



Altered Drug Membrane Permeability in a Multidrug-Resistant *Leishmania tropica* Line

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ABSTRACT. We selected a *Leishmania tropica* cell line resistant to daunomycin (DNM) that presents a multidrug-resistant (MDR) phenotype characterized by overexpression of a P-glycoprotein of 150 kDa. The resistant line overexpressed an MDR-like gene, called *ltmdr1*, located in an extrachromosomal circular DNA. DNM uptake experiments using laser flow cytometry showed a significant reduction in drug accumulation in the resistant parasites. The initial stages of the interaction of DNM with membranes from wild-type and DNM-resistant parasites were defined by a rapid kinetic stopped-flow procedure which can be described by two kinetic components. On the basis of a previous similar kinetic study with tumor cells, we ascribed the fast component to rapid interaction of DNM with membrane surface components and the slow component to passive diffusion of the drug across the membranes. The results reported here indicate that entrance of DNM into wild-type parasites was facilitated in respect to the resistant ones. We propose that resistance to DNM in *L. tropica* is a multifactorial event involving at least two complementary mechanisms: an altered drug membrane permeability and the overexpression of a protein related to P-glycoprotein that regulates drug efflux. *BIOCHEM PHARMACOL* 55;2:131–139, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. *Leishmania tropica*; resistance to daunomycin; altered permeability; MDR phenotype

It is well established that multidrug resistance (MDR)^{||} in tumor cells is a multifactorial process representing different mechanisms [1]. Among them, the overexpression of P-glycoprotein (Pgp) responsible for the MDR phenotype has been extensively documented as a very efficient mechanism to reduce intracellular drug accumulation. The decreased drug accumulation leads to an MDR phenotype characterized by a cross-resistance to a large number of chemically and structurally unrelated hydrophobic drugs [2]. However, MDR in different cell lines and tumors cannot always be explained in terms of Pgp overexpression. Additional mechanisms such as overexpression of vacuolar ATPases [3], modifications of cytoplasmic pH [4], altered detoxification pathways, and more recently, modifications in plasma membrane permeability [5, 6] have been reported.

In mammalian cells, Pgp substrates and modulators enter

cells by passive diffusion through the plasma membrane. Recently, it has been suggested that the efficiency for a specific drug to be exported by Pgp could be dependent on the rate at which the drug traverses the cell membrane [5]. Since the intracellular concentration of drugs in MDR cells results to a great extent from a balance between influx and efflux processes, it would seem important to determine the kinetics of drug influx across the cell membrane.

In parasites, specifically in *Leishmania*, the most common drug resistance mechanism described has been the decreased accumulation of drugs [7, 8] and the amplification of DNA in the form of extrachromosomal elements containing genes involved in drug resistance [9, 10]. Recent evidence has shown that the *mdr* gene is involved in drug resistance in several protozoan parasites [11–13]. However, recent reports demonstrate the existence of drug-resistant *Leishmania* in which the level of *mdr* gene expression does not correlate with the level of drug resistance [13, 14]. This suggests that other cellular alterations may participate in the resistant phenotype.

In the present study, a rapid kinetic procedure has been applied to define the initial steps in the interaction of daunomycin (DNM) with parasite membranes. This procedure provides a quantitative estimation of the kinetic differences in these interactions between wild-type (WT) and DNM-resistant parasites. We describe here that in a

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^{||} Abbreviations: CHEF, contour-clamped homogeneous electric field; DNM, daunomycin; DNM-R150, line resistant to 150 μ M of DNM; MDR, multidrug resistance; Pgp, P-glycoprotein; Rv₁, Rv₃ and Rv₆, DNM-R150 lines grown in drug absence for 1, 3 and 6 months, respectively; WT, wild-type parasites.

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DNM-resistant *Leishmania tropica* line, the overexpression of a Pgp coexists with a significantly reduced membrane permeability to DNM.

