Tropical Medicine and International Health doi:10.1111/j.1365-3156.2009.02414.x

VOLUME 15 NO 1 PP 68–76 JANUARY 2010

In vitro sensitivity of *Leishmania* (*Viannia*) *braziliensis* and *Leishmania* (*Leishmania*) *amazonensis* Brazilian isolates to meglumine antimoniate and amphotericin B

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Summary Resistance of *Leishmania* parasites to specific chemotherapy has become a well-documented problem in the Indian subcontinent in recent years but only a few studies have focused on the susceptibility of American *Leishmania* isolates. Our susceptibility assays to meglumine antimoniate were performed against intracellular amastigotes after standardizing an *in vitro* model of macrophage infection appropriate for *Leishmania (Viannia) braziliensis* isolates. For the determination of promastigote susceptibility to amphotericin B, we developed a simplified MTT-test. The sensitivity *in vitro* to meglumine antimoniate and amphotericin B of 13 isolates obtained from Brazilian patients was determined. *L. (V.) braziliensis* isolates were more susceptible to meglumine antimoniate than *Leishmania (Leishmania) amazonensis*. EC_{50}, EC_{90} and activity indexes (calculated over the sensitivity of reference strains), suggested that all isolates tested were susceptible *in vitro* to meglumine antimoniate, and did not show association with the clinical outcomes. Isolates were also uniformly susceptible *in vitro* to amphotericin B.

keywords tegumentary leishmaniasis, meglumine antimoniate, amphotericin B, sensitivity

Introduction

Leishmaniasis is a complex disease with cutaneous, diffuse, mucosal and visceral clinical manifestations. The infection is caused by protozoan parasites of the *Leishmania* genus, transmitted by the bite of phlebotomine sandflies (Murray *et al.* 2005). The disease affects more than 12 million people in 88 countries worldwide (World Health Organization). In Brazil, cases of leishmaniasis are registered in all geographic regions and, in the last decade, several reports of epidemic outbreaks in urban areas have emerged (Jeronimo *et al.* 1994; Silva *et al.* 2001; Oliveira *et al.* 2004).

Leishmania (*Viannia*) *braziliensis* is the most common species in the Americas and the most important causative agent of cutaneous and mucocutaneous leishmaniasis in Brazil, while *Leishmania* (*Leishmania*) *amazonensis* is the primary etiologic agent of the diffuse cutaneous form of the disease (Lainson & Shaw 1998).

The therapeutic arsenal routinely employed to treat patients with leishmaniasis is limited and unsatisfactory. For cutaneous leishmaniasis, pentavalent antimonials (sodium stibogluconate or meglumine antimoniate) at 20 mg/kg/day for 20–28 consecutive days are the first line therapeutic scheme recommended by WHO. These compounds are highly toxic, poorly tolerated and their effectiveness highly variable. For example, a study comparing the response to meglumine antimoniate in Brazilian patients infected with either *L.* (*V.*) *braziliensis* or *L.* (*V.*) *guyanensis* found clinical failure rates after the first treatment course of approximately 60 and 30% in *L.* (*V.*) *braziliensis* and *L.* (*V.*) *guyanensis* patients, respectively (Romero *et al.* 2001). A recently published meta-analysis of New World cutaneous leishmaniasis treatment found a mean cure rate of 71.3% in 310 Brazilian patients infected with *L.* (*V.*) *braziliensis* (Tuon *et al.* 2008).

Successful leishmaniasis treatment depends on several aspects such as the immune status of the host, clinical manifestations of the disease and the sensitivity of the causative *Leishmania*. Widespread resistance to pentavalent antimonials has been identified for *Leishmania* (*L.*) *donovani* in some regions of India and Nepal (Sundar *et al.* 2000; Rijal *et al.* 2003). However, only a few studies correlated clinical response with *in vitro* antimony

sensitivity in the New World (Grogl *et al.* 1992; Robledo *et al.* 1999; Rojas *et al.* 2006; Yardley *et al.* 2006). Up to now, only one study, based mainly on drug activity against axenic promastigotes, examined the *in vitro* sensitivity of isolates to meglumine antimoniate in Brazil (Azeredo-Coutinho *et al.* 2007).

