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Involvement of Thiol Metabolism in Resistance to Glucantime in *Leishmania Tropica*

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ABSTRACT. Clinical resistance to pentavalent antimonials, in the form of pentostam (sodium stibogluconate) or glucantime (*N*-methylglucamine antimoniate), has long been recognized as a problem in Leishmaniasis. However, the mechanisms of resistance are unclear. We selected *in vitro* a *Leishmania tropica* line resistant to 1.2 mg/mL of Sb(V) of glucantime (GLU-R10). The cell line has a stable phenotype for at least 6 months and a resistance index of 1400-fold. The resistant line has no cross-resistance to pentostam or to $SbCl_3$ and $SbCl_5$. The resistance to glucantime was reverted by buthionine sulfoximine (BSO) and chlorambucil (CLB); however, thiol analyses by HPLC of wild-type and GLU-R10 cell lines, in the presence or absence of the drug, showed no differences between these two cell lines. The resistant line had a DNA amplification shown as a circular extrachromosomal element (G-circle) of approximately 22 kb. However, the specific probes for γ-glutamyl cysteine synthetase, ornithine decarboxylase and trypanothione reductase did not recognize the G-circle amplified in the GLU-R10. The G-circle did not arise from the H region and was not related with P-glycoprotein Pgp-MDR- or Pgp-MRP-like genes. Northern blot analysis of the G-circle showed that a single transcript of approximately 6 kb was overexpressed in the resistant line. Molecular characterization of the G-circle would lead to the determination of the gene(s) involved in resistance to glucantime in *Leishmania*. BIOCHEM PHARMACOL **56**;9:1201–1208, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Leishmania tropica*; resistance to Glucantime; thiol metabolism; extrachromosomal element; buthionine sulfoximine; chlorambucil

Pentavalent antimony is the drug of choice in the treatment of *Leishmaniasis*. Clinical resistance to organic pentavalent antimonials, in the form of sodium stibogluconate (pentostam) or *N*-methylglucamine antimoniate (glucantime), has long been recognized [1, 2]. However, it is unknown whether the clinical failure of chemotherapy is attributable to the development of drug resistance mechanisms in the parasite [3, 4] or to a variety of host factors that might also contribute to low drug response [5]. The most common drug resistance mechanisms described in *Leishmania* have been the decreased accumulation of drugs and amplification of DNA as extrachromosomal elements containing genes involved in drug resistance [6, 7]. Different resistance mechanisms to pentostam have been proposed, among others modification of glycolytic enzymes, specifically phosphofructokinase [8], fatty acid β-oxidation [4], and a differential drug accumulation [9]. Recently, it has been postulated that the resistance to pentostam may require reduction of Sb(V) to Sb(III), conjugation with a thiol, and extrusion by an ATP-coupled pump [10, 11]. GSH both free and conjugated with spermidine as bis(glutathionyl)spermidine (also called $T[SH]_2$)‖, and GSH-SP, play a very important defense role in trypanosomatids [12–14]. The concentration of free and conjugated GSH seems, up to now, to be the best explanation for differences in susceptibility to nifurtimox and benznidazole among different strains of *Trypanosoma cruzi* [15, 16]. Also, the toxic effects of nifurtimox and benznidazole may be a consequence of a significant decrease in the concentrations of $T[SH]_2$ and GSH caused by conjugation with metabolites of both drugs [17]. Recently, *L. tarentolae* resistant to arsenite was found to overproduce $T[SH]_2$ 40-fold as a drug response [11]. Pretreatment of these parasites with BSO, an inhibitor of γ-GCS, can partly revert the resistant phenotype [18]. It was proposed that $T[SH]_2$ forms a complex with

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‖ *Abbreviations*: BSO, DL-buthionine [S,R]-sulfoximine; CHEF, contour-clamped homogeneous electric field; CLB, chlorambucil; GLU, Glucantime; GLU-R10, line resistant to 10 mM glucantime; GSH-SP, glutathionylspermidine; $T[SH]_2$, trypanothione; γ-GCS, γ-glutamyl cysteine synthetase; MDR, multidrug resistance; MRP, multidrug-resistance-associated protein; PCR, polymerase chain reaction; Pgp, *P*-glycoprotein; and WT, wild-type parasites.

