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Effect of glucantime on field and patient isolates of New World *Leishmania*: use of growth parameters of promastigotes to assess antimony susceptibility

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Abstract The effect of pentavalent meglumine antimoniate (glucantime) on the growth kinetics of promastigotes of 13 South American *Leishmania* strains isolated from patients, sylvatic reservoirs, and vectors was studied. Four of five *L.* (*Viannia*) *braziliensis* human isolates were obtained from drug-responsive patients and one was isolated from an unresponsive mucocutaneous-type infection. Studies involved the cell yield at the late log phase, the growth rate, and the growth-curve patterns of promastigotes in vitro. Restriction-fragment-length polymorphism, pulsed-field gel electrophoresis, and hybridization with the gene coding for a P-glycoprotein from *L.* (*V.*) *guyanensis* were used in an attempt to correlate the resistance phenotype with gene amplification. Consistent differences observed in both cell yield and growth rate among the isolates in the presence of glucantime indicated these parameters to be good criteria for the estimation of susceptibility to glucantime. Drug susceptibility varied widely between strains, indicating a great genetic heterogeneity of the isolates. Five *L.* (*V.*) *braziliensis* strains and three *L.* (*V.*) *guyanensis* strains were shown to be susceptible to glucantime. Five isolates were resistant, four of which were obtained from sylvatic vectors and one, from a patient with an unresponsive mucocutaneous infection. Molecular analyses of total DNA indicated the presence of a *pgpA*-related gene in all strains tested. No amplified sequence could be detected, suggesting that *pgpA* amplification is not involved in glucantime resistance in these wild *Leishmania* strains.

Introduction

Leishmania spp. are the causal agents of leishmaniasis, a disease distributed worldwide. Infections in humans lead to self-limited cutaneous or visceral diseases, and the latter is usually fatal if left untreated. The treatment for all forms of leishmaniasis relies mainly on the pentavalent antimonial-containing drugs stibogluconate sodium (pentostam) and meglumine antimoniate (glucantime; Olliaro and Bryceson 1993; Croft et al. 1997). Chemotherapeutic regimens for treatment have not taken into account the specific susceptibility of the parasite to the drug. *Leishmania* (*Viannia*) *braziliensis* is the species responsible for most mucocutaneous diseases in South America (Lainson 1983) and frequently shows resistance to conventional short-duration treatment or low-dose levels of antimony. Animal and cell-culture models for testing of drug susceptibility in several *Leishmania* spp. are not appropiate for *L.* (*V.*) *braziliensis* strains. A test system using promastigotes would be ideal because of the easiness of culturing these stages. Studies on the susceptibility of *Leishmania* promastigotes have been hampered by the view that promastigotes are less susceptible to antimonials than are amastigotes within macrophages (Berman and Wyler 1980; Berman et al. 1985; Croft 1986). However, Berman et al. (1989) demonstrated that amastigotes and promastigotes showed similar sensitivity to antimonials under identical growth conditions. Other groups employing different strains and conditions have demonstrated the susceptibility of promastigotes to pentostam (Grogl et al. 1989; Ullman et al. 1989; Jackson et al. 1990; Ibrahim et al. 1994; Roberts et al. 1995) or glucantime (Moreira and Petrillo-Peixoto 1991; Moreira et al. 1992). A susceptibility test based on the growth of promastigotes should take into account drug effects on promastigote growth that include the diversity among *Leishmania* strains in parameters such as cell density, growth rate, and growth-response profile along with the possible alterations induced by the drug.

The widespread clinical failures reported for visceral and mucocutaneous leishmaniasis pose serious public health challenges (Grogl et al. 1992; Ouellette and Papadopoulou 1993). Although pharmacokinetic, immunological, or physiological characteristics of the host