The second choice treatment is based on the administration of amphotericin B (AmB). This polyene antibiotic with strong antifungal activity is highly toxic and one of the major concerns on its use is nephrotoxicity and consequent renal failure (Croft & Coombs 2003). Data on *in vitro* sensitivity of clinical isolates to AmB is scarce. Nevertheless, a few reports of AmB therapeutic failure in visceral leishmaniasis patients (Mueller *et al.* 2007; Kumar *et al.* 2009), as well as the *in vitro* isolation of AmB-insensitive parasite lines (Al-Mohammed *et al.* 2005), suggests that resistance to the drug can also arise.

It is therefore evident that new studies must be conducted to evaluate the sensitivity of parasites to the major drugs currently used to treat Brazilian patients infected with *Leishmania*. A macrophage model of infection for *L. (V.) braziliensis* was established and used to determine the antimony sensitivity of 13 isolates, obtained from Brazilian patients with tegumentary leishmaniasis. The sensitivity of these isolates to AmB was also established.

Materials and methods

Parasites

Thirteen isolates of *Leishmania* were obtained from patients attending the Anuar Auad Tropical Diseases Hospital in the city of Goiânia, Goiás, Brazil. Parasite isolation was obtained from lesion biopsies performed as part of the diagnostic procedure. Skin biopsies were homogenised and inoculated into Grace's medium (Sigma-Aldrich Chem. Co., St Louis, MO, USA) supplemented with 20% fetal calf serum (FCS) and into γ-interferon knockout mice (M.A.P. Oliveira, unpublished data). The isolates were typed by PCR of ribosomal DNA and glucose-6-phosphate dehydrogenase gene as described (Uliana *et al.* 1994; Castilho *et al.* 2003) and identified as *L.* (*V.*) *braziliensis* or *L.* (*L.*) *amazonensis*. Soon after isolation, cultures were frozen and further tests were performed with freshly recovered cultures, with a low passage number (third to seventh). The patients came from different States in Brazil: Goiás (n = 8, BES6, EFSF6, GDL6, IMG3, TMB6, WSS5, EGS4, JRS1), Tocantins (n = 2, HPV6, UAF6), Pará (n = 2, RPL5, JSC6) and Bahia (n = 1, PPS6m). Of the 13 patients, only one presented mucosal lesions and reported 20 years of disease progression. The remaining 12 patients had cutaneous lesions. Nine patients had not been treated before parasite isolation and were monitored during and after treatment with meglumine antimoniate. One patient had received one course of meglumine antimoniate before the skin biopsy but had not responded to treatment. Three patients had no record of specific chemotherapy before parasite isolation but did not return for treatment (Table 1).

The study was approved by the Ethical Committee on Human and Animal Research, Hospital das Clínicas, Universidade Federal de Goiás.

Promastigotes of *L.* (*V.*) *braziliensis* (MHOM/BR/75/M2903) and *L.* (*L.*) *amazonensis* (MHOM/BR/73/M2269) reference strains and of the isolates were grown in M199 liquid medium (Sigma-Aldrich) supplemented with 10% fetal calf serum (FCS; Invitrogen) and incubated at 25 °C. *L.* (*V.*) *braziliensis* cultures were supplemented with 2% sterile male human urine.

Identification of isolates

Soon after recovery from nitrogen stocks, DNA was purified from the cultures as described (Medina-Acosta & Cross 1993) and submitted to a PCR assay targeting the *META2* gene open reading frame. The pair of primers LbM2a (5′ ATGTCCACTGCCGACGACAT 3′) and LbM2b (5′ CTAAATAGGCTCAGCGTCGT 3′) was used to amplify a 1.3 kb fragment. PCR amplification was carried out for 30 cycles of 60 s at 94 °C, 60 s at 52 °C and 60 s at 72 °C followed by a 10 min elongation at 72 °C. *Xho*I digestion of the amplified product allowed the differentiation by restriction polymorphism between *L.* (*L.*) *amazonensis* and *L.* (*V.*) *braziliensis*.

Drugs

Drugs used in the present study were meglumine antimoniate (Glucantime®, Sanofi-Aventis) and amphotericin B deoxycholate (AmB, Sigma-Aldrich). Meglumine antimoniate was stored at 4 °C and diluted at the time of incubation in RPMI 1640 medium (Invitrogen). Stock solutions of AmB (10 mM) were prepared in sterile water and kept at −20 °C.

