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Leishmania amazonensis: Multidrug Resistance in Vinblastine-Resistant Promastigotes Is Associated with Rhodamine 123 Efflux, DNA Amplification, and RNA Overexpression of a *Leishmania mdr1* Gene

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**Laboratorio de Parasitologia Molecular and †Laboratorio de Ultraestrutura Celular Hertha Meyer, Instituto de Biofísica Carlos Chagas Filho, UFRJ; ‡Departamento de Protozoologia, Instituto Oswaldo Cruz, FIOCRUZ, Rio de Janeiro, Brazil; and §Department of Tropical Public Health, Harvard School of Public Health, Boston, Massachusetts 02115, U.S.A.*

GUEIROS-FILHO, F. J., VIOLA, J. P. B., GOMES, F. C. A., FARINA, M., LINS, U., BERTHO, A. L., WIRTH, D. F., AND LOPES, U. G. 1995. *Leishmania amazonensis:* Multidrug resistance in vinblastine-resistant promastigotes is associated with rhodamine 123 efflux, DNA amplification, and RNA overexpression of a *Leishmania mdr1* gene. *Experimental Parasitology* **81,** 480–490. A vinblastine-resistant *Leishmania amazonensis* cell line (RV100) which exhibits cross-resistance to the unrelated drug adriamycin, and thus is considered to be multidrug resistant (MDR), was isolated after stepwise selection with increasing concentrations of vinblastine. This phenotype was partially reverted by the calcium channel antagonist verapamil. Drug transport studies using the hydrophobic fluorescent dye rhodamine 123 demonstrated that the MDR cell line has a reduced dye accumulation due to an increased efflux. Furthermore, DNA and RNA hybridization studies demonstrated that a gene (*lamdr1*), homologous to *ldmdr1* and *lemdr1*, was overexpressed and amplified within 27 kb extrachromosomal DNA circles (V-circles) in these cells. An independent cell line, RA5000, which was selected for resistance to adriamycin and was not cross-resistant to vinblastine, accumulated normal levels of rhodamine 123 and did not contain amplified DNA or overexpressed RNA of *mdr*-related sequences. © 1995 Academic Press, Inc.

INDEX DESCRIPTORS AND ABBREVIATIONS: *Leishmania amazonensis;* multidrug resistance (MDR); DNA amplification; P-glycoprotein (PGP); vinblastine; adriamycin; verapamil; rhodamine 123 (rho123).

INTRODUCTION

Therapeutic failure in human leishmaniasis is a relevant problem in several countries (Bryceson *et al.* 1985). An important component in treatment failure is the emergence of drug-resistant parasites. Besides the conventional treatment with antimonials, several other drugs have been tested in this infection (Berman 1988). Before new therapeutic agents are developed against leishmaniasis, it is important to investigate the possible mechanisms of resistance that occur in *Leishmania.*

DNA amplification, usually as extrachromosomal circular DNA molecules, is a common event found in many *Leishmania* species, and is associated with drug resistance in several cell lines (reviewed by Beverley 1991). In most of these systems, resistance to the drug is due to overproduction of its target enzyme mediated by an increase in copy number and, consequently, in RNA being expressed from the target enzyme gene. Besides overexpression of drug target enzymes, amplification of genes encoding PGP (P-glycoprotein)-like molecules in *Leishmania* has also been associated with the development of drug resistance. The first gene coding for a PGP homologue in *Leishmania* (*pgpA*) was described in the H region of methotrexate-resistant *Leishmania tarentolae* (Ouellette *et al.* 1990) and is also present in the H region of *Leishmania major* (Callahan and Beverley 1991). The *pgpA* gene is distantly related to the mammalian *MDR* genes and, accordingly, its overexpression does not confer a typical multidrug-resistance (MDR) phenotype but in-

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stead seems to confer low levels of resistance to arsenite and antimony (Callahan and Beverley 1991). Another class of PGP coding genes in *Leishmania* is exemplified by the *mdr1* genes of *Leishmania donovani* (Henderson *et al.* 1992) and *Leishmania enriettii* (Chow *et al.* 1993). These genes share higher sequence homology with the mammalian *MDR* genes and were found to be amplified and overexpressed in vinblastine-selected, multidrug-resistant *L. donovani* and *L. enriettii* cell lines (Henderson *et al.* 1992; Chow *et al.* 1993). Furthermore, functional assays by DNA transfection have confirmed *mdr1* as the gene responsible for the MDR phenotype (Henderson *et al.* 1992; Chow *et al.* 1993).

In mammalian cells the amplification and overexpression of *MDR* genes leads to the multidrug-resistant phenotype. The *MDR* genes encode large (170–200 kDa)-membrane glycoproteins that function as efflux pumps of broad specificity, extruding several unrelated hydrophobic natural compounds from the cytosol, thereby preventing their cytotoxic effects (reviewed by Endicott and Ling 1989; Gottesman and Pastan 1993). Several fluorescent dyes such as rhodamine 123 (rho123), which mimic the interaction of PGP with its substrates, have been used as molecular probes for the investigation of an active efflux pump in MDR cell lines. A strong correlation exists between decreased accumulation of these dyes and the MDR phenotype in mammalian cells (Neyfakh 1988; Weaver *et al.* 1991; Ludescher *et al.* 1992).