MATERIALS AND METHODS

Drugs and Chemicals

DNM and doxorubicin hydrochloride were purchased from Farmitalia Carlo Erba. Vinblastine was obtained from Lilly S.A. Puromycin was from Sigma Chemical Co. Glutathione Sepharose 4B, Protein A Sepharose CL-4B and CNBr-activated Sepharose 4B were purchased from Pharmacia LKB Biotechnology. All other reagents were of standard laboratory grade.

Parasite Culture and Selection of DNM Resistant Line

L. tropica LRC-L39 was obtained from Dr. L. F. Schnur (Kuvim Center for the Study of Infectious and Tropical Diseases, Jerusalem, Israel). The WT line used is a clone, obtained by agar plating as described [15]. Promastigotes were grown at 28° in RPMI 1640-modified medium (Gibco), as previously described [16] and supplemented with 20% heat-inactivated fetal bovine serum (Gibco). A DNM-resistant line of *L. tropica* was obtained by a stepwise selection process as described [17], using DNM concentrations of 25, 50, 100 and 150 μM . The *L. tropica* line resistant to 150 μM of DNM (DNM-R150) was maintained over the course of 1, 3 and 6 months proliferation in the continuous presence of drug. To generate revertant lines, the resistant line was grown in drug-free medium for 1, 3 and 6 months (Rv₁, Rv₃ and Rv₆, respectively). Cross-resistance of the DNM-R150 line to other unrelated drugs was determined by measuring the IC₅₀ and the resistance indexes (IC₅₀ ratio between resistant and WT cells), as described [18].

Drug Uptake by Flow Cytometry

To compare the abilities of WT and DNM-R150 lines to accumulate DNM from the culture medium, the parasites were harvested by centrifugation and washed three times in PBS. The concentration was adjusted to 10⁶ parasites in 1 mL of Dulbecco-PBS (Seromed), containing 0.1% BSA, which were incubated with 8 μM of DNM for 1 hr at 28°. Drug uptake and efflux was stopped by centrifugation. Parasites were then washed three times in ice-cold PBS before analysis. The selected time of 1 hr guaranteed that cell viability was unaffected during the assays (>95%). A concentration of 8 μM DNM gave the clearest results with the least background interference. The intracellular level of DNM was estimated by flow cytometry as described [19], in a Becton Dickinson FacScan using the intrinsic fluorescence emission of the drug scanned over the range 540–590 nm. The data analysis was performed by determining the number of parasites having a significant content of DNM (i.e., a level of drug fluorescence that excludes cell autofluorescence and drug association to cell debris).

Nucleic Acid Manipulations

Total cellular RNA was isolated from parasites in logarithmic growth phase using guanidinium isothiocyanate and phenol extraction [20]. Northern blot was performed using standard procedures [21]. The blot was hybridized with the pLa06 specific probe, which recognizes the first nucleotide binding domain of the *mdr* gene from *Leishmania amazonensis* [22]. The β -tubulin probe from *Trypanosoma cruzi* was used for normalization of the blots. Extrachromosomal circular DNA was resolved from genomic DNA by contour-clamped homogeneous electric field (CHEF) [23], using 1.5% agarose gels and a pulse time of 4 sec for 18 hr at 300 V and 13°. Extrachromosomal element was purified from agarose using the Pharmacia Sephaglass™ BandPrep Kit. An alkaline lysis method was used to enrich the extrachromosomal element as described [24]. The *ltrmdr1* gene was isolated from the circular element by subcloning a 13 kb *XhoI* fragment into pBluescript II SK⁺ vector. DNA sequence determinations were carried out on a 373A Automated DNA Sequencer from Applied Biosystems, using both universal and specific oligonucleotide primers. The complete sequence of the gene was obtained from both strands, and analyzed using the GCG (Wisconsin University) software package. The number of copies of *ltrmdr1* gene was estimated by Southern blot analysis using genomic DNA digested with *BamHI* and the pLa06 specific probe. The relative intensities of the bands were quantified using a Bio-Rad model 620 video densitometer.