In vitro infectivity of Leishmania isolates and susceptibility of intracellular amastigotes to drugs

The following types of macrophages were used in this study in order to establish an efficient model of infection by *L.* (*V.*) *braziliensis in vitro*: J774 A.1 and THP-1 lineages and BALB/c mice peritoneal (PM) or bone marrow-derived

Table 1 Activity of pentavalent antimony and amphotericin B against *Leishmania* isolates

Isolate[a]	Time of lesion[b]	Clinical form[c]	Previous treatment[d]	Treatment[e]	Clinical cure[f]	Infection rate (%)[g]	EC_{50} Sb^v (μg/ml)[h]	EC_{90} Sb^v (μg/ml)	AI[i]	EC_{50} AmB (ng/ml)[j]	EC_{90} AmB (ng/ml)	AI[i]
MHOM/BR/2006/BES^{Lb}	8 m	LC	G	G, A	No	33.0 ± 4.6	52.4 ± 5.3	203.3	1.5 ± 0.26	46 ± 1	360	0.78 ± 0.04
MHOM/BR/2006/EFSF^{Lb}	3 m	LC	None	G	Yes	33.0 ± 3.6	55.0 ± 12.2	150.2	1.6 ± 0.67	55 ± 5	650	0.85 ± 0.05
MHOM/BR/2006/GDL^{Lb}	8 m	LC	None	G	Yes	40.0 ± 2.0	31.1 ± 1.5	96.0	0.9 ± 0.06	46 ± 1	170	0.91 ± 0.08
MHOM/BR/2006/HPV^{Lb}	3 m	LC	None	G	Yes	51.0 ± 7.0	58.0 ± 13.8	299.7	1.7 ± 0.76	92 ± 4	170	1.63 ± 0.07
MHOM/BR/2003/IMG^{Lb}	1.5 m	LC	None	G	Yes	54.0 ± 19.0	58.8 ± 5.4	175.8	1.7 ± 0.27	64 ± 3	230	1.46 ± 0.22
MHOM/BR/2006/PPSm^{Lb}	20 y	MC	None	NR	NR	20.0 ± 0.5	18.5 ± 4.2	96.0	0.54 ± 0.23	36 ± 4	430	0.70 ± 0.13
MHOM/BR/2005/RPL^{Lb}	3 m	LC	None	G	Yes	85.0 ± 12.0	59.8 ± 3.2	136.2	1.7 ± 0.14	55 ± 3	110	0.86 ± 0.03
MHOM/BR/2006/TMB^{Lb}	3 m	LC	None	G	Yes	50.0 ± 10.0	29.1 ± 4.6	65.6	0.85 ± 0.25	46 ± 1	80	0.66 ± 0.01
MHOM/BR/2006/UAF^{Lb}	2 m	LC	None	G/G	No	59.0 ± 0.7	31.6 ± 9.7	148.7	0.92 ± 0.54	64 ± 1	120	1.06 ± 0.05
MHOM/BR/2005/WSS^{Lb}	8 m	LC	None	NR	NR	77.0 ± 5.6	44.8 ± 2.3	124.8	1.31 ± 0.09	64 ± 5	120	0.79 ± 0.04
MHOM/BR/2004/EGS^{La}	2 m	LC	None	NR	NR	99.0 ± 1.0	112.4 ± 3.2	238.7	2.05 ± 0.30	83 ± 6	250	1.04 ± 0.11
MHOM/BR/2001/JRS^{La}	4 m	DC	None	G	Yes	99.0 ± 1.0	112.2 ± 3.6	197.7	2.05 ± 0.28	55 ± 1	210	0.72 ± 0.01
MHOM/BR/2006/JSC^{La}	1.5 m	LC	None	G	Yes	99.0 ± 1.0	146.0 ± 5.0	296.8	2.67 ± 0.35	64 ± 1	160	0.77 ± 0.01