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have been proposed to explain the diverse clinical response, some evidence favors the assumption that inherent resistance of the parasite might contribute to the lack of response to pentavalent antimonials. In vitro studies have shown that *Leishmania* can develop resistance when cultured in the presence of pentavalent antimonials (Grogl et al. 1989; Ferreira-Pinto et al. 1996; Ephros et al. 1997) and that sensitivity to antimony correlates with the in vitro response to the treatment (Verdejo et al. 1988; Jackson et al. 1990; Faraut-Gambarelli et al. 1997). Antimonial resistance may involve a decrease in the accumulation of drug mediated by P-glycoprotein (Callahan et al. 1994; Borst and Ouellette 1995; Ullman 1995). Among the genes encoding members of the P-glycoprotein family (*pgp*), *ltpgpA* and *lmpgpA* are frequently amplified as an extrachromosomal element in resistant laboratory strains (Ellenberger and Beverley 1989; Ouellette et al. 1990). Evidence for the contribution of *pgpA* to the multidrug resistance phenotype and oxyanion resistance in *Leishmania* has been demonstrated (Callahan and Beverley 1991; Grondin et al. 1993; Dey et al. 1994; Papadopoulou et al. 1994, 1996; Ferreira-Pinto et al. 1996). Nevertheless, little is known about the biochemical alterations or molecular mechanisms by which parasites become resistant or refractory to drugs in the field (Borst and Ouellette 1995; Ullman 1995).

The present in vitro study investigates the effects of glucantime on the growth of promastigotes of several clinical and field isolates of *Leishmania* as a criterion for the assessment of susceptibility. Restriction-fragment-length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE), and hybridization with *lgpgpA* probe were employed to verify the possible involvement of *pgp*A gene amplification in the glucantime resistance of *Leishmania*.

Materials and methods

Strains and growth conditions

The following *Leishmania* strains were used: *L.* (*Viannia*) *braziliensis* strains MHOM/BR/LTB260, MHOM/BR/LTB299, MHOM/BR/LTB320, MHOM/BR/LTB259, MHOM/BR/75/M2903, MNAS/BR/85/M9554, and ISQM/BR/85/M9947; *L.* (*V.*) *guyanensis* strains IUMB/BR/85/M9957, IUMB/BR/85/M9945, and MHOM/BR/70/M1176; and *L.* (*L.*) *amazonensis* strains IFLA/BR/67/PH8, IFLA/BR/86/M10995, and IFLA/BR/86/M10996. Strains LTB320, LTB299, and LTB260 were isolated from patients with cutaneous leishmaniasis who had shown a good response after treatment with one course (17 mg/kg for 10 days, limited follow-up), two series (three glucantime ampules daily for 11 days), and three series (four glucantime ampules daily for 10 days), respectively. Strain LTB259 was isolated from an unresponsive patient (treated 10 years before with more than 600 ampules of glucantime). Strains were cryopreserved in liquid nitrogen and also maintained by serial passage at the log phase at 25 °C in a complex liquid medium (5.7 g liver-brain infusion, 5.7 g tryptose, 8.5 g NaCl, 0.5 g KCl, 9.0 g Na_2HPO_4, 2.0 g glucose per liter. pH 7.0 – after sterilization, 40% hemolyzed rabbit blood is added; Figueiredo et al. 1976).

Assessment of drug response

Glucantime (meglumine antimoniate: 85 mg pentavalent antimony/ml, lot number 020; Rhodia SA, Brazil) was stored at room temperature in darkness. Fresh drug samples were used throughout the study. The sensitivity of *Leishmania* strains was determined as described by Moreira and Petrillo-Peixoto (1991). In brief, *Leishmania* promastigotes growing at the log phase were inoculated into fresh medium (5.0 ml final volume in 50.0-ml Erlenmeyer flasks) at a concentration of 5×10^5 cells/ml in the absence (control) or presence of glucantime. The pentavalent Sb concentration in the medium ranged from 0.2 to 8.6 mg/ml. Assessment of growth was made at 24-h intervals using a Coulter Counter (Model D2; Coulter Electronics). The effect of glucantime on the cell yield was expressed as a percentage of the cell number determined in control cultures (relative growth yield) after 72 h of incubation. Linear regression was used to calculate the growth rate of controls and that of treated samples. These values were used to obtain the relative growth rate. Results were expressed as mean values for at least three experiments, each done in triplicate. Statistical significance was determined by Student's *t*-test (analysis of variance).