In this paper, we report the characterization of a vinblastine-resistant *Leishmania amazonensis* cell line (RV100) which was also resistant to adriamycin and verapamil. A sequence homologous to *ldmdr1* and *lemdr1* genes was overexpressed at the RNA level and amplified as extrachromosomal DNA circles (V-circles) in these cells. We also demonstrated that RV100 promastigotes extruded the fluorescent dye rhodamine 123.

Material and Methods

Chemicals. Vinblastine (Velbam) was purchased from Eli Lilly do Brasil Ltda. (São Paulo, SP, Brazil). Adriamycin, rhodamine 123, verapamil, and fetal calf serum were obtained from Sigma Chemical Co. (St. Louis, MO). All reagents used in the culture medium were purchased from Difco Laboratories (Detroit, MI). The restriction enzymes were obtained from New England Biolabs (Beverly, MA). All probes were labeled using [α-^{32}P]dATP purchased from Amersham International Inc. (UK).

Parasite maintenance and growth conditions. Promastigotes of *L. amazonensis* (IFLA/BR/67/PH8) were kindly provided by Dr. G. Grimaldi (Instituto Oswaldo Cruz, FIOCRUZ, Brazil) and maintained in liver infusion-trypticase (LIT) liquid medium supplemented with 10% fetal calf serum at 25°C (Jaffe *et al.* 1984). The doubling time was 12 h, and the cells were passaged weekly.

Selection of Leishmania drug-resistant cell lines. L. amazonensis promastigotes were selected by gradual increases in the concentration of the drug as described (Coderre *et al.* 1983). The RV100 cell line was grown for 2 weeks in medium supplemented with 10 μM vinblastine. The cells were then transferred to medium containing 20 μM vinblastine and allowed to grow for 2 weeks. This process was repeated every 2 weeks until the promastigotes were able to grow at 100 μM vinblastine. The RA5000 cell line was also selected as described above, starting with 0.1 μM adriamycin, and it was selected in steps until it grew normally at a concentration of 5 μM. Continuous drug pressure was maintained during all experiments.

The effective concentration of drug which inhibited the growth of the cells by 50% (EC_{50}) was calculated at late stages of exponential growth for both the wild-type and resistant cell lines (Ullman *et al.* 1989).

Reversal of drug resistance. Verapamil was used as a reversal agent at 5 μM, which did not inhibit promastigote growth. The EC_{50} value for vinblastine of RV100 cell line was obtained by means of simultaneous incubation of cells with verapamil and varying concentrations of vinblastine.

Rhodamine 123 staining and analysis. All experiments were performed with promastigote cells collected on the third day of cell growth (exponential phase). The wild-type strain, RV100, and RA5000 cell lines were incubated in liquid medium with 5 μg/ml of rho123 for 60 min at 25°C and washed with PBS (pH 7.4). The cell population analysis was performed with a Coulter EPICS 751 flow cytometer. Rho123 was excited at 488 nm by an argon ion laser and the fluorescence was collected through a 575-nm band pass filter. The cells were also observed by fluorescence and differential interference contrast (DIC) under a Zeiss Axioplan light microscope using a silicon intensifier target TV camera (SIT 66—DAGE MTI) attached to a digital image processing system (IBAS-Zeiss). The gain control of the TV camera was adjusted to a constant value to allow for direct comparison between different fluorescence intensities. The prints were obtained using identical conditions of light and developing time for different samples.

Analysis of fluorescent dye extrusion. The wild-type strain, RV100, and RA5000 cell lines were incubated for 5 min at 25°C with 20 μg/ml of rho123 and then washed with PBS (pH 7.4). The samples were resuspended in medium

without rho123 followed by flow cytometry at different time points (0, 15, 30, and 60 min) to observe cell staining. Further characterization of dye efflux was obtained by supernatant transfer of the wild-type and resistant cell lines. After 60 min of incubation as described above, the cells were collected by centrifugation and the supernatant of each sample was transferred to nontreated wild-type cells, which were incubated for 60 min and washed before analysis.

Nucleic acid extraction. Total DNA and RNA were isolated from promastigotes according to Landfear and Wirth (1985). Circular extrachromosomal DNA was purified by the alkaline lysis procedure followed by CsCl-EtBr density gradient centrifugation (Sambrook *et al.* 1989; Katakura and Chang 1989).