Antibody Preparation and Immunodetection of Ltrmdr1 Protein

The linker region of the *ltrmdr1* gene was selected for the preparation of a polyclonal antibody. The linker region is so designated because it is the link between the two homologous halves of the *Pgp* gene. Homology studies indicate that it, together with the *N* terminus, is one of the least conserved regions among the Pgps, which guarantees specificity of the antibody. A segment of DNA containing the 79 amino acids corresponding to this region (amino acids 695-774) was amplified by the polymerase chain reaction using primers with terminal *BamHI* sites. The amplified fragment was cloned into the *BamHI* site of the pGEX-2T vector (Pharmacia LKB Biotechnology) and transformed into the XL1-Blue strain (Stratagene Cloning Systems) of *Escherichia coli* for expression as a glutathione *S*-transferase fusion protein. The protein was purified using a column containing glutathione Sepharose 4B. Obtention and purification of the polyclonal antibody was as described [25], following sequential passages through columns containing protein A Sepharose CL-4B, glutathione *S*-transferase covalently coupled to CNBr-activated Sepharose 4B, and a column of fusion protein covalently coupled to CNBr-activated Sepharose 4B.

Protein determinations were performed by the method of Lowry *et al.* [26]. Plasma membrane proteins, isolated as

described below, were separated on a 6% SDS-polyacrylamide gel, and electrotransferred to nitrocellulose paper. Blots were blocked with nonfat skim milk (5%) in PBS and incubated with the anti-Pgp polyclonal antibody at a 1/1000 dilution in buffer A (PBS, 0.1% BSA, 0.1% Tween 20), for 2 hr at room temperature. After washing with buffer A, bound antibodies were detected using phosphatase-conjugated goat anti-rabbit IgG (Sigma) at a 1/5000 dilution.

Permeability of Isolated Plasma Membranes to DNM

Plasma membrane vesicles were isolated according to the protocol previously described [27]. Briefly, parasites were washed three times with PBS and resuspended at a density of 10^9 cells/mL in hypotonic lysis buffer (1 mM Tris-HCl, pH 7.9, 1 mM EDTA, 0.5 mM PMSF, 8 μ g/mL aprotinin, and 10 μ g/mL leupeptin). The cells were disrupted by three sonication pulses for 30 sec, with 30 sec intervals between each pulse. The undisrupted cells and nuclear debris were removed by centrifugation at $12,000 \times g$ for 10 sec. The lysate was diluted with 2 volumes of TMEP (50 mM Tris-HCl, pH 7.0, containing 50 mM mannitol, 2 mM EGTA, 0.5 mM PMSF, 8 μ g/mL aprotinin, 10 μ g/mL leupeptin and 2 mM β -mercaptoethanol), spun at $100,000 \times g$ for 30 min, and the resulting pellet was used as a crude membrane fraction. The purified plasma membranes were prepared by discontinuous sucrose density gradient. The purified membranes were removed from the interface between 1.23 and 1.52 M sucrose layers, diluted with TMEP buffer and recovered by centrifugation at $75,000 \times g$ for 4 hr. The purity of plasma membrane fraction was assessed by an enrichment on the enzyme marker activity acid phosphatase. The membranes were stored at -80° until needed. The permeability of membrane vesicles from WT, DNM-R150 and Rv₁ parasites to DNM was measured by determining the initial interaction of the drug with the membrane vesicles by a rapid kinetic procedure using a HiTech stopped-flow spectrophotometer model SF-51 set up under identical conditions to those described by Soto et al. [6]. Under these conditions and in the absence of drug efflux mechanisms, the interaction of DNM with the vesicles within short periods of time should reflect unidirectional transfer of the drug from the extravesicular aqueous phase to the hydrophobic vesicle environment. Thawed plasma membranes were homogenized and diluted to 1 mg of protein/mL in 10 mM Tris-HCl buffer pH 7.4. The process was initiated by mixing 0.1 mL of the membrane suspension with an equal volume of 10 μ M DNM in 10 mM Tris-HCl buffer, pH 7.4, at 25°. The traces were computer-fitted by nonlinear regression using the algorithm of Marquardt to the following general form of the equation:

$$F(t) = C + A_1[\exp(-k_1 \cdot t)] + A_2[\exp(-k_2 \cdot t)]$$

where $F(t)$ is the fluorescence at time t ; A_1 , A_2 , ... and k_1 , k_2 , ... represent the amplitudes and the apparent rate