As(III) and Sb(III) and in this form is transported by an ATP-coupled efflux pump in these parasites [11]. The present work was carried out to determine the involvement of thiol metabolism in Glucantime resistance in *L. tropica*, as well as the specific resistance mechanisms developed by the parasites in response to the drug.

MATERIALS AND METHODS

Drugs and Chemicals

GLU was obtained from Rhône–Poulenc Rorer, S.A. and Pentostam from the Wellcome Foundation. Daunomycin and doxorubicin hydrochloride were from Pharmacia-Upjohn. Methotrexate was from Cyanamid Iberica S.A. Vinblastine was obtained from Lilly S.A. $SbCl_3$ and $SbCl_5$ were from Aldrich. Puromycin, formycin B, pentamidine, pyrimethamine, berenil, amphotericin B, ketoconazole, BSO, CLB, Na_2HAsO_4, $NaAsO_2$ and *N*-methyl glucamine were from Sigma. Thiolyte was from Calbiochem.

Parasite Culture and Selection of GLU-resistant Cell Lines

L. tropica LRC-L39 (LEM 2563, Montpellier, France) was obtained from Dr. L.F. Schnur (Hebrew University). WT promastigotes were grown at 28° in RPMI 1640 modified medium (Life Technologies), as previously described [19], and supplemented with 20% heat-inactivated fetal bovine serum (Flow Laboratories). A clone of *L. tropica* was used for selection of resistance to GLU using a stepwise selection process as described previously [20], using GLU concentrations of 12, 18, 24, 60, 120 and 1,200 μg/mL ofSb(V). The *L. tropica* line resistant to 1.2 mg/mL of Sb(V) (GLU-R10) was grown in drug-free medium for 1, 3 and 6 months to determine the stability of resistance in these parasites. The growth sensitivities of WT and drug-resistant parasites to GLU and other agents were ascertained as follows. Two $\times$ 10^6 parasites/mL in 2 mL of RPMI 1640 modified medium were added to each tube containing various concentrations of growth inhibitory agent. Two control tubes lacking drug were maintained in parallel. After 48 hr of incubation, cell densities were determined on a Coulter Counter model Z1. After this period of growth, cells in control tubes typically reached a density of 3–4 $\times$ 10^7 parasites/mL. Cross-resistance of the GLU-R10 line to other unrelated drugs was determined by measuring the IC_{50} (concentration of drug which decreases the rate of cell growth by 50%) and the resistance indexes (IC_{50} ratio among resistant and WT cells).

Treatment of Parasites with BSO and CLB

BSO was added to the parasite suspensions at concentrations of 3 mM for a period of 48 hr in the presence or absence of GLU. After drug treatment, the inhibitory effect of this compound was measured as a percentage of the growth inhibition. Also, the inhibitory effect of CLB was determined on WT and resistant parasites using concentrations of 0.10, 0.20, 0.25 and 0.30 mM. Statistical differences in % growth inhibition between the different treatments with BSO and CLB were determined by using the Student's *t*-test and considered significant at $P < 0.02$.

Analysis of Thiols

The amounts of GSH, $T[SH]_2$ and GSH-SP in WT and drug-resistant parasites were determined by using the fluorescent thiol reagent monobromobimane (Thiolyte), and then separated by HPLC using a Merck–Hitachi instrument as described [21]. The amount of thiol was calculated from standard curves of pmol of reduced GSH and reduced $T[SH]_2$ vs relative area of the corresponding fluorescent peaks. GSH-SP was assayed because it gives the same fluorescence as reduced GSH.