Southern and Northern blot analysis. Southern blot analysis was performed using standard procedures (Sambrook *et al.* 1989). For Northern analysis, total RNA was fractionated in formaldehyde agarose gels and blotted onto nitrocellulose membranes according to Sambrook *et al.* (1989). Prehybridization was carried out at 42°C for at least 12 hr in a solution containing 10% dextran sulfate, 1 *M* NaCl, 1% SDS, and 50% formamide. Hybridization was carried out in the same solution in the presence of no more than 5×10^5 cpm/ml of probe. Final washes were done at 50°C with 0.1× SSC and 0.5% SDS. The probe LEMDR06 corresponded to one of the conserved nucleotide binding sites of *lemdr1*, originally obtained by PCR amplification of *L. enriettii* genomic DNA (Henderson *et al.* 1992). Nucleic acid loading in Northern and Southern analysis was standardized using an unrelated gene, the promastigote-specific glucose transporter Pro G1 (Bradley *et al.* 1989), as an internal control. All probes were labeled using [α-^{32}P]dATP by the random primer method (Feinberg and Vogelstein 1983).

Transverse alternating field electrophoresis (TAFE). *Leishmania* chromosomes were separated using the TAFE apparatus (GeneLine, Beckman). Cell plugs were prepared according to the manufacturer's protocol.

In order to investigate the physical structure of the amplified DNA molecules, chromosome plugs of the RV100 cell line were subjected to gamma irradiation (Beverley 1989). The radiation doses ranged from 3–100 krad. An ICN GR9 irradiator, with a ^{60}Co source, was employed for these experiments.

Results

Characterization of the drug resistance phenotype in the selected cell lines. In order to investigate the phenomenon of multidrug resistance in *L. amazonensis*, two independent cell lines, one resistant to vinblastine (RV100) and the other to adriamycin (RA5000), were selected by a stepwise procedure. As shown in Table I the RV100 cell line is 15-fold more resistant to vinblastine than the wild-type strain, whereas the RA5000 cell line is 16-fold more resistant to adriamycin. We tested RV100 for resistance to adriamycin and found it to be 6-fold more resistant than the wild-type parental strain (Table I). Moreover, RV100 cells are also 8-fold more resistant to verapamil than the wild-type strain (Table I). These data suggested that the RV100 cell line exhibits a MDR phenotype. In contrast, the RA5000 cell line did not exhibit significant cross-resistance to the unrelated drug vinblastine (Table I).

Multiple drug resistance mediated by PGP can be reversed by some calcium channel blockers such as verapamil. We thus tested the effect of verapamil on the resistance of RV100 to vinblastine. When verapamil was used in conjunction with vinblastine, a 2.5-fold decrease in the resistance to this drug was observed (Table I). The verapamil concentration used in these experiments was lower than the EC_{50} levels esti-

TABLE I
Drug Resistance in *Leishmania amazonensis* Cell Lines[a]

Cell lines	Drug selection	Vinblastine EC_{50}[b] (fold)[c]	Adriamycin EC_{50}[b] (fold)[c]	Verapamil EC_{50}[b] (fold)[c]	Reversion of drug resistance EC_{50}[d] (fold)[c]
Wild-type	—	12 μ*M*	0.3 μ*M*	4 μ*M*	—
RV100	Vinblastine	180 μ*M* (15)	1.7 μ*M* (6)	30 μ*M* (8)	80 μ*M* (6)[e]
RA5000	Adriamycin	18 μ*M* (1.5)	4.8 μ*M* (16)	ND[f]	ND[f]

[a] These results are representative of two sets of different experiments.
[b] Effective concentration of drug which inhibits the cell growth by 50%.
[c] Fold of drug resistance when compared with wild-type strain.
[d] Effective concentration of drug which inhibits the cell growth by 50% in presence of 5 μ*M* verapamil.
[e] Verapamil concentration did not inhibit the cell growth.
[f] Not determined.

mated for RV100 cells (Table I). In fact, RV100 cell line growth in the presence of 5 μM verapamil exhibited only 5% of inhibition (data not shown).

Drug accumulation studies using the fluorescent probe rho123. We used the hydrophobic fluorescent dye rho123 to investigate whether the drug-resistant phenotypes of RV100 and RA5000 were associated with reduced accumulation of this dye. When incubated with 5 μg/ml of rho123 both wild-type and resistant *L. amazonensis* cells accumulated the dye in a time-dependent manner until saturation. Maximal accumulation was achieved after 15 min in the case of the wild-type cells (data not shown). Fluorescence microscopic analysis of cells treated with 5 μg/ml of rho123 for 60 min demonstrated a remarkable difference in rho123 staining between wild-type and RV100 cells (Fig. 1). However, such a difference was not observed between the wild-type and RA5000 cell lines (data not shown). A more quantitative analysis of rho123 accumulation was obtained with flow cytometry, which demonstrated that only 19% of RV100 cells exhibited fluorescence above the baseline compared to the wild-type and RA5000 promastigotes at 95% and 91%, respectively (Fig. 2).

