mean	SD	Mean	SD	Mean	SD
BPK190	65.4	11.8	78.6	12.3	80.3	6.9
BPK190cl	60.2	8.1	93.6	3.7	84.2	8.3
BPK206	93.3	7.9	90.9	5.1	88.8	4.1
BPK206cl	70.7	15.6	49.7	14.5	85.5	8.3
PER002cl	<5		83.0	4.7	98.5	1.9
PER005cl	<5		87.6	5.3	96.2	2.6
PER072	<5		31.8	8.5	87.1	7.6
PER106	<5		47.7	12.3	42.2	13.9

[a] Stationary-phase cultures showing spontaneous acidification were compared to 5-day-old promastigote cultures adjusted for 24 h in MEM at pH 5.4 or Schneider's medium at pH 5.4. An adequate level of infection was set at >70%.

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

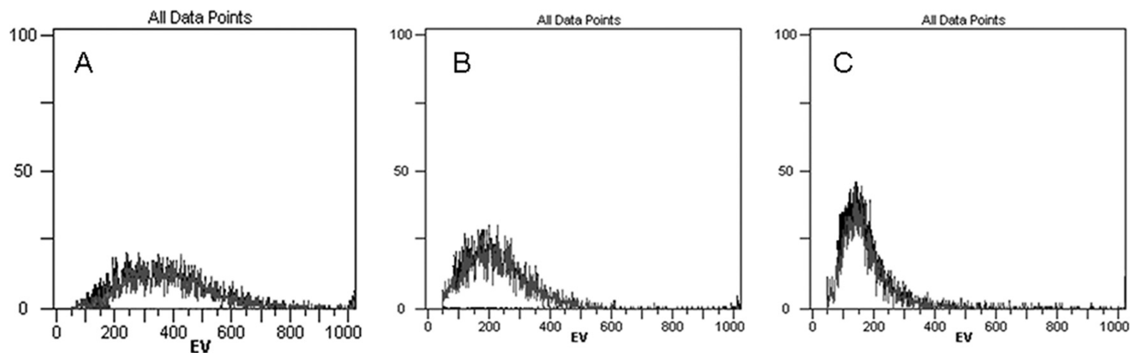

FIG. 2. Flow cytometric analysis of *L. donovani* strain BPK190. (A) Log-phase culture; (B) stationary-phase promastigotes (7-day-old cultures); (C) after 24 h of preconditioning of a 5-day-old culture in Schneider's medium at pH 5.4. EV, electronic volume.

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

rate, <5%). Preconditioning of *L. brasiliensis* produced adequate infection rates (>80%), with a further improvement being achieved when Schneider's medium was used (>90%). Conditioned promastigotes of *L. guyanensis* showed rather low and variable infectivities. Slowly growing strain PER106 failed to produce adequate infections after conditioning both in MEM (47.7% ± 12.3%) and in Schneider's medium (42.2% ± 13.9%), but it was still much better than the spontaneous stationary-phase promastigotes at producing adequate infections. Strain PER072 was adequately infective only after it was conditioned in Schneider's medium (87.1% ± 7.6%).

The process of induction of metacyclogenesis was morphologically evaluated for *L. donovani* by comparing spontaneous late-stationary-phase and preconditioned promastigotes by flow cytometry (Fig. 2) and through the use of light microscopic measurements (Fig. 3). Late-stationary-phase cultures clearly consisted of a mixed population of larger and smaller cells, with a slight shift to the left compared to the log-phase cultures (Fig. 2A and B). After preconditioning of the promastigotes for 24 h, a clear shift to the left was noted (Fig. 2C), indicative of a larger proportion of smaller cells, e.g., metacyclic promastigotes. On the basis of the cell dimensions, strain BPK206cl had only about 10% metacyclic promastigotes in stationary-phase cultures, and this amount increased to >50% after the promastigotes were conditioned. Strain BPK190 already contained about 50% metacyclic promastigotes in late stationary phase, and that amount further increased to about 64% after conditioning of the promastigotes (Fig. 3).