[a] *L. (V.) braziliensis*: ^{Lb}, *L. (L.) amazonensis*: ^{La};
[b] m = months, y = years;
[c] LC = localized cutaneous, MC = mucocutaneous, DC = disseminated cutaneous;
[d] Treatment before the isolation of strain;
[e] Treatment after the isolation of strain: (G) meglumine antimoniate 20 mg Sb^v/day for 30 days, (G/G) 2 courses of 20 mg Sb^v/day for 30 days, (A) AmB;
[f] Clinical cure: cure after meglumine antimoniate treatment; NR = patient did not return for follow up;
[g] Inhibitory concentrations against intracellular amastigotes: EC_{50} ± SD (n = 6). Values are expressed as μg/mL of pentavalent antimony [Sb^V];
[h] Percentage of infected cells ± SD (n = 6);
[i] AI = activity index (ratio between the EC_{50} of the isolate and EC_{50} of the reference strain);
[j] Inhibitory concentrations (EC_{50}) ± SD (n = 6) against promastigotes.

macrophages (BMDM). J774 A.1 and THP-1 cell lines were cultured at 37 °C in the presence of 5% CO_2 in RPMI 1640 medium supplemented with 10% FCS. Resident PM were obtained by peritoneal lavage with 3–5 ml of RPMI 1640 medium repeated three times. BMDM were obtained from BALB/c mice as described by Zamboni and Rabinovitch (2003). PM or BMDM were counted and distributed in 24-well plates on round coverslips (3×10^5 macrophages per well) containing RPMI 1640 medium with 10% FCS (and 5% L929 cell conditioned medium for BMDM) and allowed to adhere overnight at 37 °C, at 5% CO_2. Infections, for all macrophage types, were performed using ratios of 15:1 *L.* (*V.*) *braziliensis* stationary-phase promastigotes per macrophage. Infected macrophage cultures were kept at 33 °C, 5% CO_2 for 3 h in RPMI 1640 medium with 10% FCS and then washed twice with sterile PBS to remove free promastigotes. After 48 h of incubation, slides were fixed in methanol and stained with the Instant Prov kit (Newprov, Pinhais-Paraná, Brazil) for subsequent quantification of the infected cells under light microscopy.

BALB/c BMDM were used to evaluate the *in vitro* activity of meglumine antimoniate. Drug cytotoxicity to host cells was determined as previously described (Miguel *et al.* 2007). Macrophages were infected with reference and isolates of *L.* (*V.*) *braziliensis* (10:1) or *L.* (*L.*) *amazonensis* (10:1) for 3–4 h at 33 °C in an atmosphere of 5% CO_2 and then kept at 33 °C for 6 days. Treatment of infected BMDM with meglumine antimoniate (13.5, 40.5, 135 and 405 μg/ml) was performed for 6 days with an intermediate replacement of medium and drug in the third day. The number of infected macrophages/100 cells and the number of amastigotes/infected cell were counted in triplicate coverslips for each drug concentration and used to determine the 50% and 90% effective concentrations (EC_{50} and EC_{90}).

In vitro susceptibility of promastigotes to drugs

Promastigote drug susceptibility assays were performed in 96-well plates in triplicates. Approximately 3×10^6 late log-phase parasites were distributed per well, incubated with different concentrations of AmB (46, 138, 460 and 1380 ng/ml) and kept at 25 °C for 24 h. After the incubation period, 30 μl of 5 mg/ml MTT (3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide; Sigma-Aldrich) was added and cultures were kept at 25 °C for 2 h. The reaction was stopped by the addition of 50 μl 20% sodium dodecyl sulfate to each well. MTT cleavage was assessed in a multiwell scanning spectrophotometer (Labsystems; Multiskan EX) with a reference wavelength of 690 nm and a test wavelength of 595 nm. Assays were performed in triplicate and results expressed as the mean percentage reduction of parasite numbers compared with untreated control wells calculated for at least three independent experiments. The EC_{50}, EC_{90} and the activity index (AI) were determined as described below.

Statistical analyses

EC_{50} and EC_{90} were determined from sigmoidal regression of the concentration-response curves using Software ORIGIN 7.5. The AI, as defined by Yardley *et al.* (2006), was used to allow comparisons between results from different series of experiments and was obtained using the following formula: EC_{50} isolate/EC_{50} reference strain. Experiments were repeated at least 3 times and the mean and standard deviation of the AI for each isolate was calculated. To verify whether there were differences in EC_{50} among isolates, Mann–Whitney analysis was used (GraphPad Prism). Statistical significance was established at $P < 0.05$.