Determination of rho123 efflux in wild-type and drug-resistant cell lines. Reduced drug ac-

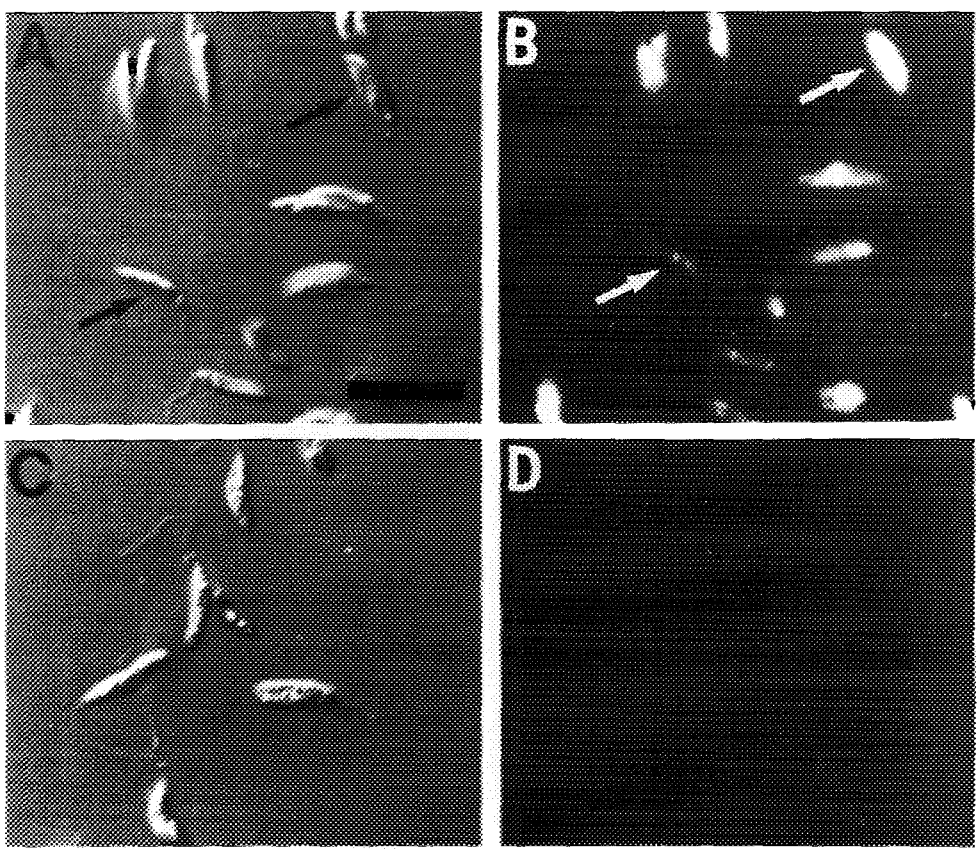

FIG. 1. Staining analysis of wild-type strain (A and B) and RV100 cell line (C and D), incubated with 5 μg/ml of rho123 for 60 min. (A and C) DIC images of a suspension of free promastigote cells. (B and D) Corresponding fluorescence images of the same fields. All the images were obtained digitally by averaging two video frames, normalizing the 255 grey value to the brightest cell of B. Arrows in A and B indicate the corresponding cells. Bar, 10 μm for A–D.

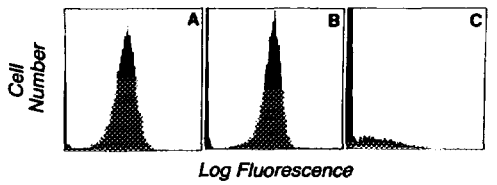

FIG. 2. Rho123 fluorescence histograms showing the dye staining of *L. amazonensis* promastigotes: wild-type strain (A), RA5000 (B), and RV100 (C) cell lines, incubated with 5 μg/ml of rho123 for 60 min. The fluorescence intensity was analyzed by flow cytometry.

cumulation in MDR cells is caused by an increased drug efflux mediated by PGP. Therefore, we tested the ability of RV100 cells to extrude rho123. Wild-type, RA5000, and RV100 promastigotes were initially loaded with 20 μg/ml of rho123 for 5 min. After washing and transferrence to rho123-free medium, the level of fluorescence was determined at different time points. A dramatic reduction of rho123 staining was observed at 30 and 60 min in RV100 cells (from 73% at time 0 to 25% and 22%, respectively) (Fig. 3A). However, wild-type and RA5000 cells sustained high levels of fluorescence comparable to the initial staining at all time points tested. The efflux of rho123 by RV100 cells was confirmed by means of transferrence of supernatant from wild-type, RA5000, and RV100 cell lines to wild-type promastigotes. As predicted, the supernatant of treated RV100 cells stained 39% of wild-type promastigotes, while only 8 and 13% of naive cells were stained by wild-type and RA5000 supernatant, respectively (Fig. 3B).

Analysis of DNA amplification and RNA overexpression of a Leishmania mdr1 gene. Southern blot analysis of *SacI*-digested DNA derived from the wild-type, RA5000, and RV100 cell lines with the probe LEMDR06 demonstrated a stronger 1.7-kb band for RV100, indicating the amplification of a *lemdr1/ldmdr1* homologue (Fig. 4). The amount of DNA from each line loaded in the gel was similar, as shown by rehybridization of the filter with the control probe Pro-G1.