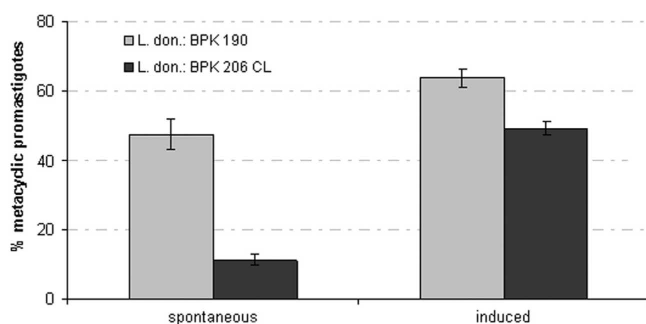

FIG. 3. Microscopic enumeration of metacyclic promastigotes in spontaneous stationary phase (day 7) and after induction on day 5 in Schneider's medium at pH 5.4 for 24 h. The morphological criteria for a metacyclic promastigote are a cell body of <8 μm and a flagellum/body ratio of >2. L. don., *L. donovani*; CL, clone.

In vitro susceptibilities of field isolates. After adoption of the established conditioning protocol, the in vitro susceptibilities of a larger set of clinical field isolates of *L donovani*, *L. guyanensis*, and *L. braziliensis* were determined (Table 1). Use of the reference compound PX-6518 confirmed the adequate transformation of the promastigotes into intracellular amastigotes, as demonstrated by an IC_{50} of <1 μg/ml for VL strains and an IC_{50} of <2 μg/ml for CL and MCL strains. Since the treatment outcome was also known for each isolate, information on the predictive value of the intracellular amastigote susceptibility test for the determination of Sb resistance could be gained. In addition, MIL was included as a reference, and full susceptibility to MIL was observed (IC_{50}s, <5 μM for VL strains and <10 μM for CL and MCL strains) since the patients had never been treated with the drug.

For *L. donovani*, strain BPK043 was included as the sensitive reference strain in each experiment (Sb^V IC_{50}, 13.3 μg/ml equivalents [μg/ml eq.]; Sb^{III} IC_{50}, 6 μg/ml eq.) and to allow calculation of the AI. By combining the AI values for Sb^V and SB^{III}, strains BPK177 and BPK190 clearly showed decreased sensitivity, which also fully corresponded to the clinical profile as a nonresponder or a relapse. BPK206, derived from a cured patient, showed marginally decreased sensitivity to Sb^V (AI = 3.6), but it was still fully sensitive to Sb^{III} (SI = 1.8). Its clone had about the same profile (Sb^V AI = 5.5, Sb^{III} AI = 0.8). Strains BPK091 and BPK178 were derived from a cured patient and just fell within the sensitive range (AI < 4). Similar results were obtained with *L. guyanensis*, all strains of which (except PER072) were sensitive to both Sb^V and Sb^{III}, thereby reflecting the clinical outcome of a definite cure. Since only clones of the *L. braziliensis* strains were available, the Sb^V-Sb^{III} susceptibility outcome does not reflect the clinical outcome.

DISCUSSION

In vitro susceptibility testing with live *Leishmania* stages still remains the primary tool for epidemiological mapping of drug resistance, as molecular resistance markers have not yet been identified or fully validated (17, 21, 25). Evaluation of the susceptibility of isolates from patient-derived diagnostic material is still hampered by the lack of reliable and reproducible in

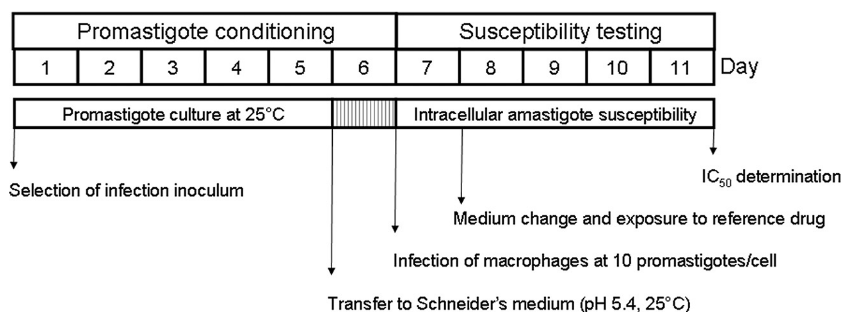

FIG. 4. Flowchart of promastigote preconditioning and testing of the susceptibilities of the *Leishmania* field isolates.

