Pergamon

Biochemical Pharmacology, Vol. 47, No. 11, pp. 1939–1947, 1994.

0006-2952/94 $7.00 + 0.00

0006-2952(94)E0100-Y

P-GLYCOPROTEIN OVEREXPRESSION IN METHOTREXATE-RESISTANT *LEISHMANIA TROPICA*

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(*Received* 30 *September* 1993; *accepted* 18 *February* 1994)

Abstract—A methotrexate (MTX)-resistant *Leishmania tropica* line develops a stable drug-resistant phenotype in which the resistance mechanism is associated with a significant reduction in MTX accumulation. After a 2 hr exposure to [^{3}H]MTX, a *L. tropica* line resistant to 1000 μM of MTX did not accumulate more than 3% of the amount of drug incorporated by wild-type cells. The same resistant cell line was found to be cross-resistant to several unrelated drugs. The monoclonal antibody C219, directed against the cytoplasmic domain of mammalian *P*-glycoproteins, recognized a putative *P*-glycoprotein of 240 kDa overexpressed in the resistant line. Also, this resistant line showed the overexpression of the putative homolog of the *ltpgpE* gene, as determined by northern blot analysis using gene-specific probes for the *P*-glycoprotein genes of *Leishmania tarentolae*. This overexpression was not correlated with a proportional increase in the copy number of the gene, but Southern blot analysis suggested that the *ltpgpE* homolog was overexpressed as a consequence of gene rearrangement. This would be considered as an epiphenomenon that probably does not arise from the same MTX-resistant mechanism.

Key words: Leishmania tropica; methotrexate resistant; altered transport; *P*-glycoprotein; gene overexpression

Drug resistance in parasites can be caused by at least three different mechanisms: decreased uptake of the drug [1–4], amplification of the gene for the drug target enzyme [5–8], and structural and functional changes in the target enzyme [9–12].

In the protozoan parasite *Leishmania*, resistance to the dihydrofolate reductase inhibitor MTX§ may be due to a different independent mechanism such as decreased uptake of MTX [1, 2], amplification of the gene coding for the bifunctional dihydrofolate reductase–thymidylate synthase (DHFR–TS) [5, 6] and, finally, the amplification of extrachromosomal H circles [13–19].

At least two drug-resistant genes are present on the H circle. The first gene described, *ltpgpA* in *Leishmania tarentolae* [20] and *lmpgpA* in *Leishmania major* [21], is related to the mammalian *P*-glycoprotein involved in the multidrug resistance (MDR) phenotype in cancer cells [22]. The second gene, *ltdh* in *L. tarentolae* [23] and *hmtx* in *L. major* [24], is a short chain dehydrogenase involved in high level antifolate resistance. The *P*-glycoproteins *ltpgpA* and *lmpgpA* have been associated with low level resistance to oxyanions, but not with MTX resistance [21]. *P*-glycoprotein gene amplification has also been noted in chloroquine-resistant *Plasmodium falciparum* [25–28], in emetine-resistant *Entamoeba histolytica* [29] and in vinblastine-resistant *Leishmania donovani* [30].

In this study, we describe a *Leishmania tropica* cell line selected for resistance to MTX that develops significantly decreased MTX accumulation as a resistance mechanism. Also, the same MTX-resistant cell line developed resistance to a number of unrelated drugs, some of which are part of the MDR spectrum. This putative MDR phenotype was correlated with the overexpression of a 6 kb transcript, hybridizing with a specific probe for the *ltpgpE* gene of *L. tarentolae* and to the overproduction of a protein recognized by the anti-*P*-glycoprotein mammalian monoclonal antibody C219 [31]. We suggest that the overexpression of this putative *P*-glycoprotein is not due to gene amplification but to gene rearrangement, and it may be considered as an epiphenomenon that is associated with the MTX resistance.

MATERIALS AND METHODS

Parasites and maintenance of culture. L. tropica LRC-L39 was obtained from Dr L. F. Schnur (Hebrew University, Jerusalem, Israel). Promastigotes were grown at 28° in RPMI 1640 modified medium (Gibco, Middlesex, U.K.), as previously described [32], and supplemented with 20% heat-inactivated fetal bovine serum (Flow Laboratories, U.K.).

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§ Abbreviations: MTX, methotrexate; MTX-R1000, resistant line to 1000 μM of MTX; Rv_3 and Rv_6, MTX-R1000 lines grown in drug absence over 3 and 6 months, respectively.