DNA isolation and Southern hybridization

Extraction and quantification of DNA were performed as previously described (Petrillo-Peixoto and Beverley 1988). Total DNA was digested with restriction endonucleases and electrophoresed in 1% agarose gels (TRIS/acetate buffer at 5 V/cm). The intensity of K-DNA bands and of DNA smears present in ethidium-bromide-stained gels was used to monitor the amount of DNA loaded in each lane. PFGE, 10^7 cells were embedded in 0.5% low-melting-point agarose blocks as described by Van Der Ploeg et al. (1984). Blocks were treated with 1% sodium dodecyl sulfate (SDS), 1 mg pronase/ml, and 0.5 *M* ethylenediaminetetraacetic acid (EDTA, pH 8.0) at 42 °C for 48 h and then stored at 4 °C in 0.5 *M* EDTA (pH 8.0). Electrophoresis was performed in 0.5× TBE (22.5 m*M* TRIS, 22.5 m*M* boric acid, 0.625 m*M* EDTA, pH 9.0) in a CHEF (clamped homogeneous electric field electrophoresis) apparatus (homemade) using different running conditions. DNA was transferred onto nylon membranes (Southern 1975) according to the manufacturer's instructions (Gene Screen Plus; New England Nuclear, Boston, Mass.) except that 10× SSPE (1.5 *M* NaCl, 0.1 *M* NaH_2PO_4, 10 m*M* Na_2EDTA, pH 7.0) was used instead of 10× SSC. A DNA fragment containing the open reading frame of the *lgpgpA* gene from the *L.* (*V.*) *guyanensis* H circle (LB7 probe – 6.7-kb *Eco* RI/*Eco* RI fragment; Ferreira-Pinto et al. 1996) was labeled with [α^{32}-P]-deoxy cytidine *triphosphate* by random priming and was used as a probe (Feinberg and Vogelstein 1983). Blots were hybridized at 56 °C (Church and Gilbert 1984) and washed at low stringency (2 × SSPE) at room temperature for 15 min.

Results

Strains of the *Leishmania (Viannia) braziliensis* complex are difficult to grow in liquid media as compared with the ease of cultivation of *L.*(*L.*) *mexicana* and *L.*(*L.*) *donovani* complexes. With the liquid medium used in this study a high yield of promastigotes was achieved. Differences in growth rates and peak densities were observed for the *Leishmania* strains. Table 1 shows the cell density and growth rates recorded for all strains. *L.* (*L.*) *amazonensis* strains had luxuriant growth, showing a cell density of $3.72–5.04 \times 10^7$/ml. Parasite populations of $0.56–3.16 \times 10^7$/ml were observed among the *L.* (*V.*) *braziliensis* strains. *L.* (*V.*) *guyanensis* strains grew to levels intermediate to the other two groups. Variability

Table 1 Growth parameters of *Leishmania* strains in the absence of glucantime[a]

Strain	Cell density ($\times 10^{-5}$/ml)	Growth rate (±SEM)
L (*Viannia*) *braziliensis* LTB260	94	1.45 ± 0.10
L (*V.*) *braziliensis* LTB 299	143	1.95 ± 0.05
L (*V.*) *braziliensis* LTB 320	102	1.84 ± 0.16
L (*V.*) *braziliensis* LTB259	56	1.56 ± 0.09
L (*V.*) *braziliensis* M9554	79	1.38 ± 0.06
L (*V.*) *braziliensis* M2903	157	2.30 ± 0.11
L (*V.*) *braziliensis* M9947	317	2.95 ± 0.01
L (*V.*) *guyanensis* M9957	92	2.30 ± 0.13
L (*V.*) *guyanensis* M9945	117	2.22 ± 0.07
L (*V.*) *guyanensis* M1176	110	2.53 ± 0.12
L (*L.*) *amazonensis* PH8	373	3.02 ± 0.21
L (*L.*) *amazonensis* M10995	419	2.92 ± 0.01
L (*L.*) *amazonensis* M10996	505	3.18 ± 0.02

[a] Data represent mean values for at least 6 determinations

between experiments was low, ranging from 0.04% to 8% for growth rates and from 7% to 35% for peak densities.