To evaluate the expression of the *mdr1* gene in *L. amazonensis* wild-type and resistant cell lines, Northern blot analysis was performed. As shown in Fig. 5A, the LEMDR06 probe recognized a 12-kb RNA expressed at high levels in the RV100 cell line, similar in size to the one overexpressed in MDR *L. donovani* and *L. enrietti* (Henderson *et al.* 1992; Wong *et al.* 1994). The membrane was subsequently hybrid-

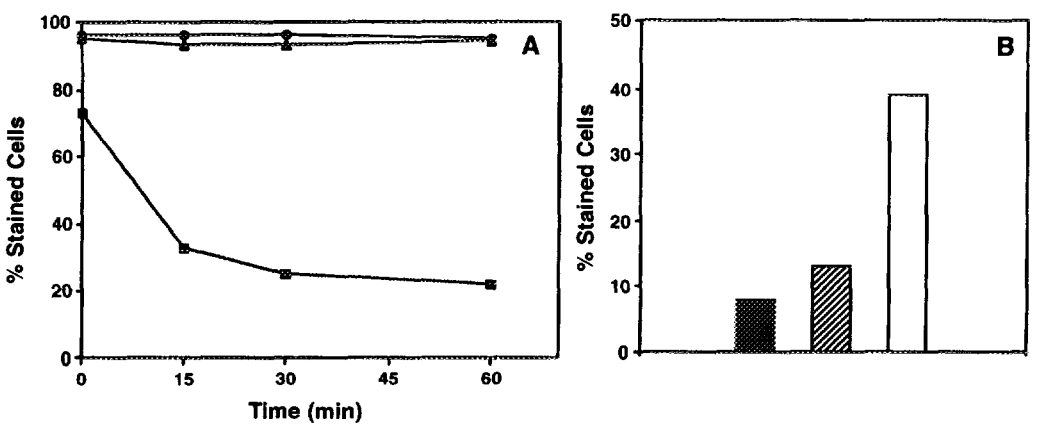

FIG. 3. Rho123 efflux experiment of *L. amazonensis* drug-sensitive and drug-resistant cell lines. (A) Wild-type strain (●), RA5000 (▲), and RV100 (■) cell lines were incubated with 20 μg/ml of rho123 for 5 min, washed with PBS (pH 7.4), and resuspended in rho123-free medium followed by incubation for increasing time points. (B) The cell-free conditioned medium at the 60-min time point of A, from wild type (filled bar), RA5000 (hatched bar), and RV100 (open bar), were transferred to recipient untreated wild-type promastigotes which were incubated for a further 60 min. The fluorescence intensity was analyzed by flow cytometry. These results are representative of two sets of different experiments.

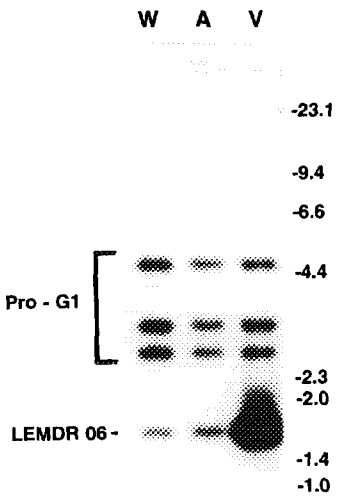

FIG. 4. Southern blot analysis of wild-type and drug-resistant *L. amazonensis*. Total DNA (5 μg) was digested with *Sac*I and separated by 0.8% agarose gel. The gel was blotted onto a nylon membrane and hybridized, at high stringency, with the PGP-specific probe LEMDR06 and subsequently with the Pro-G1 DNA fragment. W, wild type; A, RA5000; V, RV100. Pro-G1 and LEMDR06 indicate the specific bands recognized by these probes. The molecular weight markers (kb) are indicated on the right.

ized with the Pro-G1 probe (Fig. 5B). Thus, the MDR phenotype in the RV100 cell line seems to be associated with amplification and overexpression of the *L. amazonensis* homologue of *mdr1*, hereafter designated *lamdr1*.

Pulsed-field analysis and purification of the amplified DNA. Amplified DNAs in drug-resistant *Leishmania* are frequently found as extrachromosomal circles (Beverley 1991). These molecules display a particular eletrophoretic profile in pulsed-field electrophoresis and therefore can be easily identified in chromosome gels (Beverley 1988). The chromosomal location and physical nature of the amplified DNA in the RV100 cell line was investigated using TAFE gels. Two high-molecular-weight class chromosomes of 1.05 and 0.915 Mb hybridized with the LEMDR06 probe in the wild-type, RA5000, and RV100 cell lines (Fig. 6A). In RV100 DNA an additional hybridization band migrated between the 0.375 and 0.45-Mb molecular weight markers. This band showed a pattern of smeared hybridization with material retained in the gel slot compatible with the one displayed by extrachromosomal circular DNA (Fig. 6A). To confirm the structure of the amplified DNA, chromosomal plugs of RV100 were submitted to increasing doses of gamma irradiation and analyzed by TAFE. A shift in the mobility of the bands hybridizing to LEMDR06 was observed consistent with the conversion of closed circular to linear DNA (Fig. 6B). Figure 6B also shows that the circular amplified DNA of RV100 is composed of two species: one faster and less abundant and one slower and more abundant. The two species are also seen in shorter exposures of the blot in Fig. 6A (data not shown). These may represent either circles of different sizes or multimers. The size of the lighter band (Fig. 6B, 100 krad), around 27 kb, is approximately half the size of the heavier band, 50 kb.