Results

Identification of *Leishmania* isolates

The PCR molecular technique has been shown to be highly effective in the detection and identification of *Leishmania* parasites (Reithinger & Dujardin 2007). To keep track of the cultures and assure proper handling of the isolates, a new typing method was employed. Comparison of the nucleotide sequence of the *META 2* gene in *L.* (*L.*) *amazonensis* (Ramos *et al.* 2004) and *L.* (*V.*) *braziliensis* (available at http://www.genedb.org) revealed the existence of restriction sites for the enzyme *Xho*I which allows the differentiation of these two species (Figure 1a). Amplification of the *META 2* open reading frame followed by analysis of restriction polymorphism confirmed the typing of the isolates BES6, EFSF6, GDL6, HPV6, IMG3, PPS6m, RPL5, TMB6, UAF6 and WSS5 as *L.* (*V.*) *braziliensis* (Figure 1b,d). Isolates EGS4, JRS1 and JSC6 were identified as *L.* (*L.*) *amazonensis* (Figure 1c,e).

Infectivity of L. (V.) braziliensis to different types of macrophages

As pentavalent antimony (Sb^v) must be converted by the host cell to its trivalent form to be fully active against *Leishmania* (Croft *et al.* 2006), the activity of meglumine antimoniate was tested against intracellular amastigotes. Experimental infections with *L.* (*V.*) *braziliensis* are usually referred to as a difficult model to evaluate the efficacy of antileishmanial compounds *in vitro* once parasites of this species exhibit low infectivity to macrophages *in vitro* and

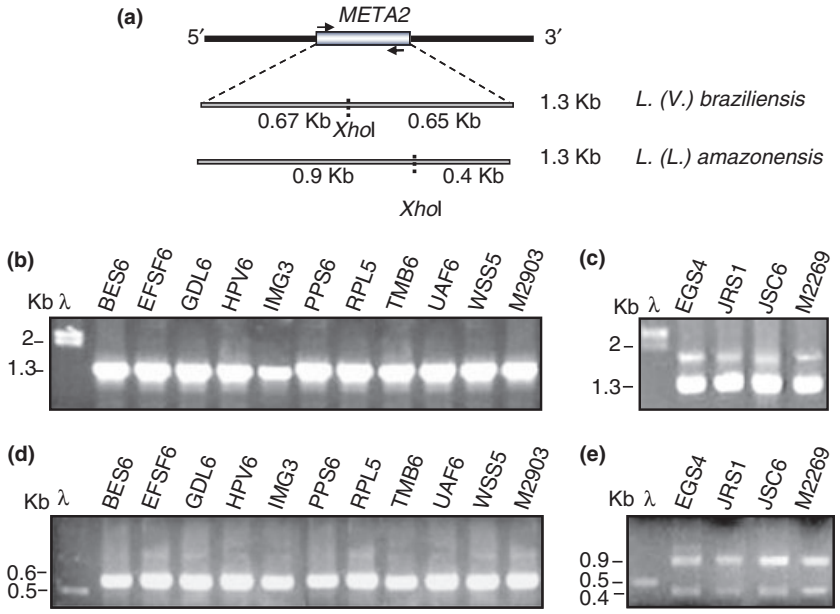

Figure 1 Identification of *Leishmania* isolates by *META2* gene amplification. (a) Schematic representation of the *META2* genes and the position of *Xho*I restriction sites. (b and c) PCR amplification of the 1.3 kb META2 ORF from genomic DNA of the isolates. (d and e) Restriction fragment analysis of the amplified product, after digestion with *Xho*I. Ethidium bromide stained 2.0% agarose gels.

infected host cells exhibit small parasitophorous vacuoles with one or a small number of amastigotes per vacuole. Prior to investigating the *in vitro* sensitivity to meglumine antimoniate of *Leishmania* isolates (mostly typed as *L.* (*V.*) *braziliensis*) we had to establish an effective assay for detecting drug activity against intracellular amastigotes in this model. We evaluated the susceptibility of four different types of macrophages to infection with the *L.* (*V.*) *braziliensis* reference strain. After 48 hours, THP-1, J774.A1 and PM presented low rates of infection, of approximately 10, 18 and 15%, respectively. On the other hand, BALB/c BMDM showed an infection rate of 84% (Figure 2a). The microscopic evaluation of the various types of infected macrophages also showed that the morphology of infected BMDM, with their expanded cytoplasm, was appropriate for the observation of amastigotes (Figure 2b). BMDM were then chosen as the model of infection for the susceptibility study.