For the determination of glucantime inhibitory concentrations, dose-response curves were performed. Figure 1 shows the dose-response curves generated for six strains – expressed either as the relative growth yield (Fig. 1a) or as the relative growth rate (Fig. 1b). Two different survival-curve patterns were clearly distinguished with both indices: pattern 1, whereby the dose-response line decreased with glucantime concentrations of up to 2.4 mg Sb/ml (strains LTB299, M2903, and M9554); and pattern 2, whereby growth inhibition was observed only at concentrations exceeding 2.4 mg Sb/ml (LTB 259, M9947, and PH8). Results recorded for the relative growth yield showed that the M2903 strain was more sensitive than the M9554 and LTB299 strains (highest growth inhibition 60% for strains M9554 and LTB299 and 86% for strain M2903), whereas the strains could not be distinguished on the basis of the relative growth rate.

On the basis of the dose-response curves, two drug concentrations (0.2 and 2.0 mg Sb/ml) were chosen to study the effects of glucantime on the growth indices of a larger number of strains. For most of the strains a glucantime concentration of 0.2 mg Sb/ml had little effect on growth (Table 2). The results obtained with 2.0 mg Sb/ml (relative growth yield varying from 5% to 100%) distinguished sensitive from resistant strains better. The growth yield at 2.0 mg Sb/ml was then used as a criterion for susceptibility to glucantime. As part of the criterion, strains that showed a significant difference in growth yield ($P < 0.01$) relative to the control value were considered sensitive to the drug (generally lower than 70%). Eight strains were considered sensitive (Table 2). Four other strains were found to be resistant. Strain PH8 was intermediate in drug susceptibility; the differences in growth yield were significant only at the 95% level.

If the relative growth rate is used as a second criterion, similar patterns of sensitivity/resistance are found. Exceptions to the pattern were observed for strains that showed peculiar growth curves, such as a biphasic growth pattern. Standard growth-curve patterns of a sensitive (M2903) or a resistant strain (M10995) in the absence and in the presence of the drug

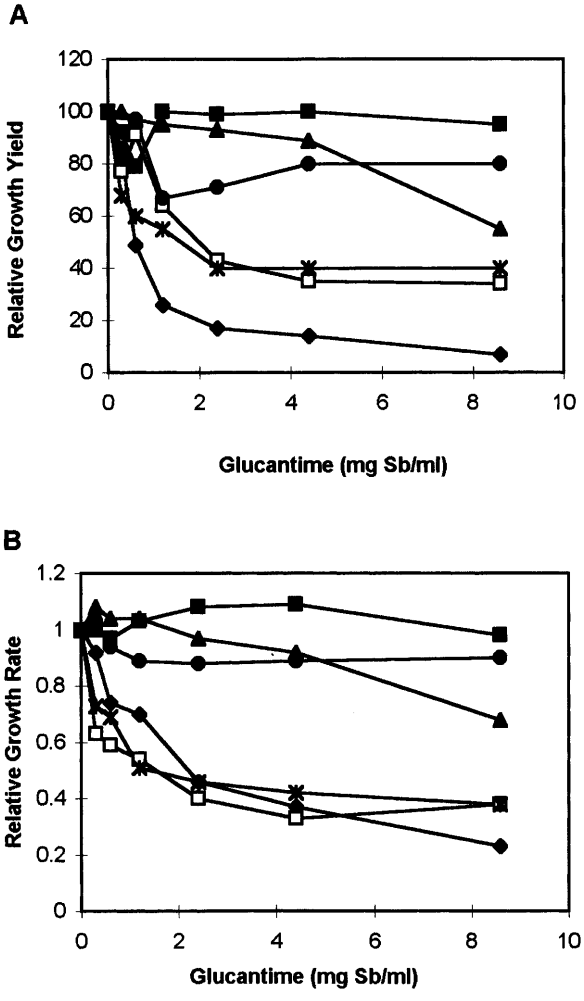

Fig. 1A, B Concentration-response profile of six *Leishmania* strains to glucantime. **A** Relative growth yield. **B** Relative growth rate. (□ *L.*(*V.*) *braziliensis* LTB299, ◆ *L.*(*V.*) *braziliensis* M2903, * *L.*(*V.*) *braziliensis* M9554, ▲ *L.*(*V.*) *braziliensis* LTB259, ■ *L.*(*V.*) *braziliensis* M9947, ● *L.*(*L.*) *amazonensis* PH8)

Table 2 Effect of glucantime on the growth of *Leishmania* strains (*ND* Not done)