TABLE 1. Cross-resistance profile of *L. tropica* lines resistant to daunomycin

| Drugs [†] | IC ₅₀ (μ M)* | | |
|--------------------|------------------------------|-------------------------|----------------------|
| | WT | DNM-R150 | Rv ₁ |
| DNM | 2.6 \pm 0.3 | 162.0 \pm 16.2 (62.3) | 10.1 \pm 1.5 (3.9) |
| PUR | 3.8 \pm 0.4 | 45.1 \pm 6.7 (11.8) | 3.9 \pm 0.4 (1.0) |
| VIN | 14.6 \pm 1.5 | 170.4 \pm 17.1 (11.5) | 13.2 \pm 1.5 (0.9) |
| DOX | 5.1 \pm 0.5 | 160.3 \pm 24.1 (31.4) | 10.4 \pm 4.6 (2.0) |

* The IC₅₀, concentration of drug which decreases the rate of cell growth by 50%, was determined for WT, DNM-R150 and revertant line Rv₁. The mean \pm errors of five independent experiments is given.

[†] Abbreviations for drugs used are: DNM, daunomycin; PUR, puromycin; VIN, vinblastine; DOX, doxorubicin. The resistance index, indicated between parentheses, is the ratio of IC₅₀ for the resistant/revertant lines and the IC₅₀ for the WT line.

constants, respectively, corresponding to each of the terms associated to equation (A); C is the extrapolated fluorescence of DNM at equilibrium.

Statistical Analysis

Statistical differences between the kinetic data were evaluated by Student's *t*-test and considered significant at $P < 0.05$.

RESULTS

Characterization of the MDR Phenotype in a *L. tropica* Line

The *L. tropica* DNM-R150 line was generated *in vitro* using a stepwise selection process initiated with a concentration of 25 μ M DNM. The time required to induce resistance at the maximum concentration of 150 μ M DNM was approximately 5 months. The resistance index at the maximum DNM concentration was 62.3-fold (Table 1). In Rv₁, Rv₃ and Rv₆ parasites, the IC₅₀ values for DNM reverted to those for WT, ranging from 10 μ M in the Rv₁ (Table 1) to 6 μ M in the Rv₃ and 3 μ M in the Rv₆ line. These results suggest that the loss of resistance occurred rapidly and was greatly reduced by 1 month. At 1, 2 and 3 weeks after drug withdrawal, the IC₅₀ values were 50, 30 and 20 μ M, respectively.

In addition, DNM-R150 parasites were maintained over 1, 3 and 6 months in the presence of DNM, with a similar level of resistance to DNM. The results of the cross-resistance profile of DNM-R150 parasites to structurally and functionally unrelated drugs are summarized in Table 1. Significant cross-resistance was observed towards puromycin, vinblastine and doxorubicin, with a resistance index over 10. In mammalian cells, the MDR phenotype is reversed by exposure to cyclosporin A [28] and verapamil [29]. However, these compounds failed to reverse cross-resistance to different drugs in the DNM-R150 line, even at concentrations that inhibited the rate of cell growth by 30% (not shown).

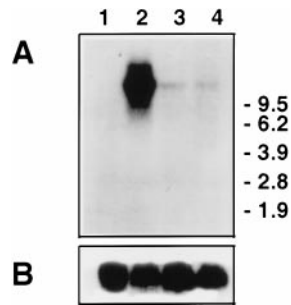


FIG. 1. Northern blot analysis of WT and DNM-resistant *L. tropica* lines. A, 20 μ g of total RNA from WT (1), DNM-R150 (2), Rv₁ (3) and Rv₃ (4) lines were electrophoresed on a 1% agarose/2.2 M formaldehyde gel, blotted and hybridized with the pLa06 probe. B, The blot was rehybridized with the β -tubulin probe from *Trypanosoma cruzi* to monitor the amounts of RNA layered on the gel. The size marker (kb) was the RNA ladder from Promega.