In vitro susceptibility to pentavalent antimony and AmB

The rate of macrophage infection for the different isolates was evaluated by determining the percentage of infected macrophages and the number of amastigotes per macrophage in control groups, kept without drug. Infection rates ranged between 19.5 and 97.0% with an average number of five amastigotes per macrophage for *L.* (*V.*) *braziliensis* isolates and between 98 and 100% for *L.* (*L.*) *amazonensis* isolates, with approximately 11 amastigotes per macrophage (Table 1). In all experiments, the reference strain was tested in parallel with the isolates. The EC_{50} for the reference strains in different experiments ranged from 29.1 ± 3.6 to 39.1 ± 3.9 μg/ml for *L. (V.) braziliensis* and 49.1 ± 4.2 and 60.1 ± 5.7 μg/ml for *L. (L.) amazonensis*. Susceptibility assays were performed in triplicate for each isolate and experiments were repeated at least twice. It is important to mention that meglumine antimoniate's cytotoxicity was evaluated in parallel and the cytotoxic concentration for 50% of cells (CC_{50}) was greater than 810 μg/ml of Sb^v. Therefore, the doses used in our study were not toxic to macrophages.

The Sb^v EC_{50} for *L. (V.) braziliensis* isolates ranged from 18.5 ± 4.2 to 59.8 ± 3.2 μg/ml while values between 112.2 ± 3.6 and 146.0 ± 5.0 μg/ml were obtained for *L.* (*L.*) *amazonensis* isolates (Table 1). So, for the isolates studied here, *L.* (*V.*) *braziliensis* cultures were significantly more sensitive to antimonials than *L. (L.) amazonensis* (Mann–Whitney, $P = 0.007$). On the other hand, the values for Sb^v EC_{90} showed a wide distribution between *L. (V.) braziliensis* isolates, some of which with EC_{90} very similar to *L.* (*L.*) *amazonensis* parasites (for example, 299.7 μg/ml for HPV6 and 296.8 μg/ml for JSC6). For EC_{90} values, there were no significant differences between *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* isolates.

The AIs for *L.* (*V.*) *braziliensis* isolates varied between 0.54 ± 0.23 and 1.7 ± 0.76. Therefore, both EC_{50} and AI values indicated similar *in vitro* sensitivity of the test strains to meglumine antimoniate as compared with the reference strain. AIs for *L.* (*L.*) *amazonensis* isolates ranged between 2.05 ± 0.30 and 2.67 ± 0.35.