The circular DNA molecules, named V-cir-

FIG. 5. Analysis of *lamdr1* expression level. Total RNA (15 μg) of *L. amazonensis* cell lines was extracted and separated on a 0.9% agarose–formaldehyde (1.2%) gel. The gel was blotted onto a nylon membrane and hybridized, at high stringency, with LEMDR06 (A), striped, and rehybridized with Pro-G1 (B). W, wild type; A, RA5000; V, RV100. The migration of molecular weight markers (kb) is indicated.

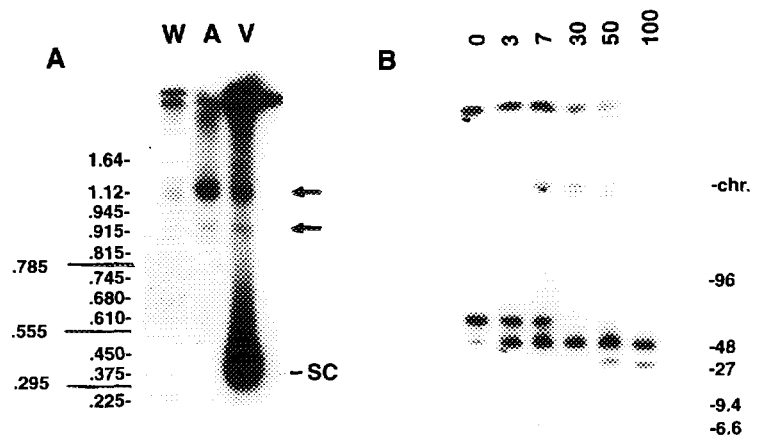

FIG. 6. (A) Chromosomal Southern blot of *L. amazonensis* cell lines hybridized with LEMDR06. Chromosomes of *L. amazonensis* were separated by TAFE electrophoresis for 22 hr, with 1′ pulse time. W, wild type; A, RA5000; V, RV100. The DNAs marked by arrows correspond to the *lamdr1* chromosomal copies. SC indicates the position of the circular DNAs (supercoiled). Yeast chromosomes size markers are indicated on the left (Mb). (B) Southern blot of TAFE electrophoresis of irradiated chromosome blocks of RV100, hybridized with LEMDR06. Numbers above the lanes correspond to radiation dose (krad). Electrophoresis was carried out for 12 hr, with a 4-s pulse time. Molecular weight markers are indicated on the right (kb). Chromosomes position (chr).

cles, were purified by the alkaline lysis method, followed by a CsCl-EtBr gradient. The supercoiled fraction was collected, digested with *Bgl*II, and then separated in a 0.7% agarose gel (Fig. 7A, left panel, lane C). The restriction digestion profile of the purified circle matched that of the extra bands shown in the *Bgl*II-digested total DNA, derived from the RV100 cell line (Fig. 7A, left panel, lane V). The size of the amplicon, as measured by the sum of the restriction fragments of the circle digested with *Bgl*II (Fig. 7A, left panel, lane C) and other restriction enzymes (data not shown), is calculated to be approximately 25 kb, in agreement with the size found above, after the gamma irradiation. The gel in Fig. 7A (left panel) was transferred and hybridized to LEMDR06 in order to confirm that the purified circular DNA corresponded to the amplified DNA of RV100 (Fig. 7A, right panel). Purified circular DNA and cloned fragments derived from it were used for the construction of the restriction site map of the V-circle shown in Fig. 7B.

DISCUSSION

We have developed two independent *L. amazonensis* cell lines: RV100 and RA5000, resistant to vinblastine and adriamycin, respectively (Table I). The RV100 cell line exhibited cross-resistance to the chemically unrelated drug adriamycin and was also resistant to the chemosensitizing agent verapamil. In contrast, the RA5000 lineage was solely resistant to adriamycin (Table I). Verapamil is a substrate for PGP and seems to share closely related binding sites with antitumor drugs (Cornwell *et al.* 1987; Kiyama *et al.* 1988; Safa 1988). It has also been demonstrated that MDR cell lines exhibit a reduced intracellular accumulation of verapamil, and collateral resistance against this calcium channel antagonist has been observed in MDR mouse tumor cells (Yusa and Tsuruo, 1989; Reeve *et al.* 1989). Our data are in good agreement with these findings.