Nucleic Acid Manipulations

Total DNA from *L. tropica* lines was obtained by phenol extraction [22]. DNA digestion, dephosphorylation, ligation and bacterial transformation were carried out according to established procedures [23]. Total RNA was extracted using the Chomczynsky method [24], and the polyadenylated fraction of the RNA was obtained by affinity chromatography on an oligo(dT)-cellulose column [25]. Northern blot was performed using standard procedures [23]. The blots were hybridized with different specific probes. γ-GCS was obtained by PCR amplification, using degenerated oligonucleotide primers corresponding to conserved amino acid sequences in other γ-GCS proteins. The sense and antisense primers were based on amino acid sequences ^{241}MGFGMG^{246} to ^{422}WRVEFR^{427}, respectively (positions corresponding to the human γ-GCS). The oligonucleotide primers used for the amplification were 5′-ATGGG(C/G)TT(C/T)GG(C/G)ATGGG-3′ for the sense strand and 5′-CG(A/G)AACTC(C/G)AC(C/G)CGCCA-3′ for the antisense strand. The degeneracy of the primers was based on the codon bias found in *Leishmania* genes. Primers were incubated with *L. tropica* genomic DNA, following a standard PCR protocol under the following conditions: 30 cycles of 1 min at 95°, 2 min at 48° and 1 min at 72°. The PCR product was subcloned into pGEM-T vector (Promega) and sequenced in both directions on a 373A Automated DNA Sequencer (Applied Biosystem). The nucleotide sequence data were submitted to the Genbank™ data base with the accession number U95955. Comparison of the amino acid sequence revealed an identity of 54.5% with the human γ-GCS. The ornithine decarboxylase specific probe was obtained by PCR amplification, using oligonucleotide primers corresponding to the previously described *L. donovani* ornithine decarboxylase [26]. Another specific probe used was the trypanothione reductase from *L. donovani* [27], which catalyzes the NADPH-dependent reduction of $T[SH]_2$. The *ltrmdr1* probe, that recognizes the first nucleotide binding domain of the Pgp-MDR-like gene from *L. tropica* [28], was also

TABLE 1. Cross-resistance profile of *L. tropica* line resistant to Glucantime

Drugs	IC_{50} (μM)* WT	GLU-R10	
Glucantime*	25.2 ± 2.3	36000 ± 3240	(1429)
Pentostam†	19.1 ± 1.8	25.8 ± 2.7	(1.4)
Puromycin	3.8 ± 0.7	10.2 ± 1.5	(2.7)
Ketoconazole	18.8 ± 1.3	25.1 ± 2.4	(1.3)
Methotrexate	22.6 ± 1.2	39.5 ± 2.2	(1.7)
Vinblastine	14.6 ± 0.7	12.3 ± 1.0	(0.9)
Doxorubicin	31.1 ± 7.8	83.2 ± 3.6	(2.7)
Daunorubicin	5.3 ± 0.6	5.6 ± 0.3	(1.1)
$SbCl_3$	44.9 ± 0.1	90.0 ± 1.7	(2.0)
$SbCl_5$	60.0 ± 2.8	80.1 ± 5.8	(1.3)
Na_2HAsO_4	5.5 ± 0.3	13.5 ± 1.2	(2.5)
$NaAsO_2$	6.0 ± 0.4	7.5 ± 0.8	(1.3)
Berenil	1.3 ± 0.1	1.1 ± 0.1	(0.9)
Pyrimethamine	31.8 ± 1.7	37.5 ± 2.0	(1.2)
Amphotericin B	0.2 ± 0.01	0.3 ± 0.03	(1.5)
Formycin B	0.01 ± 0.001	0.01 ± 0.001	(1.0)
Pentamidine	1.2 ± 0.1	1.4 ± 0.1	(1.2)
N-methyl Glucamine	292000 ± 40880	404000 ± 56360	(1.4)

*The IC_{50} is the concentration (μM) which decreases the rate of cell growth by 50%. The means ± SD of 3 independent experiments are given. The resistance index, indicated between parentheses, is the ratio of IC_{50} for GLU-R10 and WT cells.
†Values are expressed as μg/mL Sb(V).

applied. The *nbs*A probe, which covers the first nucleotide binding domain of the Pgp gene A (*ltpgp*A) from *L. tarentolae* and recognizes the Pgp-MRP-like gene in *Kinetoplastida* [29], was employed as well. Finally, the β-tubulin probe from *T. cruzi* was used for normalization of the blots. Bands were visualized by autoradiography and the relative intensities quantified using a Bio-Rad model 620 video densitometer.