Drugs and reagents. Methotrexate was purchased from Cyanamid Iberica S.A. (Division Lederle, Madrid, Spain). Glucantime was purchased from Rhône-Poulenc Farma, S.A.E. (Madrid, Spain). Vinblastine was purchased from Lilly S.A. (Madrid, Spain); doxorubicin hydrochloride and daunorubicin were from Farmitalia Carlo Erba (Madrid, Spain). Ketoconazole, puromycin, sodium arsenate and antimony trichloride were from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Drugs were dissolved in the culture medium on the day of use and filtered through a 0.2 μm membrane filter (Millipore, Bedford, U.S.A.). [3,4-^{3}H]Methotrexate ([^{3}H]MTX) (48.7 Ci/mmol) was purchased from Du Pont de Nemours (Germany) GmbH, NEN Division. All other chemicals were of the best available grade.

Selection of MTX-resistant cell lines. An MTX-resistant line of *L. tropica* was obtained using a stepwise selection process previously described [5], using MTX concentrations of 5, 10, 20, 50, 100, 500 and 1000 μM. Logarithmic phase cells were seeded at a concentration of 4×10^6 cells/mL in medium containing MTX. The culture densities were determined by daily counting in a hemocytometer chamber where the parasites were previously fixed with 0.36% formaldehyde in phosphate-buffered saline (PBS: 1.2 mM KH_2PO_4, 8.1 mM Na_2HPO_4, 130 mM NaCl, 2.6 mM KCl adjusted to pH 7.4). Cells were seeded into the next higher concentration of MTX when the cell doubling time was stabilized, usually after five passages (3 generations each).

Cross-resistance to other drugs. The MTX-R1000 *L. tropica* line was analyzed for resistance to other unrelated drugs such as puromycin, ketoconazole, glucantime, vinblastine, doxorubicin, daunorubicin, antimony trichloride and sodium arsenate. The IC_{50} (concentration of drug which decreases the rate of cell growth by 50%) and the resistance indexes (average ratio of resistant cell line IC_{50} divided by the wild-type IC_{50}) were determined for both the wild-type and MTX-R1000 as previously described [18].

MTX uptake studies. To compare the abilities of wild-type and MTX-R1000 to take up [^{3}H]MTX from the culture medium, cells were harvested by centrifugation, washed with PBS and resuspended in folate-deficient medium [33], at a density of 10^7 cells/mL. The uptake measurements were initiated by mixing, in an Eppendorf microfuge tube, 100 μL of 200 nM [^{3}H]MTX in folate-deficient medium, with 100 μL of cells, and layered over 200 μL of chemically inert dibutyl phthalate (Sigma). Aliquots obtained at intervals of 15, 30, 60 and 120 min of incubation at 28° or 0°, were centrifuged through the dibutyl phthalate at 12,000 *g* for 1 min. The upper aqueous phase was removed by aspiration and the walls of the tube were washed three times with PBS. The dibutyl phthalate was aspirated and the cell pellets were treated as previously described [2]. Radioactive MTX was determined by liquid scintillation counting.

In order to estimate the transport kinetic parameters such as the apparent affinity for MTX and the maximal velocity of influx, we used the Lineweaver–Burk analysis, as described for *Leishmania donovani* by Kaur *et al.* [2]. In this analysis, each value is the average of 3 data points obtained after exposure of cells to different concentrations (0.125, 0.25, 0.5, 0.75 and 1 μM) of [^{3}H]MTX in folate-deficient medium. The assay was then terminated at 1 and 2 min time intervals by separation of the cells from the exogenous radioactive ligand by the method described above.

Metabolic labelling of parasites. Parasites at a concentration of 2×10^7/mL, were labelled at 28° for 2 hr in Hanks' balanced salt solution containing 25 mM Hepes (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) and L-[^{35}S]methionine (Du Pont-New England Nuclear; specific activity 1000 Ci/mmol) at 10 μCi/mL. After labelling, parasites were washed five times with PBS. Radioactivity was equalized for individual samples before loading into sodium dodecyl sulfate (SDS) 6% polyacrylamide gel [34]. After electrophoresis, the gels were dried under vacuum and autoradiographed.

Preparation of the crude membrane fraction. For preparation of the crude membrane fraction, the method of Ghosh *et al.* [35] was followed with some modifications. Cells were washed with PBS, suspended at 10^9 cells/mL in hypotonic lysis buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 100 μg/mL aprotinin, 100 μg/mL leupeptin), and disrupted by three freeze–thaw cycles. The lysate was centrifuged at 12,000 *g* for 10 sec, the supernatant was diluted with 2 volumes of lysis buffer, centrifuged at 100,000 *g* for 30 min, and the resulting pellet was used as a crude membrane fraction. The membrane preparation was suspended in lysis buffer containing 10% (v/v) glycerol and stored at −80° until use. The enrichment in plasma membrane fraction was determined by the enzyme marker activity acid phosphatase, which was previously described and demonstrated to be a useful marker for such membranes [36]. Acid phosphatase activity was measured according to the method previously described [36].