vitro models, and prediction of the treatment outcome is even more controversial (26, 32). While the use of amastigotes in macrophages is still considered the gold standard for all drugs, the use of axenic amastigotes for the evaluation of susceptibility to MIL and amphotericin B may also be appropriate (31). The present study aimed to provide a standardized method for infection of macrophages with clinical isolates causing VL (*L. donovani*), CL (*L. guayensis*), and MCL (*L. braziliensis*) and attempted to link Sb susceptibility in vitro to the clinical outcome. From the knowledge that macrophages can be adequately infected with either metacyclic promastigotes or amastigotes, both options were addressed.

As the products of metabolism reduce the pH in promastigote cultures, most investigators have used spontaneous acidification in the stationary phase to obtain infective metacyclic forms (6, 12). However, considerable variation in the efficiency of metacyclogenesis between different species and strains has been observed (13, 15). For example, rapidly dividing strains will show enhanced metacyclogenesis compared to that of slowly growing strains when no adequate acidification of the medium can be attained. In our study, this was particularly the case for the faster-growing VL strains than the slower-growing CL and MCL strains. Even among the VL strains, strain BPK190 entered into stationary phase 1 day earlier than strain BPK206; thereafter, parasite numbers and viability started to decline, affecting the infectivity of the strains for macrophages. Moreover, the rate of spontaneous metacyclogenesis can still vary a lot during different subcultivation cycles; for example, for BPK206cl it was quite low (<20%) during an early passage (Fig. 3) and improved a lot (>70%) in the next passage (Table 2). Therefore, the use of spontaneously acidified stationary-phase cultures is not practical, as it requires daily monitoring for acidification and the metacyclogenesis state and may vary with every species, isolate, and even subpassage.

The first option for improvement was to trigger log-phase promastigotes to transform into amastigotes by adopting the conditioning factors that had been used for the generation of axenic amastigotes (14, 16, 18, 29). Acidification of the growth medium to pH 5.4 or elevation of the temperature from 26°C to 34°C or 37°C was sufficient to induce promastigote transformation (19, 30). The combined effect of temperature and acidity is more effective for promastigote transformation to axenic amastigotes (3, 34). However, the rounded stages obtained in our study were poorly infective for macrophages, particularly after they were conditioned at a high temperature (37°C or 34°C) (Fig. 1). It has also been suggested that a stepwise adaptation to temperature would ameliorate amastigote transformation (14), but this method would be far too time-consuming for adoption for use with field isolates. Moreover, a selection process favoring those organisms that are best adapted for in vitro transformation and growth would occur.

The second option was to condition the process of promastigote metacyclogenesis, which is triggered mainly by a drop in the external pH, which alters the timing of in vitro events by causing, first, the differentiation into nondividing metacyclics and, subsequently, entry into stationary phase earlier than might occur otherwise (6). Several authors indeed cultured promastigotes at a lower pH (1, 33) and generated more homogeneous populations of metacyclic promastigotes for multiple species (33). However, it was also observed that lowering of the pH induced an earlier entry into stationary phase and lower final viable cell densities (6). In this study, cultures were first grown under conventional conditions and by taking into account the fact that a certain synchrony could already be promoted by adapting the infection inoculum, i.e., by using a small inoculum for fast-growing strains and a larger inoculum for slow-growing strains. In this way, all VL and CL strains reached early stationary phase after about 5 days, which was considered the most appropriate time to start conditioning. In cases in which stationary phase was not yet attained, for example, with strain BPK206, enforced acidification resulted in a marked increase in the population of metacyclic promastigotes, while for those already in stationary phase (strain BPK190), a further increase could be observed (Fig. 3). Other than the enforced drop to pH 5.4, the duration of conditioning was also critical, with 24 h being the most appropriate for *L. donovani* and *L. braziliensis*. The external acid environment was apparently not sufficient to trigger the adequate differentiation of *L. guyanensis*, underlining the need for at least a minimal growth rate for successful preconditioning. Besides the drop in the external pH, the culture medium may also influence developmental changes (15). In our study, acidified MEM and Schneider's medium (pH 5.4) were compared and were able to trigger differentiation in almost similar ways, with >80% infection rates for all species except *L. guyanensis* being achieved (Table 1).