Strain	Relative growth yield[a]		Relative growth rate[b]	
	0.2 mg Sb/ml	2.0 mg Sb/ml	0.2 mg Sb/ml	2.0 mg Sb/ml
Sensitive strains:				
LTB 260	72*	43**	1.03	0.53**
LTB 299	75	34**	0.94	0.58**
LTB 320	57**	11**	0.85	0.09**
M2903	37**	5**	0.47**	0.15**
M9554	80*	40**	0.99	0.85*
M9957	79	62**	0.90	0.81**
M9945	77	47**	0.89*	0.69**
M1176	ND	60**	ND	0.94
Intermediately resistant strain:				
PH8	91	69*	0.96	0.86*
Resistant strains:				
LTB 259	100	93	1.03	0.97
M9947	75	80	0.94	0.88
M10995	90	76	0.96	0.93
M10996	89	74	0.96	0.96

* $P \leq 0.05$; ** $P \leq 0.01$ versus the respective control (Student's *t*-test)
[a] Percentage of growth at the given drug concentration (mg Sb/ml) in relation to the control cell number at the late log phase
[b] Relative growth rate normalized to the rate obtained in the respective control without the drug

(2.0 mg Sb/ml) are represented in Fig. 2a. Strains M9945 and M9957, both of which were sensitive, showed a biphasic growth pattern in the absence of the drug; the growth rate was higher in the first phase (0–48 h; $P < 0.01$) than in the second phase (48–96 h). In the presence of the drug, strains M9957 and M9945 (Fig. 2b) showed a decrease in growth rate in the early phase and grew like the control in the second phase. A marked reduction in cell numbers indicated the effect of glucantime on growth yield for strains M1176 and M9554 (Fig. 2c). Strain M10996 (Fig. 2d) was considered resistant on the basis of the relative growth yield. However, some inhibitory effect ($P < 0.05$) was observed after 48 h of incubation.

Molecular analyses of all strains were used in an attempt to correlate the resistance phenotype with genomic alterations. When total DNA was digested with *Eco* RI, *Hind* III, and *Pst* I, the RFLP patterns of the 13 *Leishmania* strains were similar among sensitive and resistant isolates. Southern analysis using a *lgpgpA* probe failed to show any quantitative difference among the strains (data not shown). When *Leishmania* chromosomes were separated using different running conditions of CHEF electrophoresis, 19 DNA bands ranging in size from 0.2 to 2 Mb and showing variable staining intensities were resolved. Figure 3 shows a gel run at a 90-s pulse time and an autoradiograph obtained after hybridization with the *LbpgpA* probe. Sequences homologous to *pgpA* were present in all strains studied. At least two bands of apparent sizes of 0.85 and 1.6 Mb were recognized (Fig. 3B, arrows) besides the signal at the origin and at the compression zone. The band at 0.85 Mb corresponds to the chromosomal location of *pgpA* (and the H region) and *pgpB*/*C* genes; a second band of 1.6 Mb is probably due to cross-hybridization of *pgpE* and *pgpF* genes (Legaré et al. 1994). The stronger signals observed in lanes 2, 5, and 10 of Fig. 3 were reduced after the amount of DNA applied had been normalized. However, differences in intensity between bands located in the same lane (lanes 7–9) can be observed.

Discussion

The marked differences in growth yield and growth rate observed in the absence of glucantime for the several strains studied show that *Leishmania* are highly heterogeneous. This observation also indicates the need for additional care in the performance of comparative studies of drug effect based on growth parameters. Different degrees of susceptibility to glucantime were observed for 13 *Leishmania* strains on the basis of the concentration-response data. The relative cell yield expressed the level of inhibition more clearly than did the relative growth rate, although the latter was more reproducible. A lower growth yield reflects the decrease in cell numbers at the early growth phase (0–24 h) generally associated with lower growth rates. However, for some strains (M1176 and M9554) the decrease in cell numbers observed in the early phase cannot be demonstrated when a reduction in growth rate is used as a criterion for susceptibility. To avoid an arbitrary demarcation between sensitive and resistant strains the statistically significant reduction in growth yield observed at the late log phase at 2.0–2.4 mg Sb/ml should be used as a criterion for susceptibility. When applied to cell yield values recorded after 72 h of incubation, the same criterion also indicates drug susceptibility; hence, it may be used for preliminary tests. In cases in which one