Immunoblots and indirect immunofluorescence. Protein determinations were performed by the method of Lowry *et al.* [37]. Membrane proteins (15 μg) and total cell proteins (50 μg) were separated on a 6% SDS–polyacrylamide gel, and electrotransferred to nitrocellulose paper [38]. Blocking was performed with skimmed milk (5% in PBS). The blots were incubated with 2 μg/mL of the anti-*P*-glycoprotein monoclonal antibody C219 (kindly supplied by Dr Victor Ling, Ontario Cancer Institute, Canada), diluted in buffer A (PBS, 0.1% bovine serum albumin, 0.1% Tween 20), for 5 hr at room temperature. After washing with buffer A, bound antibodies were detected as described [39] using horseradish peroxidase-conjugated rabbit anti-mouse IgG (Nordic Immunol. Lab., CA, U.S.A.), at a 1/1000 dilution and 4-chloro-1-naphthol as substrate. To visualize the subcellular localization of the antigen recognized by the monoclonal antibody, an indirect immunofluorescence was performed in wild-type and MTX-R1000 lines according to the procedure previously described [40], using C219 monoclonal antibody (2 μg/mL). After 2 hr incubation with the monoclonal antibody, the cells were washed three times with PBS at 4° and incubated

Table 1. Drug resistance in *L. tropica* lines

Drugs†	IC_{50} (μM)* WT	IC_{50} (μM)* MTX-R1000	Relative drug resistance‡
MTX	20.0 ± 6.7	15,900 ± 550	794 ± 269¶
PUR	3.8 ± 0.7	16.2 ± 1.5	4.2 ± 0.8‖
KET	18.8 ± 1.3	29.8 ± 2.4	1.6 ± 0.1
GLU	212 ± 12	221 ± 14	1.0 ± 0.1
VIN	14.8 ± 0.7	36.7 ± 1.0	2.5 ± 0.1§
DOX	36.1 ± 7.8	94.1 ± 3.6	2.6 ± 0.6§
DAU	34.2 ± 2.4	32.6 ± 5.8	0.9 ± 0.2
$SbCl_3$	48.1 ± 0.1	60.6 ± 1.7	1.3 ± 3.4
Na_2HAsO_4	17.3 ± 3.5	73.1 ± 1.4	4.2 ± 0.9‖

* Values are means ± SD (N = 3).

† Abbreviations for drugs used are MTX, methotrexate; PUR, puromycin; KET, ketoconazol; GLU, glucantime; VIN, vinblastine; DOX, doxorubicin; DAU, daunorubicin; $SbCl_3$, antimony trichloride.

‡ Values significantly different from the wild-type (WT) value by Student's *t* test are designated by the following symbols (§$P < 0.0005$; ‖$P < 0.0002$, ¶$P < 0.0001$).

for 1 hr in the presence of affinity-purified fluorescein-conjugated sheep anti-mouse IgG (20 μg/mL in 0.01% Evans Blue/PBS) (Boehringer Mannheim). Slides were washed with PBS, dried, mounted in glycerol and photographed using a fluorescence microscope.

Nucleic acid isolation. Total DNA was isolated as previously described [5]. Total cellular RNA was isolated from logarithmic growing parasites by cell lysis in guanidinium isothiocyanate and phenol extraction [41]. Poly(A)$^+$RNA was purified from total RNA by chromatography on oligo(dT)-cellulose as previously described [42].

Southern and northern hybridizations. DNA from wild-type and MTX-R1000 lines were digested with the restriction endonucleases *Eco* RI and *Bam* HI. The digested DNA was electrophoresed in a 1% agarose gel and transferred onto Hybond-N membranes (Amersham Corp.) by the method of Southern [43]. For identification of specific transcripts, poly(A)$^+$RNA of wild-type and MTX-R1000 lines of *L. tropica* were electrophoresed on 1% agarose/2.2 M formaldehyde gels and transferred onto nylon membranes. Blots were hybridized with different probes: the nbsA probe, which covers the first nucleotide binding sites of the *P*-glycoprotein gene A (*ltpgpA*) from *L. tarentolae* and recognizes members of the *P*-glycoprotein gene family in *Kinetoplastida* [20]; the specific probes for *ltpgpA*, *ltpgpD* and *ltpgpE* genes, and a probe that recognizes *ltpgpB* and *C* genes [19]; the pLa06 probe, from *Leishmania amazonensis* [44], homolog to the ldmdr1 probe from *L. donovani* [30], and the specific ltdh probe, from the *ltdh* gene of *L. tarentolae* [23]. The *DHFR–TS* gene from *L. tropica* (generously provided by Dr L. M. Ruiz-Perez, Instituto de Parasitología Lopez-Neyra, CSIC, Granada, Spain), was employed as a single copy probe to determine the expression and the copy numbers of the drug target enzyme on northern and Southern blots from wild-type and MTX-R1000 lines. The β *tubulin* gene from *Trypanosoma cruzi* (generously provided by Dr. Antonio Gonzalez, Instituto de Parasitología Lopez-Neyra, CSIC, Granada, Spain) was employed for normalization of wild-type and MTX-R1000 hybridization signals on Southern and northern blots. Hybond-N membranes containing either fragmented DNA or fractionated poly(A)$^+$RNAs were pre-hybridized in 50% formamide, 5× Denhardt solution, 0.1% SDS, 5× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 100 μg/mL salmon sperm DNA for 4 hr at 42°. Filters were then hybridized as previously described [45], to DNA probes labelled with (α-^{32}P)dCTP by random priming [46], for 16–20 hr at 42°. For both the DNA and RNA blots, final post-hybridization washes were in 2× SSC plus 0.1% SDS at 42°. All blots were visualized by autoradiography and the relative intensities of the bands were quantified using a Bio-Rad model 620 video densitometer.