Pulse-Field Gel Electrophoresis Analysis

Extrachromosomal circular DNA was resolved from genomic DNA by contour-clamped homogeneous electric field (CHEF) [30], using 1.5% agarose gels and a pulse time of 4 sec for 18 hr at 300 V and 13°. The method of alkaline lysis was used to enrich extrachromosomal elements, as previously described [31].

RESULTS

Characterization of a GLU-resistant L. Tropica *Line*

A clone of *L. tropica* was used for in vitro selection of resistance to GLU using a stepwise selection process, starting with a concentration of 12 μg/mL of Sb(V). The resistance index at the maximum GLU concentration was greater than 1000-fold (Table 1). The results of stability of GLU resistance using GLU-R10 parasites maintained in drug-free medium for 1, 3 and 6 months showed that the IC_{50} values for GLU did not differ significantly from the value for GLU-R10 parasites (not shown), suggesting that the resistant phenotype is stable without drug pressure. We studied the cross-resistance profile of GLU-R10 parasites to structurally and functionally unrelated drugs. The results are summarized in Table 1 and show no significant cross-resistance toward different drugs such as Pentostam (the other drug of choice in the treatment of leishmaniasis), $SbCl_3$ and $SbCl_5$.

Reversion of GLU Resistance by BSO and CLB

BSO is an inhibitor of γ-GCS [32], the enzyme that catalyzes the first step in GSH synthesis. The results show that in the absence of GLU, BSO (3 mM) had no significant toxic effect on the WT and GLU-R10 lines (Table 2). However, in the presence of 2.4 mg/mL of Sb(V), the toxic effects of BSO increased substantially in the resistant parasites (Table 2). These results are dose-

TABLE 2. Effect of BSO on cytotoxicity of Glucantime in *L. tropica* lines

Lines	Treatment GLU (μg/mL Sb^5)	BSO (mM)	% Growth Inhibition†
WT	8.4	—	7.4 ± 0.8
	—	3	6.2 ± 0.5
	8.4	3	13.1 ± 1.7
GLU-R10	1,200	—	5.3 ± 0.5
	—	3	8.0 ± 1.0
	120	3	37.7 ± 4.5*
	600	3	48.1 ± 6.7**
	1,200	3	74.8 ± 8.9***
	2,400	3	86.2 ± 10.3***

†The values represent the percentage of growth inhibition relative to control growth in absence of inhibitory agent. The data shown are the average of three independent experiments ± SD. Values significantly different by Student's *t* test are designated by an asterisk (*$P < 0.02$, **$P < 0.01$, ***$P < 0.002$).

TABLE 3. Effect of CLB on cytotoxicity of Glucantime in *L. tropica* lines

Lines	Treatment: GLU (μg/mL Sb^5)	Treatment: CLB (mM)	% Growth Inhibition†
WT	8.4	—	7.4 ± 0.8
	—	0.30	5.6 ± 0.6
	8.4	0.10	7.1 ± 0.3
	8.4	0.20	9.2 ± 0.7
	8.4	0.25	17.3 ± 2.0
	8.4	0.30	20.7 ± 2.3
GLU-R10	1,200	—	5.3 ± 0.6
	—	0.30	12.0 ± 1.8
	1,200	0.10	45.7 ± 5.3*
	1,200	0.20	61.3 ± 7.7*
	1,200	0.25	67.4 ± 8.6**
	1,200	0.30	73.2 ± 9.3**

†The values represent the percentage of growth inhibition relative to control growth in absence of inhibitory agent. The data shown are the average of three independent experiments ± SD. Values significantly different by Student's *t* test are designated by an asterisk (* $P < 0.02$, ** $P < 0.01$).

dependent on GLU concentration. Also, the results confirm the reversion of the IC_{50} to GLU in the resistant line from 36 to 0.6 mg/mL of Sb(V) in the presence of BSO. In addition, the involvement of thiol metabolism as a cellular detoxification mechanism to antimony is supported by the results obtained with CLB, a compound characterized by alkylation of cellular targets such as DNA, RNA and proteins in eukaryotic cells and for its binding to GSH spontaneously or through GSH S-transferase [33]. The effect of different concentrations of CLB on WT and drug-resistant parasites is shown in Table 3. Although no significant toxic effects were observed on WT parasites, a substantial increase in the % growth inhibition was observed in the resistant parasites.