In summary, the following preconditioning protocol is proposed (Fig. 4). Promastigotes are cultured in conventional MEM until day 5 and are then transferred into Schneider's medium (pH 5.4) for 24 h at 25°C, prior to infection of macrophages at a 10:1 infection ratio. Infected macrophages are washed 12 to 24 h later to remove noninternalized promasti-

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

gotes. In the susceptibility protocol, compounds are added at this time point, and evaluation of parasite burdens is done 5 days later.

Ideally, in vitro models should consider not only the internalization of metacyclic promastigotes in the host cell but also the subsequent transformation and multiplication of the amastigotes (23). For this reason, the experimental drug PX-6518 was included as an internal reference because of its exclusive action on dividing intracellular amastigotes (22, 31). To challenge the proposed preconditioning protocol (Fig. 4), testing for susceptibility to both Sb^{V} and Sb^{III} was then performed with a larger panel of clinical field isolates. Since the treatment outcome for each isolate is known (Table 2), a preliminary assessment of the predictive value may also become possible, despite the reservations of some investigators (26). The three phenotypes previously reported were also observed in our study (26, 32): 11 isolates were Sb^{V} sensitive and Sb^{III} sensitive (the 5S3S phenotype), 4 isolates were Sb^{V} resistant and Sb^{III} sensitive (the 5R3S phenotype), and 3 isolates were resistant to both Sb^{V} and Sb^{III} (the 5R3R phenotype). Combination of the AI values for both Sb^{V} and Sb^{III} appeared to be necessary to achieve a full correspondence of susceptibility to Sb^{V} and Sb^{III} with the listed clinical profile of the listed species: AI values of $\geq$4 for both Sb^{V} and SB^{III} were always linked to relapses or no response; an AI of $\geq$4 for Sb^{V} only could still be linked to a cure. Although strains BPK091 and BPK178 were both derived from a cured patient, the AI values fell only just within the sensitive range, and it could be speculated that resistance and clinical failure may indeed ultimately develop. However, in view of the relatively low number of clinical isolates that was evaluated, particularly isolates of *L. guyanensis* and *L. braziliensis*, more-extensive studies with many more *Leishmania* species and strains are needed before a proper judgment on the predictive value of the results of in vitro susceptibility tests for treatment failure can be made.

In conclusion, the proposed conditioning protocol allows the more reproducible infection of macrophages and enhances the validity of in vitro susceptibility testing. The use of AI values for phenotypic characterization (26, 32) may allow a better comparison of the results between different series of experiments and between different laboratories. Hence, both tools are recommended for use for in vitro susceptibility testing and resistance profiling of clinical field isolates.

ACKNOWLEDGMENTS

This work was supported by grants from FWO Flanders (project G.0103.06), the University of Antwerp Research Council (grant BOF-NOI 20282), and the UNDP/World Bank/WHO Special Programme for Research and Training in Tropical Diseases. Paul Cos is a postdoctoral researcher who received a grant from the Fund for Scientific Research (FWO Flanders, Flanders, Belgium).

An Matheeussen, Kristel Kuypers, and Natascha Van Pelt performed most of the technical laboratory work.