RESULTS

Induction, selection and stability of MTX-resistant L. tropica *lines* in vitro

The MTX-R1000 line was generated *in vitro* using a stepwise selection process, starting at an initial drug concentration of 5 μM. Cells transferred into this concentration showed initially slower growth, but were stabilized at normal growth rate after five passages. The resistant line had a doubling time and a cell density similar to what is observed in wild-type cells in the absence of drug (21.80 and 22.96 hr for wild-type and MTX-R1000, respectively). The time required to induce MTX resistance *in vitro*, at the maximum concentration (1000 μM), was 188 days. The resistance index at the maximum MTX concentration was 794-fold (Table 1). To determine the stability of resistance to MTX in the absence of drug pressure, MTX-R1000 cells were grown in drug-free medium for 3 and 6 months (Rv_3 and Rv_6). The IC_{50} values for MTX remained unaltered in Rv_3; but in Rv_6 the IC_{50} values for MTX reverted to wild-type ones.

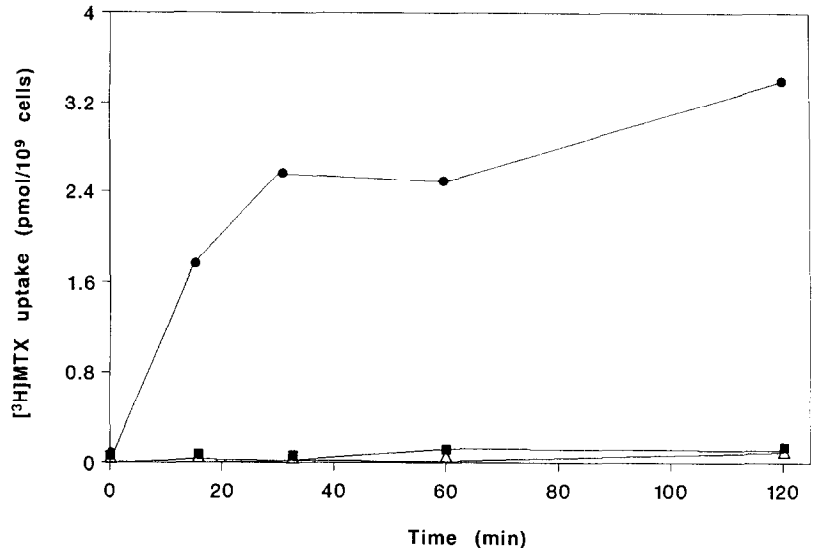

Fig. 1. [^{3}H]MTX uptake by *L. tropica*. The abilities of wild-type (●), MTX-R1000 (■) and revertant 3 months (△) *L. tropica* to incorporate [^{3}H]MTX from the culture medium were determined as described in Materials and Methods. Ten million cells were incubated in 200 μL RPMI 1640 folate-deficient medium containing 100 nM [^{3}H]MTX. The data shown are the differences observed between 28° and 0°. The results are those of a single experiment, which has been repeated two other times with virtually the same results.

Table 2. MTX transport kinetics in *L. tropica* lines

Cell line	MTX influx	
	K_t*	V_{max}†
Wild type	0.46 ± 0.07	3.52 ± 0.53
MTX-R1000	0.40 ± 0.06	1.21 ± 0.07

The abilities of wild-type and MTX-R1000 lines to transport increasing concentrations of [^{3}H]MTX was determined as described in Materials and Methods. Each value presented is the average of three independent experiments ± SD, obtained at 1 and 2 min.

* Apparent affinity for MTX, μM.

† Maximal velocity of MTX influx, pmol/min/[10^9 cells].

Cross-resistance to other drugs

We studied the cross-resistance profile of a number of structurally and functionally unrelated drugs in the MTX-R1000 cell line. The results of the cross-resistance experiments are summarized in Table 1. Significant cross-resistance was observed towards puromycin, vinblastine, doxorubicin and sodium arsenate. The first three drugs are commonly associated with mammalian MDR. The cross-resistance observed in Rv_3 was similar to that observed in MTX-R1000 but the values of cross-resistance reverted to the wild-type values in Rv_6.