The RV100 cells demonstrated a partial reversal of resistance by verapamil at 5 μM (Table I). The 2.5-fold reversal of drug resistance observed in RV100 cells was comparable to those described in MDR mammalian cell lines, in which verapamil exhibits a wide range of chemosensitizing activity (reviewed by Ford and Hait 1990). The fact that in those assays we

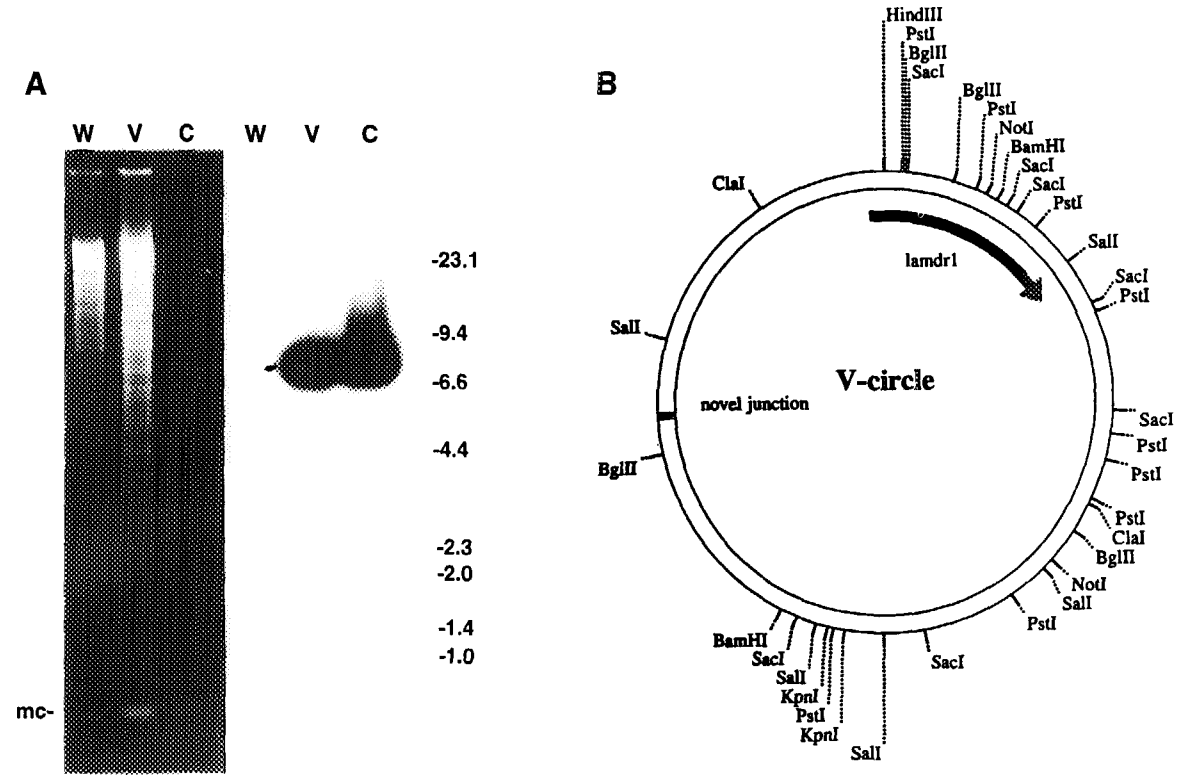

FIG. 7. (A) Alkaline lysis purification of the V-circle. The V-circle was isolated by alkaline lysis followed by CsCl-EtBr gradient centrifugation digested with *Bgl*II and separated on a 0.7% agarose gel (left panel). The gel was blotted onto a nylon membrane and hybridized, at high strigency, with the LEMDR06 probe (right panel). W, wild-type total DNA; V, RV100 total DNA; C, alkaline lysis purified fraction of RV100 DNA. Minicircles that contaminate the V-circle preparation (mc) are indicated. Molecular weights (kb) are indicated on the right. (B) Restriction site map of purified V-circle. Note that not every site for *Pst*I and *Sac*I enzymes could be assigned. The recombination site (novel junction) and localization of *lamdr1* gene are indicated.

used a concentration of verapamil well below the EC_{50} (30 μM) exhibited by RV100 suggests that the reversal is not due to combined toxicity of the two drugs used in the experiment. Our data, however, cannot rule out that the verapamil effect is due to an inespecific alteration in the permeability of RV100 cells. It is interesting to note that other groups which described MDR *Leishmania* strains could not find this chemosensitization effect, and the toxic effect of verapamil in these selected lineages has been emphasized (Henderson *et al.* 1992; Chow *et al.* 1993). However, reversal of drug resistance by verapamil in kinetoplastids is not unprecedented and has been observed in *Trypanosoma cruzi* and *L. donovani* (Neal *et al.* 1989). This apparent contradiction may reflect differences between our *L. amazonensis* MDR line and the species and strains used in such studies.

Neyfakh (1988) demonstrated the extrusion of hydrophobic fluorescent dyes such as rho123 from mammalian drug-resistant cell lines. Moreover, transfection of human *MDR1* gene in NIH3T3 cell lines leads to the extrusion of rho123 by PGP (Weaver *et al.* 1991). In fact, rho123 seems to be an excellent PGP substrate (reviewed by Gottesman and Pastan 1993). Recently, Nare *et al.* (1994), using a photoactive derivative of rho123, demonstrated the direct binding of this compound to PGP and mapped the binding region as a 6-kDa peptide which is also targeted by other hydrophobic PGP substrates such as vinblastine and verapamil. Using rho123 as a model substrate, we could clearly demonstrate that the MDR phenotype of RV100 promastigotes correlates with a very poor accumulation of rho123 (Figs. 1 and 2). The reduced accumulation of rho123 in RV100 cells is caused by an increased efflux of this dye, as determined by the fluorescence levels of rho123-loaded cells at several time points after incubation in dye-free medium (Fig. 3A). Confirming that the decrease in staining of RV100 cell lines was due to efflux and not to some type of metabolization of the dye, the supernatant of RV100 cells stained untreated wild-type cells (Fig. 3B). Taken together, these data demonstrated that the MDR phenotype exhibited by RV100 is likely to be caused by increased extrusion of hydrophobic compounds.