Analysis of Thiols in L. Tropica *Lines*

The reversal effects of BSO and CLB on Glucantime resistance suggested the involvement of thiol metabolism in resistance to GLU. Based on this information, we determined the GSH-$T[SH]_2$ status and the effect upon it of BSO in *L. tropica* lines. Table 4 shows the concentrations of GSH, GSH-SP and $T[SH]_2$ in the WT and GLU-R10 lines. The total amount of reduced free and conjugated GSH did not vary between the different control lines, and most of the GSH (over 70%) was present as $T[SH]_2$. When the parasites were treated with 3 mM BSO, the total thiol concentrations in the WT and resistant parasites decreased by over 50% (Table 4). The above results suggest that the GLU resistance in the GLU-R10 line was not due to an increase in thiol concentrations of the parasites.

Molecular Mechanism of Resistance to GLU in the GLU-R10 Line

To determine if the resistant line exhibited amplification of some genes of thiol metabolism, Southern blot analysis was performed using specific probes for γ-GCS, ornithine decarboxylase and trypanothione reductase. The results showed no differences between the WT and GLU-R10 lines at the DNA level (not shown). Also, Southern blot analysis with the specific probes for *Leishmania* Pgp-MDR- and Pgp-MRP-like genes showed no amplification of these ATP-binding cassette transporters in GLU resistance (not shown).

In *Leishmania sp*., amplified genes frequently emerge as extrachromosomal circular elements [6, 34]. CHEF analysis, used at conditions that favor the separation and resolution of extrachromosomal amplicons, revealed the existence of a band whose apparent molecular size changed with pulse frequency and had characteristics of supercoiled DNA in the resistant parasites (Fig. 1). The amplification of the G-circle was five-fold in the GLU-R10 line and in parasites maintained without drug pressure (Fig. 2). Additionally, the G-circle recognized two nonspecific bands below 2 kb corresponding to kinetoplast minicircle DNA (kDNA) of the parasite, probably due to the contamination of kDNA circles in the purification process of the G-circle. The extrachromosomal G-circle, purified by alkaline lysis and restriction mapped with different enzymes, had a size of approximately 22 kb (Fig. 3). We propose that the amplicon probably contains repeated sequences, as deduced from the presence of 2- and three-fold intensity of some bands (Fig. 3B). Northern blot analysis, using the G-circle as a specific probe, shows a single transcript of approximately 6 kb in the resistant parasites (Fig. 4).

DISCUSSION

The resistance mechanisms to pentavalent antimonials are unclear. A recent hypothesis suggests that in *Leishmania*

TABLE 4. Thiol concentrations in WT and GLU-R10 *L. tropica* lines: Effect of BSO

Lines	Treatment	GSH	GSH-SP	$T[SH]_2$	Total Thiols
WT	Control	2.21 ± 0.80	1.93 ± 0.90	10.77 ± 0.67	14.91 (100)
	BSO	1.29 ± 0.39	0.65 ± 0.10	6.02 ± 0.23	7.96 (53)
GLU-R10	Control	1.86 ± 0.30	1.46 ± 0.80	10.09 ± 0.42	13.41 (90)
	BSO	1.65 ± 0.24	0.63 ± 0.13	5.70 ± 0.80	7.98 (54)

Thiol concentrations are expressed as nmol of GSH equivalent per mg of parasite total protein. Parasites were treated with BSO 3 mM for 18 hr prior to thiol determination. The results are expressed as the means ± SD of 3 independent experiments. Data in parentheses correspond to the percentage with respect to the total thiols in WT.