MTX uptake measurements

As described in Refs 1 and 2, parasitic protozoa can become resistant to MTX by their failure to take up the drug efficiently. The MTX-R1000, wild-type and Rv_3 abilities to accumulate [^{3}H]MTX were compared and are described in Fig. 1. At 0° the rate of accumulation was greatly reduced. The MTX-R1000 and Rv_3 cells showed slower MTX accumulation, since after 2 hr of exposure to [^{3}H]MTX these cell lines accumulated less than 3% of the amount of drug incorporated by wild-type cells. We examined the transport of MTX in wild-type and resistant lines by Lineweaver–Burk analysis. The data (Table 2) indicate that the apparent affinity for MTX was similar in wild-type and MTX-R1000 lines, whereas the maximal velocity of influx was significantly lower in the resistant line. The apparent affinity values for MTX were similar to those obtained by Kaur *et al.* [2] in *L. donovani*, and slightly lower than those obtained by Ellenberger and Beverley [3] with *Leishmania major*. However, the maximal velocity value for wild-type was significantly lower than those described for *L. donovani* [2] and *L. major* [3].

*Protein changes in MTX-R*1000 L. tropica

SDS–polyacrylamide gel analysis of total cell proteins from [^{35}S]methionine labelled parasites revealed the presence of an overexpressed protein of 240 kDa in MTX-R1000 and Rv_3 (Fig. 2). Western blot analysis of total cell proteins, using the anti-*P*-glycoprotein monoclonal antibody C219, showed that the 240 kDa protein reacts with that antibody (Fig. 3). This putative *P*-glycoprotein was absent in Rv_6, a cell line that has lost its drug resistance. Interestingly, cell fractionation studies revealed that in MTX-R1000 and Rv_3 lines C219 detects the putative *P*-glycoprotein in the plasma membrane fraction (Fig. 3) and not in the cytosol fraction (data not shown). The purity of the plasma membrane fraction was assessed by an enrichment on the

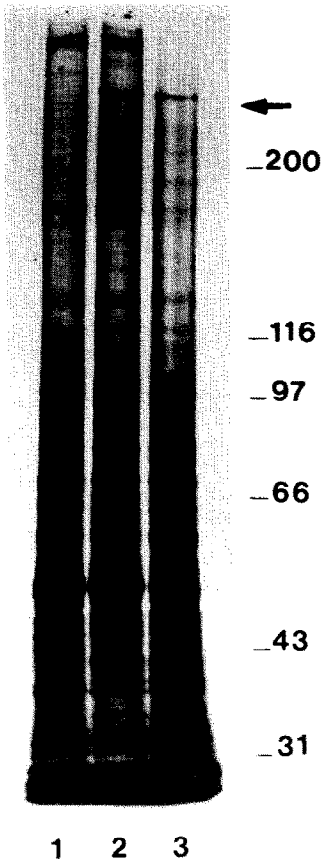

Fig. 2. SDS–polyacrylamide gel analysis of [^{35}S]methionine labelled *L. tropica* lines. Samples (10^5 cpm each) were separated on 6% SDS–polyacrylamide gel. The arrow indicates the position where a protein is overproduced in the MTX-resistant strain. The lanes contain: wild-type cells, lane 1; MTX-R1000, lane 2; Rv_3, lane 3. Molecular mass standards (kDa) used, indicated in the margin, are: myosin, 200; β-galactosidase, 116; phosphorylase b, 97; bovine serum albumin, 66; ovalbumin, 43; carbonic anhydrase, 31.

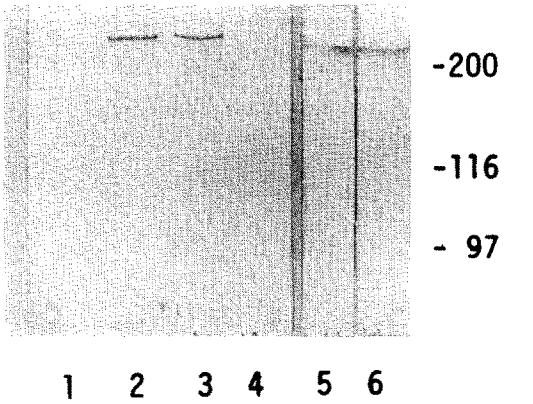

Fig. 3. Western blot analysis of *P*-glycoprotein-like peptides in *L. tropica* lines. Samples were separated on a 6% SDS–polyacrylamide gel transferred to nitrocellulose, and reacted with the monoclonal antibody C219. The lanes 1 to 4 contain whole cells (50 μg): wild-type, lane 1; MTX-R1000, lane 2; revertant 3 months, lane 3; revertant 6 months, lane 4; lanes 5–6 contain membranes (15 μg): MTX-R1000, lane 5; revertant 3 months, lane 6. Molecular mass standards (kDa), indicated in the margin, are as described in Fig. 2.