REFERENCES

1. **Abou el Naga, I. F., and I. A. El Moneim Sharaf.** 2000. Metacyclogenesis of *Leishmania major* in an acidic medium. J. Egypt. Soc. Parasitol. **30:**431–440.
2. **Bandyopadhyay, P., D. K. Ghosh, A. De, K. N. Ghosh, P. Cheudhuri, P. Das, and A. Bhattacharya.** 1991. Metacyclogenesis of Leishmania spp: species-specific *in vitro* transformation, complement resistance, and cell surface carbohydrate and protein profiles. J. Parasitol. **77:**411–416.
3. **Barak, E., S. Amin-Spector, E. Gerliak, S. Goyard, N. Holland, and D. Zilberstein.** 2005. Differentiation of *Leishmania donovani* in host-free system: analysis of signal perception and response. Mol. Biochem. Parasitol. **141:**99–108.
4. **Basu, J. M., A. Mookerjee, P. Sen, S. Bhaumik, P. Sen, S. Banerjee, K. Naskar, S. K. Choudhuri, B. Saha, S. Raha, and S. Roy.** 2006. Sodium antimony gluconate induces generation of reactive oxygen species and nitric oxide via phosphoinositide 3-kinase and mitogen-activated protein kinase activation in *Leishmania donovani*-infected macrophages. Antimicrob. Agents Chemother. **50:**1788–1797.
5. **Bates, P. A.** 1994. Complete developmental cycle of *Leishmania mexicana* in axenic culture. Parasitology **108:**1–9.
6. **Bates, P. A., and L. Tetley.** 1993. *Leishmania mexicana*: induction of metacyclogenesis by cultivation of promastigotes at acidic pH. Exp. Parasitol. **76:**412–423.
7. **Berman, J.** 2003. Current treatment approaches to leishmaniasis. Curr. Opin. Infect. Dis. **16:**397–401.
8. **Carrió, J., M. de Colmenares, C. Riera, M. Gállego, M. Arboix, and M. Portús.** 2000. *Leishmania infantum*: stage-specific activity of pentavalent antimony related with the assay conditions. Exp. Parasitol. **95:**209–214.
9. **Croft, S. L., S. Sundar, and A. H. Fairlamb.** 2006. Drug resistance in leishmaniasis. Clin. Microbiol. Rev. **19:**111–126.
10. **Croft, S. L.** 2001. Monitoring drug resistance in leishmaniasis. Trop. Med. Int. Health **6:**899–905.
11. **Cunningham, I.** 1977. New culture medium for maintenance of tsetse tissues and growth of trypanosomatids. J. Protozool. **24:**325–329.
12. **Cysne-Finkelstein, L., R. M. Temporal, F. Aguiar Alves, and L. L. Leon.** 1998. *Leishmania amazonensis*: long-term cultivation of axenic amastigotes is associated to metacyclogenesis of promastigotes. Exp. Parasitol. **89:**58–62.
13. **Da Silva, R., and D. L. Sacks.** 1987. Metacyclogenesis is a major determinant of *Leishmania* promastigote virulence and attenuation. Infect. Immun. **55:** 2802–2806.
14. **Debrabant, A., M. B. Joshi, P. F. P. Pimenta, and D. M. Dwyer.** 2004. Generation of *Leishmania donovani* axenic amastigotes: their growth and biological characteristics. Int. J. Parasitol. **34:**205–217.
15. **Dey, T., F. Afrin, K. Anam, and N. Ali.** 2002. Infectivity and virulence of *Leishmania donovani* promastigotes: a role for media, source, and strain of parasite. J. Eukaryot. Microbiol. **49:**270–274.
16. **Ephros, M., A. Bitnun, P. Shaked, E. Waldman, and D. Zilberstein.** 1999. Stage-specific activity of pentavalent antimony against *Leishmania donovani* axenic amastigotes. Antimicrob. Agents Chemother. **43:**278–282.
17. **Gourbal, B., N. Sonuc, H. Bhattacharjee, D. Legare, S. Sundar, M. Ouellette, B. P. Rosen, and R. Mukhopadhyay.** 2004. Drug uptake and modulation of drug resistance in Leishmania by an aquaglyceroporin. J. Biol. Chem. **279:** 31010–31017.
18. **Gupta, N., N. Goyal, and A. K. Rastogi.** 2001. *In vitro* cultivation and characterization of axenic amastigotes of Leishmania. Trends Parasitol. **17:**150–153.
19. **Hunter, K. W., C. L. Cook, and S. A. Hensen.** 1982. Temperature-induced in vitro transformation of *Leishmania mexicana*. Acta Trop. **39:**143–150.
20. **Lira, R., S. Méndez, L. Carrera, C. Jaffe, F. Neva, and D. Sacks.** 1998. *Leishmania tropica*: the identification and purification of metacyclic promastigotes and use in establishing mouse and hamster models of cutaneous and visceral disease. Exp. Parasitol. **89:**331–342.
21. **Loiseau, P. M., and C. Bories.** 2006. Mechanisms of drug action and drug resistance in Leishmania as basis for therapeutic target identification and design of antileishmanial modulators. Curr. Top. Med. Chem. **6:**539–550.
22. **Maes, L., D. Vanden Berghe, N. Geromprez, L. Quirijnen, P. Cos, N. De Kimpe, and L. Van Puyvelde.** 2004. In vitro and in vivo activities of a triterpenoid saponin extract (PX-6518) from the plant *Maesa balansae* against visceral *Leishmania* species. Antimicrob. Agents Chemother. **48:**130–136.
23. **Méndez, S., M. Nell, and J. M. Alunda.** 1996. *Leishmania infantum*: infection of macrophages *in vitro* with promastigotes. Int. J. Parasitol. **26:**619–622.
24. **Murray, H. W.** 2001. Clinical and experimental advances in treatment of visceral leishmaniasis. Antimicrob. Agents Chemother. **45:**2185–2197.
25. **Natera, S., C. Machuca, M. Padrón-Nieves, A. Romero, E. Díaz, and A. Ponte-Sucre.** 2007. *Leishmania* spp.: proficiency of drug-resistant parasites. Int. J. Antimicrob. Agents **29:**637–642.
26. **Rijal, S., V. Yardley, F. Chappuis, S. Decuypere, B. Khanal, R. Singh, M. Boelaert, S. De Doncker, S. Croft, and J. C. Dujardin.** 2007. Antimonial treatment of visceral leishmaniasis: are current *in vitro* susceptibility assays adequate for prognosis of *in vivo* therapy outcome? Microbes Infect. **9:**529–535.
27. **Sacks, D. L.** 1989. Metacyclogenesis in *Leishmania* promastigotes. Exp. Parasitol. **69:**100–103.
28. **Saraiva, E. M., L. H. Pinto-da-Silva, J. L. Wanderley, A. C. Bonomo, M. A. Barcinski, and M. E. Moreira.** 2005. Flow cytometric assessment of *Leishmania* spp. metacyclic differentiation: validation by morphological features and specific markers. Exp. Parasitol. **110:**39–47.
29. **Sereno, D., M. Cavaleyra, K. Zemzoumi, S. Maquaire, A. Ouaissi, and J. L. Lemesre.** 1998. Axenically grown amastigotes of *Leishmania infantum* used as an in vitro model to investigate the pentavalent antimony mode of action. Antimicrob. Agents Chemother. **42:**3097–3102.