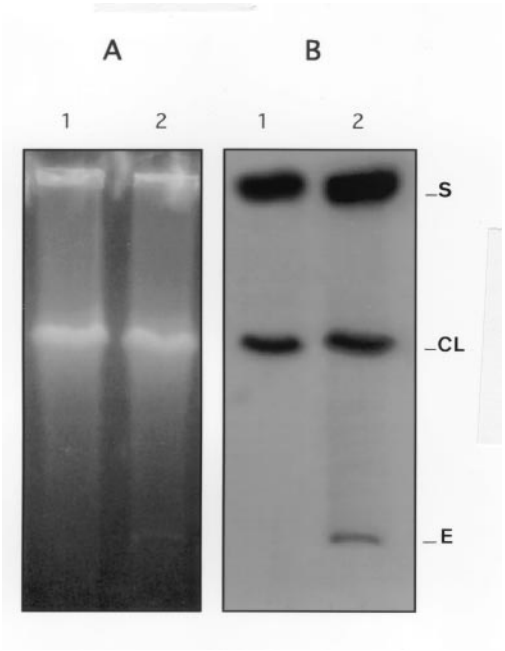

FIG. 1. CHEF analysis of extrachromosomal elements in GLU-R10 *L. tropica*. CHEF was developed using a pulse time of 4 sec over a period of 18 hr at 300 V, to favor the separation of extrachromosomal amplicons of *Leishmania*. (A) Ethidium bromide-stained gel of WT parasites (lane 1) and GLU-R10 parasites (lane 2). The Southern blot of the gel was hybridized with the G-circle as a specific probe (B). Bands visible in the autoradiogram are: S, slot location; CL, chromosomal location; E, extrachromosomal element.

Sb(V) is reduced to Sb(III), conjugated to $T[SH]_2$ and extruded by an ATP-coupled pump [11]. We were interested in the resistance mechanisms to GLU in a clone of the *L. tropica* line resistant to GLU *in vitro*. The resistant phenotype is stable for at least 6 months. In addition, the resistant line did not show cross-resistance to Pentostam, but a low level of resistance to arsenicals and antimonials was observed. The absence of cross-resistance between GLU and Pentostam has been previously described [35, 36]. These results would suggest that the resistance to GLU is related to the chemical structure of the drug and is probably associated with an efficient mechanism of reduction of the intracellular drug accumulation.

GSH has been shown to be an important molecule for protecting organisms from free radicals and toxic compounds [12]. In trypanosomatids, $T[SH]_2$ is the major thiol involved in the maintenance of intracellular thiol states and the defense against oxidative damage [13]. Recently, the presence of ovothiol A has been reported in Trypanosomatids [37, 38]. Ovothiol A has good antioxidant properties, similar to GSH and $T[SH]_2$, and represents approximately 30% of the total thiols in *Leishmania* [37]. *L. tarentolae* resistant to arsenite overproduced $T[SH]_2$, yielding a complex with As(III) and Sb(III) that is transported by an ATP-coupled efflux pump [11]. In *T. cruzi*, the different susceptibilities of several strains to nifurtimox and benznidazole was related to the concentrations of reduced thiols of these strains [15]. The toxic effect of nifurtimox and benznidazole may be explained, in part, as a consequence of a significant decrease in the concentrations of $T[SH]_2$ and GSH by their conjugation with metabolites of both drugs [17]. Cellular resistance to some anticancer drugs has been shown to be reversed by treatment of the cells with BSO. In *L. tarentolae*, resistant to arsenite, BSO can partly revert the resistant phenotype [18]. We have observed here that the treatment of parasites with BSO produced a thiol depletion that was accompanied by a substantial increase in the chemosensitiviy to GLU in the resistant parasites. However, the thiol analyses of the WT and GLU-R10 lines by HPLC, in the presence or absence of the drug, showed no differences in the concentrations of reduced thiols between these lines. The presence and quantity of ovothiol A in *L. tropica* was not determined. It is difficult to say whether ovothiol A is involved in the resistance to GLU, since reversion of GLU resistance was obtained after treatment with BSO, which has no effect on ovothiol metabolism. The absence of a relationship between the level of thiols and the reversion of GLU resistance after treatment with BSO was supported by Southern blot analysis using specific probes for γ-GCS, ornithine decarboxylase and trypanothione reductase genes. At the moment, we suggest that the resistant mechanism to GLU could be related to modifications in the membrane permeability of resistant parasites and/or the expression of a transporter involved in drug extrusion or in drug accumulation in intracellular organelles. The result of an increased sensitivity to CLB in resistant parasites supports the involvement of thiol metabolism as a cellular detoxification mechanism to antimony. The toxicity of