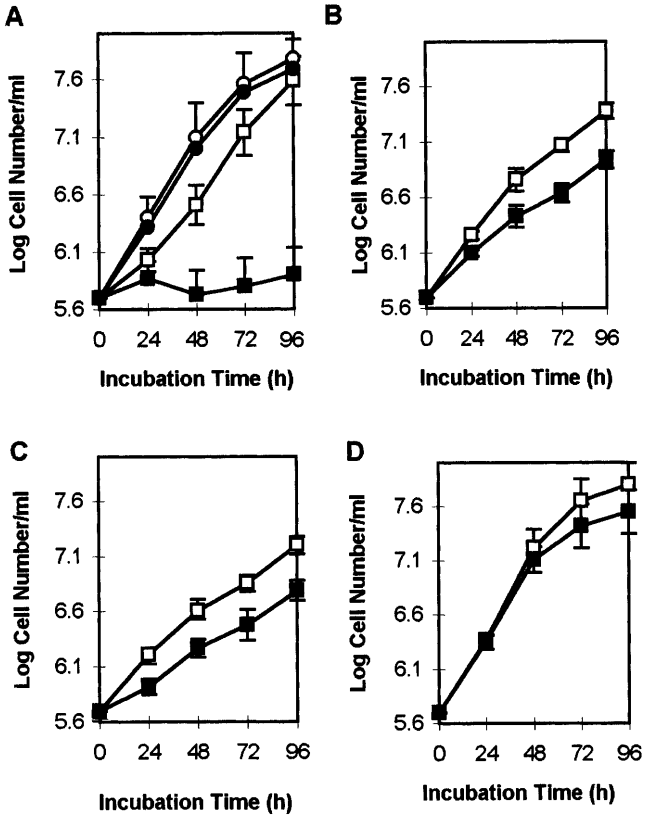

Fig. 2A–D Growth kinetics of *Leishmania* strains in the absence (*white symbols*) and presence (*black symbols*) of 2.0 mg Sb/ml glucantime. **A** *L.*(*V.*) *braziliensis* M2903 (*squares*), *L.*(*L.*) *amazonensis* M10995 (*circles*). **B** *L.*(*V.*) *guyanensis* M9945. **C** *L.*(*V.*) *braziliensis* M9554. **D** *L.*(*L.*) *amazonensis* M10996