enzyme marker activity acid phosphatase, with a 3.5-fold increase in the specific activity of the plasma membrane fraction as compared to the initial homogenate. The molecular mass of the putative *P*-glycoprotein overexpressed in MTX-R1000 (240 kDa) is larger than that described in MDR mammalian cell lines (170 kDa) [47], *P. falciparum* [48] and *Leishmania panamensis* [40]. Comparative indirect immunofluorescent staining of wild-type and MTX-R1000 lines, using the monoclonal antibody C219, are shown in Fig. 4. A stronger membrane-bound immunofluorescence was observed in the resistant parasites (Fig. 4B), confirming the membrane localization of the putative *P*-glycoprotein.

P-*glycoprotein overexpression in* L. tropica *MTX-R1000*

Poly(A)$^+$RNA from wild-type and MTX-R1000 lines was isolated, fractionated by size and hybridized to different specific probes from *L. tarentolae P*-glycoprotein genes [19], with the pLa06-specific probe for a gene of *L. amazonensis* involved in a MDR phenotype [44], with the specific probes for the antifolate resistant *ltdh* gene from *L. tarentolae* [23] and with a probe encoding the *DHFR–TS* gene from *L. tropica*. Whereas no transcripts were detected in northern blot analyses probed with the pLa06 probe and ltdh probe, a single transcript of approximately 6.0 kb was recognized by the ltpgpE-specific probe for *P*-glycoprotein E gene [19] in the MTX-R1000 line (Fig. 5A). A probe encoding the *β tubulin* gene from *T. cruzi* was employed for normalization of the amount of RNA in the wild-type and MTX-R1000 samples (Fig. 5B). Thus, the MDR phenotype of *L. tropica* MTX-R1000 was associated with the overexpression of only one of the six *L. tropica P*-glycoprotein genes for which we have probes. The size of the putative *P*-glycoprotein mRNA is similar to that of the mRNAs reported for the mammalian MDR gene [44], emetine-resistant *E. histolytica* [29] and MTX-resistant *L. tarentolae* [20] and smaller than the 7–8 kb MDR-like mRNAs described in *P. falciparum* [27] and the 12.5 kb mRNA described in vinblastine-resistant *L. donovani* [30]. Also, no difference in mRNA levels of the *DHFR–TS* gene of *L. tropica* was observed between wild-type and MTX-R1000 lines (data not shown).

P-*glycoprotein gene rearrangement in* L. tropica *MTX-R1000*

Southern blot analysis revealed that several fragments from *L. tropica* hybridize to a probe that recognized the *P*-glycoprotein gene family of *L. tarentolae* (data not shown) but no gene amplification was observed in the MTX-R1000 line. However, both in the *Bam* HI and *Eco* RI digests, the resistant cell line showed a novel fragment hybridizing with the ltpgpE-specific probe (Fig. 6A) and with a nbsA