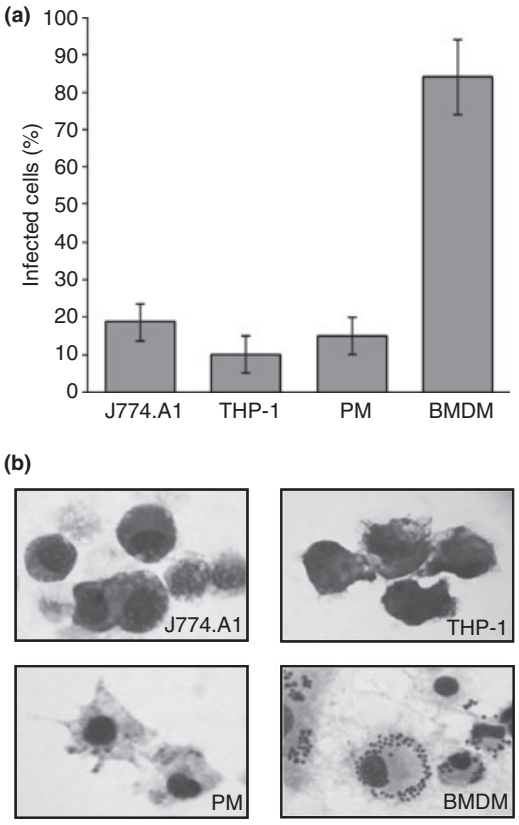

Figure 2 Susceptibility of macrophages to infection with *L. (V.) braziliensis*. J774.A1, THP-1, PM or BMDM were infected with stationary phase promastigotes of *L. (V.) braziliensis* reference strain (MHOM/BR/75/M2903), using a ratio of 15 parasites/macrophage for 3 h. After 48 h incubation, slides were fixed and stained. (a) Percentage of infected cells, evaluated in triplicate coverslips and in two independent experiments expressed as the mean ± SD. (b) Morphology of infected cells (1000 ×).

Since the susceptibility of *Leishmania* promastigotes and intracellular amastigotes to AmB is reportedly similar (Escobar *et al.* 2002; Sharief *et al.* 2006), we assessed the sensitivity of promastigote cultures of all isolates to the drug using a modified MTT test. The reference strains were included in all experiments. AmB EC_{50} for the reference strains in different experiments varied from 36 ± 4 to 92 ± 4 ng/ml for *L. (V.) braziliensis* and 55 ± 1 and 83 ± 6 ng/ml for *L. (L.) amazonensis*. AI values ranged between 0.66 ± 0.01 and 1.63 ± 0.07, revealing homogeneous susceptibility of all tested isolates. No significant differences were found between *L.* (*V.*) *braziliensis* and *L.* (*L.*) *amazonensis* isolates regarding susceptibility to AmB.

Of 10 patients with treatment follow up, two did not respond well to the first course of meglumine antimoniate (20 mg/kg/day for 20 days). One (UAF6) was treated with a second course of antimonial, which healed the lesions. The isolate from the second patient (BES6) was obtained after the first course of treatment, to which there was only a partial response. The second course of meglumine antimoniate was then administered, with clinical improvement. However, new skin lesions were present 2 months later and the patient was successfully treated with AmB. The isolates from these 2 patients were sensitive *in vitro* to meglumine antimoniate (EC_{50} of 31.6 ± 9.7 and 52.4 ± 5.3 μg/ml and AI of 0.92 ± 0.54 and 1.5 ± 0.26, respectively) suggesting, for these two isolates, no correlation between *in vitro* tests and response to treatment.

In this series, there was only one patient with mucosal leishmaniasis and a long time of disease progression, but without record of previous treatment. From this patient, an antimonial susceptible *L.* (*V.*) *braziliensis* isolate (PPS6m) was obtained, with EC_{50} of 18.5 ± 4.2 μg/ml and AI of 0.54 ± 0.23. Response to treatment, however, could not be documented (Table 1).

Discussion

Treatment outcome with antimony has been shown to be influenced by the causative species, with a high rate of success registered for *L.* (*V.*) *braziliensis* infections in Guatemala (Navin *et al.* 1992). However, one has to keep in mind the great intraspecific genetic heterogeneity among *L. (V.) braziliensis* (Cupolillo *et al.* 2003). Sensitivity to Sb^v *in vitro* varies depending on the *Leishmania* species (Croft *et al.* 2006). The now well-established primary resistance of *L.* (*L.*) *donovani* in India was initially documented as a progressively more frequent refractoriness to clinical treatment with antimonials. Keeping in mind that treatment failure in leishmaniasis can be ascribed to host factors, as well as to parasite resistance, it is a cause for concern that treatment failure for *L. (V.) braziliensis* infections can be as high as 29% in Brazil (Tuon *et al.* 2008).

Data available in the literature about *Leishmania* antimonial drug resistance is often difficult to generalize, once different experimental conditions and evaluation parameters are used. In this study, we chose to test antimonial susceptibility against the intracellular amastigote stage and reported the results on the basis of EC_{50}/EC_{90} as well as AI, to standardize data between experiments as previously done for the evaluation of Peruvian isolates (Yardley *et al.* 2006).