Molecular analysis of the RV100 cell line demonstrated a DNA amplification containing a gene homologous to the *Leishmania mdr1* genes which were found amplified in MDR *L. donovani* and *L. enriettii* cell lines initially selected with vinblastine (Henderson *et al.* 1992; Chow *et al.* 1993) (Fig. 4). This amplified DNA is not related to other amplifications such as the R and H regions identified in methotrexate-resistant *Leishmania* (Beverley *et al.* 1984) (data not shown). The characterization of the *L. amazonensis mdr1* counterpart, *lamdr1*, is being carried out and the partial sequence analysis of this gene revealed a high homology (85–90% at the amino acid level) with the other *Leishmania mdr1* members (Araripe and Lopes, unpublished).

The DNA amplification observed in the RV100 cell line is a circular extrachromosomal element. This was determined by migration in TAFE gels under different pulse conditions and after gamma irradiation (Fig. 6). The estimated molecular weight of the smallest amplified species after linearization by gamma rays is basically the same as the molecular weight estimated by restriction analysis of purified circular DNA (27 vs 25 kb). This strongly suggests that the two species of amplified DNA detected in pulsed-field gels are monomers and dimers of an amplification unit. Furthermore, it indicates that the V-circle is a direct amplification, similar in overall structure to most of the amplified DNAs found in *Leishmania* (Beverley 1991). The V-circle of *L. enriettii* contains 35–40 kb of amplified DNA (Chow *et al.* 1993), whereas the V-circle of *L. amazonensis* characterized here encompasses only 25–27 kb of chromosomal DNA. It is currently unknown how much homology exists between these amplified elements beyond the *mdr1* genes.

Northern blot analysis demonstrated the overexpression of a 12-kb RNA molecule in RV100 cells which hybridized with the LEMDR06 probe. This transcript was also detected at low levels in both wild-type and RA5000 RNAs, suggesting expression of this gene in wild-type *Leishmania* cells. Strikingly,

this RNA is larger than the 4.5- to 5.0-kb transcripts encoding mammalian *MDR* genes (Chen *et al.* 1986; Gros *et al.* 1986). A similarly large (10.5–12 kb) *mdr1* transcript was also found in vinblastine-resistant *L. donovani* (Henderson *et al.* 1992) and *L. enriettii* (Wong *et al.* 1994). In *Plasmodium falciparum*, *pfmdr1* transcripts are also much larger than the predicted coding region of the gene (8 vs 4.5 kb) (Foote *et al.* 1989; Wilson *et al.* 1989). The nature of these large *mdr* RNAs in protozoan parasites is unclear and may contain extensive noncoding sequences. The coding region of the *mdr1* gene in *L. enriettii* is 3.84 kb (Chow *et al.* 1993).

The amplification of distinct PGP sequences found in *Leishmania* is associated with different drug-resistant phenotypes. Transfection experiments with the *L. major* counterpart of *pgpA* showed that this gene, when overexpressed, is able to confer resistance to oxyanions such as arsenite and trivalent antimony, but not to methotrexate or vinblastine (Callahan and Beverley 1991). The *ldmdr1* and *lemdr1* genes, on the other hand, were implicated by transfection as responsible for the resistance to hydrophobic natural compounds such as *Vinca* alkaloids and anthracyclins shown by MDR *L. donovani* and *L. enriettii* (Henderson *et al.* 1992; Chow *et al.* 1993). Despite the lack of transfection assays for *lamdr1* function, we assume, based on the similarity between the phenotype of RV100 and the phenotype of the other MDR *Leishmania* and on the high level of homology between the three *mdr1* genes, that *lamdr1* overexpression is ultimately responsible for the RV100 MDR phenotype. In addition, using probes of other putative *Leishmania mdr* genes, such as the PCR-amplified nucleotide binding sites pLa08 (Gueiros-Filho *et al.* 1992) and LEMDRF2 (Henderson *et al.* 1992), we did not detect any other *mdr* amplification in the RV100 genome that could explain its resistance profile (data not shown).

The adriomycin-resistant RA5000 cell line did not demonstrate cross-resistance to the unrelated drug vinblastine and did not extrude the fluorescent probe rho123. Furthermore, this resistant cell line did not exhibit amplification or overexpression of any known *mdr*-like *Leishmania* genes. The resistance of RA5000 cell line to adriamycin, which is also a substrate for PGP, could be due to a PGP-independent mechanism described in other systems (Deffie *et al.* 1988).

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