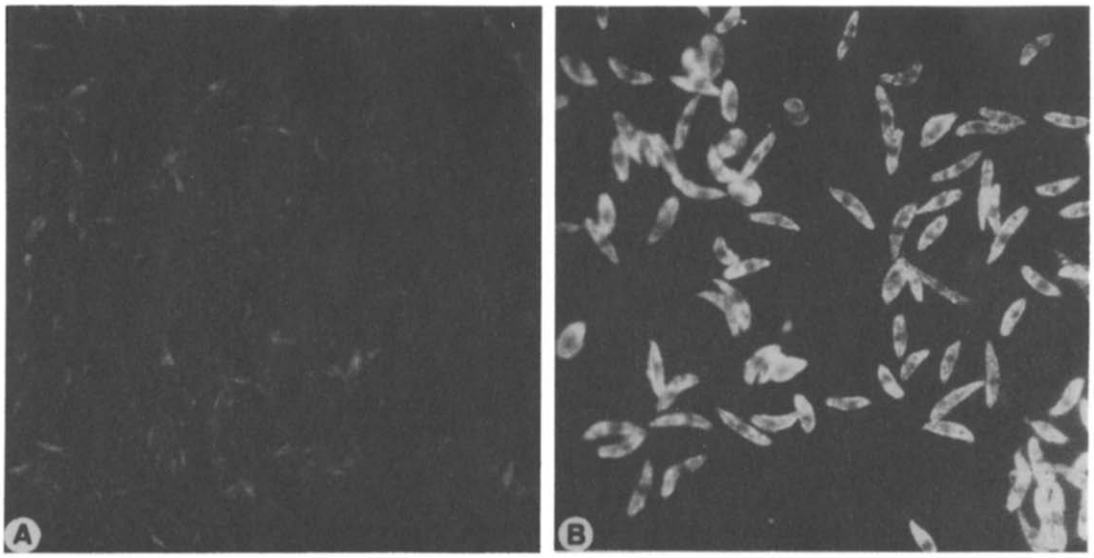

Fig. 4. Indirect immunofluorescence of wild-type and MTX-R1000 *L. tropica* lines with C219 monoclonal antibody. A, wild-type parasites. B, MTX-R1000 parasites. The slides containing fixed parasites were prepared as described in Materials and Methods. The cells were fixed in 95% ethanol and then in acetone, each for 5 min at −20° and reacted with the monoclonal antibody C219 (2 μg/mL) followed by a fluorescein-conjugated anti-mouse IgG (20 μg/mL), diluted in 0.01% Evans Blue/PBS.

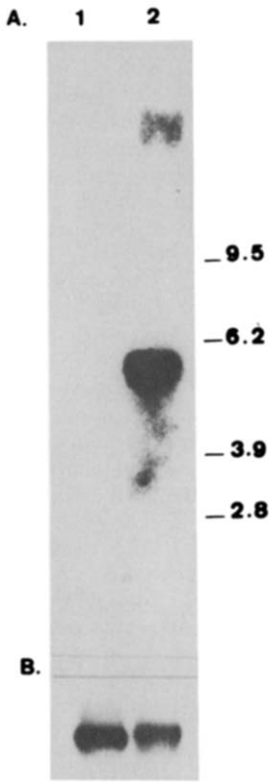

Fig. 5. Expression of the *ltpgp E* homolog in *L. tropica* wild type and MTX-R1000 lines. (A) 7 μg of poly (A)$^+$RNA of wild type (lane 1) and MTX-R1000 (lane 2) was electrophoresed on a 1% agarose/2.2 M formaldehyde gel, blotted and hybridized with the ltpgpE probe. (B) The gel was rehybridized with the β tubulin probe to monitor the amounts of RNA layered on the gel. The size marker (kb) was the RNA ladder from Promega.

probe, derived from the *ltpgpA* gene, that recognized five *P*-glycoprotein genes in *Leishmania* [20] (data not shown). A probe encoding the *DHFR–TS* gene of *L. tropica* was used to rehybridize the Southern blots and determine the copy numbers of the gene for the drug target enzyme. The results show that no modification in the gene copy number was observed in both cell lines (Fig. 6B). Also, a probe encoding the *β tubulin* gene from *T. cruzi* was employed to normalize the amount of DNA in the wild-type and MTX-R1000 samples (Fig. 6C).

DISCUSSION

The most common mechanisms of MTX resistance described in *Leishmania* have been a decreased accumulation of the drug [2, 3], amplification of the gene for the drug target enzyme [5–8] and amplification of extrachromosomal H circles [13–19].

To help our studies on drug resistance mechanisms in *Leishmania*, we have selected *in vitro* a *L. tropica* LRC-L39 strain for resistance to MTX, using a stepwise selection process. This MTX-R1000 line showed stable MTX resistance, growing in drug-free medium, for at least 3 months. In our *Leishmania* MTX-resistant line, the *DHFR-TS* gene, coding for the drug target enzyme, was not amplified or overexpressed. In addition, the same MTX resistant cell line had no extrachromosomal H circles, as deduced by the absence of amplification of the homolog genes *ltdh* and *ltpgpA*, localized in the H circle. The *ltdh* gene of *L. tarentolae* has been involved in the MTX resistance [23]; however, using a specific probe, we have not detected expression of this gene in our MTX resistant cell line. This resistant line shows a significantly decreased uptake of MTX when compared to wild-type parasites. Significant reduction in the maximal velocity for MTX influx is

Fig. 6. Putative DNA rearrangement in MTX-R1000 *L. tropica* line. (A) 2 μg of wild-type and MTX-R1000 DNAs were digested, electrophoresed in a 1% agarose gel, blotted and hybridized in 50% formamide to the labelled specific probe for *ltpgpE*. Wild-type and MTX-R1000 DNA digested with *Eco* RI, lanes 1 and 2; wild-type and MTX-R1000 DNA digested with *Bam* HI, lanes 3 and 4; wild-type and MTX-R1000 DNA digested with *Eco* RI/*Bam* HI, lanes 5 and 6. The probe was stripped and the same filter was rehybridized first with the *DHFR-TS* gene of *L. tropica* (B), and then with the *β tubulin* gene of *T. cruzi* (C), to monitor the amount of DNA loaded. The blot in panel C was cut to reduce the size of the figure. The size marker (kb) was derived from lambda phage DNA digested with the restriction endonuclease *Hind* III.

observed in the absence of alterations in the apparent affinity for MTX. The decreased accumulation is possibly so efficient that the resistant cells do not require the other MTX-resistant mechanisms to adapt to higher drug concentration.