Amplification and Overexpression of a P-Glycoprotein

Northern blot analysis was performed using total RNA from WT, DNM-R150 and Rv₁ and Rv₃ parasites and hybridized to the pLa06 specific probe. A single transcript of approximately 13 kb was recognized in the resistant and revertant parasites (Fig. 1A). The size of this transcript is larger than that found in mammalian cells [30] or *P. falciparum* [11] and similar to that reported in vinblastine-resistant *Leishmania donovani*, which overexpresses the *ldm1* gene [13]. The low mRNA levels in the revertant parasites (Rv₁ and Rv₃) support the IC₅₀ results, indicating that Rv₁ and Rv₃ parasites revert to the WT phenotype.

In *Leishmania* sp., amplified genes frequently emerge as extrachromosomal circular elements [9, 10]. CHEF was used at conditions that favor the separation and resolution of extrachromosomal amplicons. Chromosomal Southern blot analysis of the CHEF revealed the existence in resistant parasites of a band whose apparent molecular size changed with pulse frequency and had characteristics of supercoiled DNA (Fig. 2). This extrachromosomal circular element, called DN circle, contained the *ltrmdr1* gene, was present at low DNM concentrations and disappeared in the revertant parasites (Fig. 2, lanes 1–8 and 9–11, respectively). The extrachromosomal DN circle, purified from agarose gel or by alkaline lysis and restriction mapped with *NotI* and *BglII*, had a size of approximately 30 kb (not shown). The amplification of DN circle seemed to increase with the degree of resistance of the parasite lines upon exposure to different DNM concentrations (50, 100 and 150 μ M), being 20-fold in the DNM-R150 line. The association of the DN extrachromosomal element to DNM resistance was evidenced by the parallel of the IC₅₀ index and Pgp expression.

The *ltrmdr1* gene was isolated from the extrachromosomal element. Analysis of the nucleotide sequence of *ltrmdr1* gene revealed an open reading frame of 4,023 bp that encoded a polypeptide of 1,341 amino acids with an estimated molecular mass of 147 kDa (not shown). These

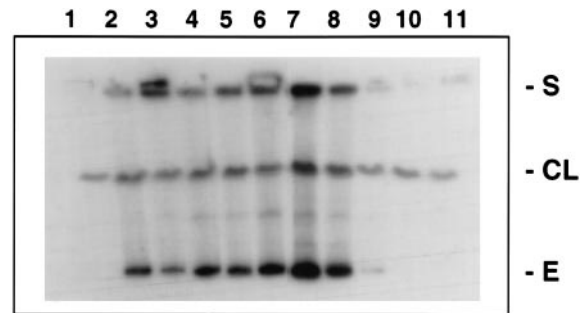


FIG. 2. CHEF analysis in DNM-resistant *L. tropica* lines. CHEF was developed using a pulse time of 4 sec for 18 hr at 300 V to favor the separation of extrachromosomal amplicons of *Leishmania*. Each lane contains intact chromosomal DNA from 10⁷ parasites of: lane 1, WT parasites; lanes 2–5, parasites resistant to different DNM concentrations (25, 50, 100 and 150 μ M, respectively); lanes 6–8, DNM-R150 parasites grown in the presence of 150 μ M DNM at time intervals of 1, 3 and 6 months, respectively; lanes 9–11, DNM-R150 parasites grown in the absence of DNM for 1, 3 and 6 months, respectively. The Southern blot of the gel was hybridized with the pLa06 probe. Bands visible in the autoradiogram are: S, slot location; CL, chromosomal location; E, extrachromosomal element.

nucleotide sequence data were submitted to the GenBank™ data base with the accession number U63320. Comparison of the *ltrmdr1* gene sequence with the GenBank™ data base revealed a high homology with other *Leishmania mdr1* genes, i.e., 95 and 82% sequence identity at the amino acid level with *L. donovani lmdr1* and *Leishmania enriettii lemdr1*, respectively, and 37% sequence identity with human *mdr1*.