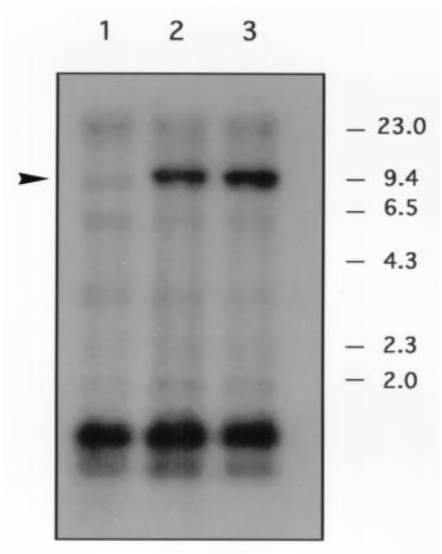

FIG. 2. Gene amplification in GLU-R10 *L. tropica* line. Three μg of genomic DNA from: WT parasites (lane 1), GLU-R10 parasites (lane 2) and GLU-R10 parasites grown in the absence of GLU for 1 month (lane 3) were digested with *Xho*I, electrophoresed in a 0.8% agarose gel and hybridized to the labeled G-circle. The arrow indicates the position of the amplified fragment.

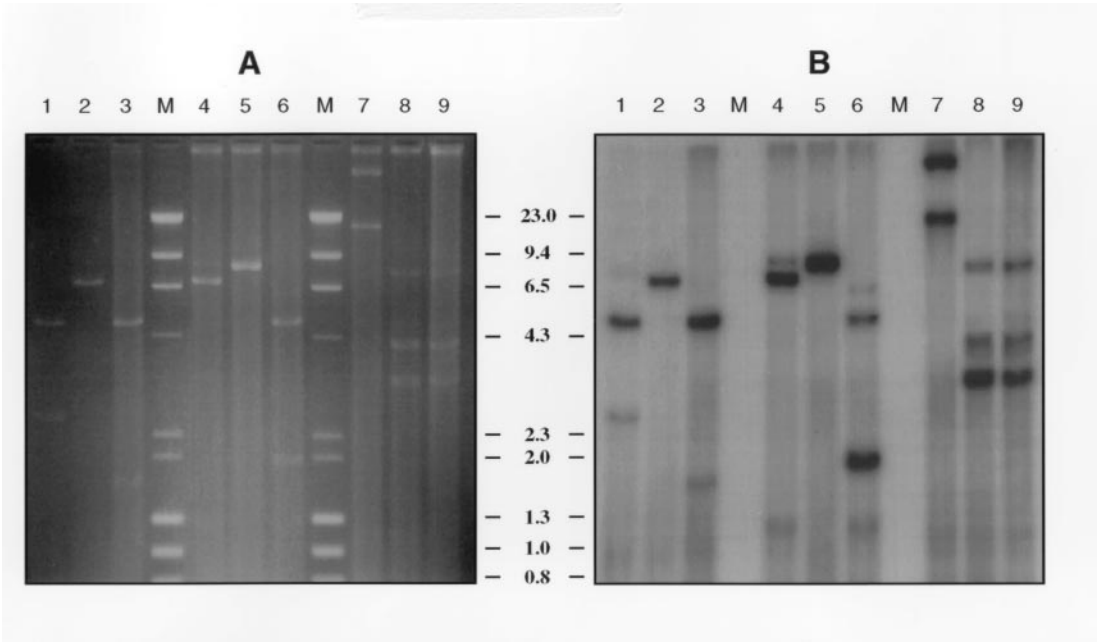

FIG. 3. Restriction map of G-circle from GLU-R10 *L. tropica* line. (A) Ethidium bromide-stained gel containing 0.1 μg of G-circle digested with different endonucleases, electrophoresed in a 0.8% agarose gel and hybridized to the labeled G-circle (B). Lane 1, *Apa*I; Lane 2, *Sac*I; lane 3, *Apa*I-*Sac*I; lane 4, *Sal*I; lane 5, *Xho*I; lane 6, *Sal*I-*Xho*I; lane 7, *Spe*I; lane 8, *Ksp*I; lane 9, *Spe*I-*Ksp*I; M, markers (kb) were derived from lambda phage DNA digested with the restriction endonuclease *Hin*dIII and from Ø phage DNA digested with *Hae*III.

CLB decreases when it is conjugated with GSH [33]. The increased sensitivity of resistant parasites to CLB is probably due to competition between CLB and Sb(III) for conjugation to GSH.

On the other hand, the MRP is an ATP-coupled transport pump that has been shown to extrude GSH conjugates from lung cancer cells [39, 40]. MRP confers arsenite resistance [41], and it has been postulated that MRP transports a GSH conjugate of arsenite [40]. In *Leishmania*, the transport properties of antimony-resistant mutants are indistinguishable from those of arsenite-resistant mutants [42]. Transfection experiments with *ltpgp*A from *L. tarentolae* give low levels of resistance to Pentostam, suggesting that mechanisms other than PgpA amplification could be involved in the resistance to pentostam [43]. Dey *et al.