Our MTX-resistant cells also exhibited cross-resistance to a wide range of drugs, some of which are part of the mammalian MDR spectrum. Thus, this *in vitro*-induced MTX-resistant *L. tropica* exhibits a moderate cross-resistance to drugs that is reminiscent of the MDR phenotype found in cancer cells. The MDR phenotype described in mammalian cells is characterized by multiple biochemical changes (such as change in drug transport, altered expression of various membrane and cytosolic proteins, cross-resistance to other structurally and functionally unrelated drugs) [49]. Molecular studies indicate that the most frequent change that occurs in the MDR cell lines is amplification of a gene, responsible for the increased expression of a *P*-glycoprotein. To test whether a *P*-glycoprotein was overexpressed in these cells, we have employed a mouse monoclonal antibody directed against the cytoplasmic domain of the mammalian *P*-glycoprotein [31]. Monoclonal antibody C219 reacts on western blots with a protein of 240 kDa, which is substantially larger than the *P*-glycoproteins described [40, 47, 48]. Post-translational modifications (N-glycosylation and phosphorylation) may account for this large *P*-glycoprotein. The amount of this protein, estimated by SDS–polyacrylamide gel of total proteins labelled with [^{35}S]methionine, was approximately 1% of total proteins. Similar results were obtained with a Coomassie Blue stained gel. Subcellular localization by indirect immunofluorescence and western blots, using the monoclonal antibody C219, revealed that this protein is enriched in the plasma membrane fraction relative to whole cells and that it is absent in the cytosol fraction. This phenotype is stable for at least 3 months in the absence of drug pressure.

In mammalian systems, there are multiple *P*-glycoprotein genes (three in rodents and two in humans) [49]; in *P. falciparum* two different genes have been described as members of a closely related family [27]. The *P*-glycoprotein-related gene *ltpgpA*/*lmpgpA* found on the H circle of *Leishmania* [20, 21] is part of a large gene family [19, 50], which includes the B, C, D and E *P*-glycoprotein genes [19]. However, the sequence of these genes has been found to be divergent from that of the mammalian mdr1 [19, 20]. A novel *P*-glycoprotein gene in vinblastine-resistant *L. donovani* [30] and *L. amazonensis* [44] had been described associated with a MDR phenotype. Based on their sequences and functional role, this gene has been considered as a genuine *Leishmania* homolog of the mammalian mdr1 [30]. Using *P*-glycoprotein gene-specific probes [19, 44], we have observed that only a transcript molecule of 6 kb was detected with the ltpgpE-specific probe. Overexpression of genes in *Leishmania* has always been linked to gene amplification. Although no amplification of the homologous *ltpgpE* gene was observed, the level of RNA produced was several-fold higher compared to the wild-type. This disproportionate amount of RNA compared to the copy number of the gene could be explained by either a higher rate of transcription, or increased stability of the RNA or other post-transcriptional events that act at the maturation level or delivery of the RNA to the cytoplasm [51, 52]. It is tempting to speculate that this increase in RNA level in mutant MTX-R1000 is due to a gene rearrangement as suggested by our interpretation of the Southern blot. Gene rearrangements have often been implicated in activating the expression of genes [53].

In conclusion, in this study we have observed that *L. tropica* resistant to MTX develop a significantly reduced MTX accumulation. Co-existing with this mechanism, there is an independent phenomenon characterized by the overexpression of a homologous

ltpgpE gene. Until now, there is no evidence that the *Leishmania P*-glycoprotein genes described are involved in MTX resistance [20, 21, 30]. Therefore, we suggest that the overexpression of the homologous *ltpgpE* gene is an epiphenomenon which, for some unknown reason, is associated with MTX resistance. We are now testing this hypothesis and we are addressing directly, by transfection experiments, whether the *ltpgpE* homolog gene is responsible for the MDR phenotype that we have observed in *L. tropica* MTX-R1000, and whether it corresponds to the 240 kDa overproduced protein.

Acknowledgements—We thank Dr Victor Ling (Ontario Cancer Institute, Canada) for providing the C219 monoclonal antibody, Dr Ulisses Gazos (Federal University of Rio de Janeiro, Brazil) for the generous gift of the pLa06 probe and Drs L. M. Ruiz-Perez and D. Gonzalez for their interest and advice in this work. We are thankful to Mrs P. Navarro and Mr Gaétan Roy for their technical assistance. This work was supported by the Fondo de Investigaciones Sanitarias Grant No. 93/0342 (F. G.) and Grant No. FAR91-0538 (S. C.). It was also supported in part by the Plan Andaluz de Investigación, Research Group No. 3062.

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