Azeredo-Coutinho *et al.* (2007) established EC_{50} values ranging from 19 and 55 μg/ml of Sb^v for *L. (V.) braziliensis* isolates obtained from patients in Rio de Janeiro, Brazil. These values are on the same order of magnitude as

those found in our work. In that study, isolates from patients who responded to treatment had significantly lower EC_{50} than those that were poorly responsive to therapy. This correlation between *in vitro* sensitivity and clinical response to Sb^v was not verified in our study. Only one patient (BES6) showed no clinical improvement after receiving two courses of treatment with Sb^v. In this case, the EC_{50} value was similar to other isolates of *L.* (*V.*) *braziliensis* and up to three times lower than the EC_{50} value determined for isolates of *L.* (*L.*) *amazonensis*. This patient was clinically cured after treatment with AmB (Table 1).

Resistance to Sb^v *in vitro* (defined as an AI >6) was verified in approximately 85% of *L. (V.) braziliensis* Peruvian isolates tested, but no significant association was found between *in vitro* sensitivity and clinical outcome (Yardley *et al.* 2006). Two of three *L. (V.) braziliensis* isolates studied in Colombia were classified as resistant (EC_{50} values higher than 128 μg/ml of Sb^v) and found to be able to develop secondary resistance (Rojas *et al.* 2006). Interestingly, for *L. (V.) braziliensis* isolates in our study, the highest AI was 1.7 while the maximum EC_{50} was 59.8 ± 3.2 μg/ml of Sb^v. Our sample was composed of isolates from spread out geographical origins, as were the isolates tested by Yardley *et al.* (2006). Data from our study and from the analysis of parasites isolated in Rio de Janeiro (Azeredo-Coutinho *et al.* 2007) would suggest that *L. (V.) braziliensis* isolates in Brazil are susceptible *in vitro* to meglumine antimoniate. Obviously, a larger number of isolates is necessary to confirm this observation.

This is the first report on the analysis of *in vitro* susceptibility of *L.* (*V.*) *braziliensis* and *L. (L.) amazonensis* isolates to AmB. *Leishmania* sensitivity to AmB also seems to be dependent upon the species (Escobar *et al.* 2002): EC_{50} between 0.50 and 0.96 μg/ml were determined for *L.* (*L.*) *major* isolates (Yardley & Croft 1997), and between 0.31 and 1.62 μg/ml for *L.* (*L.*) *donovani* isolates (Kumar *et al.* 2009). In our study, the highest AmB EC_{50} determined for an isolate was 0.092 ± 0.004 μg/ml for *L. (V.) braziliensis* and 0.083 ± 0.006 μg/ml for *L. (L.) amazonensis*. This could indicate a higher sensitivity of these two species to AmB as compared with *L.* (*L.*) *donovani* and *L. (L.) major*. Testing of the different species under the same experimental conditions would be required to confirm this hypothesis. There is some evidence towards the occurrence of decreased susceptibility *in vitro* of *L.* (*L.*) *infantum* isolates from patients submitted to several courses of treatment (Di Giorgio *et al.* 1999). On the other hand, sensitivity of isolates obtained over time from HIV-*Leishmania* co-infected patients submitted to several courses of treatment with AmB did not seem to change (Lachaud *et al.* 2009). In our study, the susceptibility to AmB was similar among all isolates of *Leishmania* evaluated.

In Latin America reports of effective treatment of cutaneous leishmaniasis with meglumine antimoniate have shown divergent outcomes with 10% treatment failure in Bolivia (Bermúdez *et al.* 2006), up to 39% in Colombia (Palacios *et al.* 2001) and 29% in Brazil (Tuon *et al.* 2008). We did not find evidence that treatment failure in Brazil is due to parasite primary resistance. A study with a larger number of isolates will be essential to validate these findings and evaluate the activity of drugs routinely used in the treatment of leishmaniasis. These data could boost the search for novel chemotherapeutic compounds and would consist in a valuable tool in the research focused in explaining therapeutic failure in many areas where leishmaniasis ravages communities, generally in countries where this has become a neglected public health problem.

Acknowledgements

We thank Dr Lucile M. Floeter-Winter for assistance with the isolates' identification and Dr. Jeffrey J. Shaw for helpful comments and discussions. This work has received financial support from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). R.C.Z-N. and D.C.M. were recipients of CNPq and FAPESP fellowships, respectively.

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