* (1996) have postulated that one possible mechanism of resistance to Pentostam may require reduction to Sb(III), conjugation with a thiol and extrusion of the complex by an ATP-coupled pump [10]. Recently, co-amplification of the γ-*GCS* and *Pgp*A genes in *L. tarentolae* arsenite-independent revertant parasites showed higher resistance levels to arsenite than expected from the individual contribution of both genes [18]. These results suggest that PgpA is a pump that transports As-thiol conjugates [18]. In the GLU-resistant line, the P-gps are not involved in resistance, as suggested by Southern blot analysis using specific probes for *Leishmania* Pgp-MDR- and Pgp-MRP-like genes. Pgp-MDR-like genes of *Leishmania* extrude hydrophobic drugs but not hydrophilic compounds such as Sb(III) and pentostam [44]. Also, Pgp-MRP-like genes transport hydrophilic compounds such as Sb(III), but the high level of resistance in *Leishmania* is associated with a significant increase in GSH levels [18]. The absence of amplification of MDR- and MRP-like genes in the resistant line does not prove that the corresponding proteins are not involved in GLU resistance, since mutations in the protein could increase the affinity for this compound. CHEF analysis reveals the presence in the resistant line of one circular extrachromosomal element, called the G-circle,

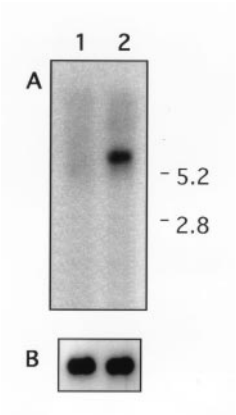

FIG. 4. Northern blot analysis of G-circle expression in GLU-R10 *L. tropica* line. (A) Poly(A)$^+$RNA (2 μg) from WT parasites (lane 1) and GLU-R10 parasites (lane 2) were electrophoresed on a 1% agarose/2.2 M formaldehyde gel, blotted and hybridized with the labeled G-circle. Then, the blot was stripped and hybridized with the β-tubulin probe from *T. cruzi* to monitor the amount of RNA in each lane (B). The RNA molecular weight marker (Kb) was from Promega.

that codes for a single transcript of 6 kb overexpressed in the resistant line. Experiments are in progress to identify the gene(s) located in the G-circle. This data will help us to elucidate the molecular mechanism involved in resistance to GLU in *L. tropica* as well as to design strategies to overcome the action of the coded proteins.

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