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009

30. **Teixeira, M. C. A., R. de Jesus Santos, R. B. Sampaio, L. Pontes-de-Carvalho, and W. L. C. dos-Santos.** 2002. A simple and reproducible method to obtain large numbers of axenic amastigotes of different *Leishmania* species. Parasitol. Res. **88:**936–968.
31. **Vermeersch, M., R. A. Inocêncio da Luz, K. Toté, J. P. Timmermans, P. Cos, and L. Maes.** 2009. In vitro susceptibilities of *Leishmania donovani* promastigote and amastigote stages to antileishmanial reference drugs: practical relevance of stage-specific differences. Antimicrob. Agents Chemother. **53:**3855–3859.
32. **Yardley, V., N. Ortuño, A. Lianos-Cuentas, F. Chappuis, S. De Donker, L. Ramirez, S. L. Croft, J. Arevalo, V. Adaui, H. Bermudez, S. Decuypere, and J. C. Dujardin.** 2006. American tegumentary leishmaniasis: is antimonial treatment outcome related to parasite drug susceptibility? J. Infect. Dis. **194:**1168–1175.
33. **Zakai, H. A., M. L. Chance, and P. A. Bates.** 1998. *In vitro* stimulation of metacyclogenesis in *Leishmania braziliensis*, *L. donovani*, *L. major* and *L. mexicana*. Parasitology **116:**305–309.
34. **Zilberstein, D.** 1994. The role of pH and temperature in the development of *Leishmania* parasites. Annu. Rev. Microbiol. **48:**449–470.

Downloaded from aac.asm.org at INSTITUTE OF TROPICAL MEDICINE on November 23, 2009