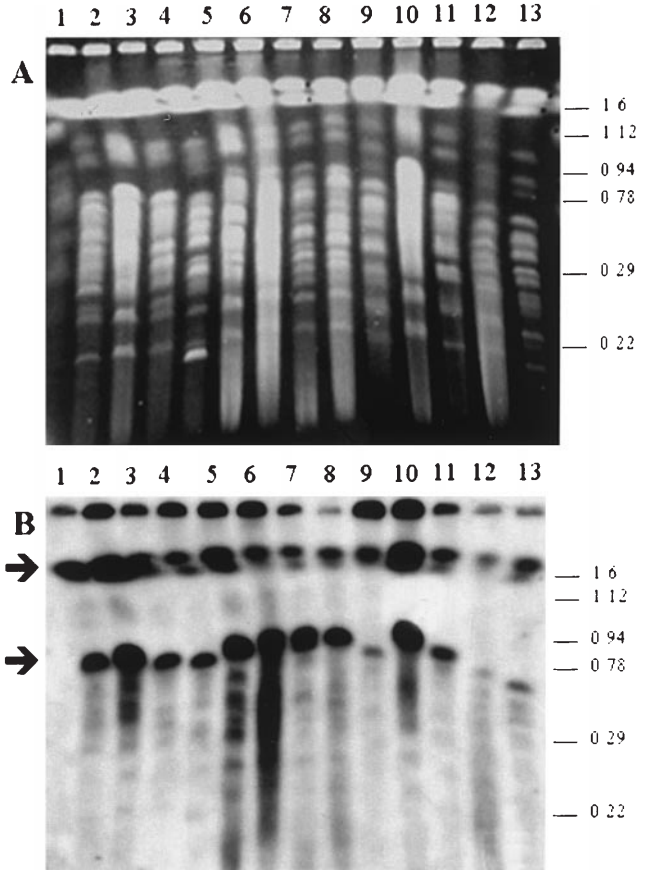

Fig. 3A, B PFGE and Southern-blot analyses of *Leishmania* chromosomes **A.** Chromosomes of 13 *Leishmania* isolates separated by CHEF using a 90-s pulse time. **B** Autoradiograph after hybridization with the *lgpgpA* probe. Sizes markers were derived from chromosomes of *Saccharomyces cerevisiae*. (*1 L.*(*V.*) *braziliensis* LTB260, *2 L.*(*V.*) *braziliensis* LTB299, *3 L.*(*V.*) *braziliensis* LTB320, *4 L.*(*V.*) braziliensis M2903, *5 L.*(*V.*) *braziliensis* M9554, *6 L.*(*V.*) *guyanensis* M9957, *7 L.*(*V.*) guyanensis M9945, *8 L.*(*V.*) *guyanensis* M1176, *9 L.*(*L.*) *amazonensis* PH8, *10 L.*(*V.*) *braziliensis* LTB259, *11 L.*(*V.*) *braziliensis* M9947 *12 L.*(*L.*) *amazonensis* M10995, *13 L.*(*L.*) *amazonensis* M10996)

cannot resolve the susceptibility of a strain it would be necessary to determine the growth curve up to exponential growth.

By application of the proposed criteria a good correlation was observed between clinical data and the results obtained in this study for clinical stocks that had not been subjected to extensive serial passages in culture (LTB260, LTB299, LTB320, and LTB259). The two other isolates from humans (M1176 and M2903) were included as reference strains. They are not discussed in this regard because of their long-term maintenance in culture. These data indicate that it is possible to measure the susceptibility of *Leishmania* isolates to glucantime and that this test can predict the clinical outcome (cure or failure). It is also noteworthy that drug resistance was detected in strains isolated from sylvatic vectors. Similar results have been obtained with *Trypanosoma* strains isolated from wild reservoirs and sylvatic vectors in which previous contact with the drugs can be excluded (Filardi and Brener 1987). The differences in the susceptibility to glucantime shown here are consistent with results obtained when the activity of pentavalent antimonial drug was evaluated in primary isolates from leishmaniasis patients (Grogl et al. 1989; Jackson et al. 1990; Faraut-Gambarelli et al. 1997).

Considering the heterogeneity of the Sb response observed among different *Leishmania* strains as an innate property or as acquired antimony resistance, the potential outcome of resistance due to suboptimal treatment conditions could explain previous reports of Sb treatment failure. Reports of differences in susceptibility to antimony among clones derived from the same strain (Berman et al. 1989; Grogl et al. 1989; Moreira and Petrillo-Peixoto 1991) and among antimony-resistant *Leishmania* promastigotes obtained in vitro (Ullman et al. 1989; Grogl et al. 1992; Ferreira-Pinto et al. 1996; Ephros et al. 1997) support the assumption that there is indeed a selection for less sensitive parasites. It has not been established whether the changes induced in vitro correspond to the changes believed to precede the expression of drug resistance in nature or to the event leading to unresponsiveness in patients. In this regard the isolation of parasites from unresponsive patients and

the study of drug response in in vitro systems remain as an important approach to the better understanding of antimony resistance in *Leishmania*.

RFLP and PFGE analyses are used to detect molecular differences between sensitive and resistant parasites when gene amplification is involved as the major resistance mechanism (Coderre et al. 1983; Beverley 1991). In this study a significant part of the genome of all *Leishmania* strains could be resolved, although the estimated genome size of the New World species is larger than that of *L. infantum* (Wincker et al. 1996). Even though karyotype polymorphism was observed among the *Leishmania* strains studied, hydridization data indicate that the chromosomal location of the *pgpA* gene seems to be conserved. Moreover, the data obtained with specific probe hybridization suggest that *pgpA* amplification is not involved in glucantime resistance in these wild *Leishmania* stocks. Several oxyanion-resistant lines failed to show any gene amplification, and some clinical isolates can be more resistant than drug-resistant strains obtained by in vitro selection (Ouellette and Papadopoulou 1993; Dey et al. 1994; Ferreira-Pinto et al. 1996). These data indicate that mechanisms other than *pgpA* gene amplification can result in oxyanion resistance. Further work will be required to establish the molecular basis involved in antimony resistance in *Leishmania* in the field.

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