The specificity of the polyclonal antibody directed against the linker region of the *ltrmdr1* gene product was observed in Western blot analysis of membrane extracts from WT, DNM-R150 and Rv₁ parasites. The blot revealed the presence of a band of approximately 150 kDa in resistant parasites which was undetected in those from WT and Rv₁ (Fig. 3). The size of the band corresponds to the molecular weight predicted from the nucleotide sequence, suggesting that this protein is not N-glycosylated.

DNM Accumulation Experiments

To evaluate whether the drug resistance of DNM-R150 parasites could be ascribed to a failure to accumulate drug, DNM uptake experiments were carried out using laser flow cytometry. Recorded data are displayed as histograms of cell number versus fluorescence intensity (Fig. 4). A typical fluorescence histogram of WT and resistant parasites incubated without drug is also displayed in Figs. 4A and B, respectively (white profile). The peak of fluorescence distribution of DNM is significantly shifted to the right in the Wt parasites. The resistant line exhibits a significantly lower DNM accumulation (23% of fluorescent-positive cells) compared to that of WT (85% of fluorescent-positive cells). Concentrations of verapamil and cyclosporin A over 10-fold the IC₅₀ significantly increased the DNM accumu-

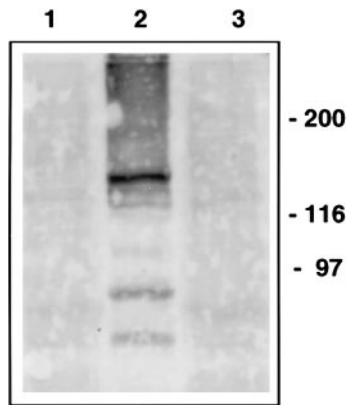


FIG. 3. Western blot analysis of a P-glycoprotein in *L. tropica* lines. Plasma membrane proteins (50 μ g) were separated on a 6% SDS-polyacrylamide gel, transferred to nitrocellulose and reacted with the polyclonal antibody directed against the linker region of the *ltrmdr1* gene. Wild-type, lane 1; DNM-R150, lane 2; Rv₁, lane 3. Molecular mass standards (kDa) are from Bio-Rad.

lation in the resistant line (not shown). However, the high concentration of the chemosensitizers necessary to increase the accumulation in the resistant parasites suggests that these modulators are not efficient reversal agents for MDR phenotype in these parasites.

Diffusion of DNM in Isolated Membranes

We studied whether other mechanisms, such as alterations in the passive permeability of plasma membranes, could contribute to the MDR phenotype. Incorporation of anthracyclines to hydrophobic environments such as the lipid

matrix of the membranes, is accompanied by a change in the fluorescence quantum yield of the drugs [31]. This property has been exploited to monitor unidirectional influx of DNM in tumor cells, allowing for the quantitative determination of the kinetic parameters defining the process [6]. On this basis, we used an identical rapid kinetic procedure (stopped-flow) to analyze the inward diffusion of DNM across isolated membrane vesicles from WT, DNM-R150 and Rv₁ parasites. Fig. 5 shows the fluorescence decay corresponding to the initial stages of the interaction of 5 μ M DNM with membranes from WT and DNM-R150 parasites. The interaction of DNM with the Rv₁ membranes has been omitted to better visualize the differences between WT and DNM-R150 membranes, since in this case the trace describing the process falls between the other two. Figure 5 shows that the process was very rapid (only 2 sec were necessary for its detection) and reveals kinetic differences between WT and resistant membranes in their interaction with DNM. Fitting and statistical analysis of the traces of Fig. 5 to equation A (see "Materials and Methods") indicates that regardless of the type of membrane, the process required at least two components to be satisfactorily described. This is illustrated in Fig. 6, which shows both the experimental traces and the corresponding fits to equation A containing one (Fig. 6A) or the sum of two exponential components (Fig. 6B). Table 2 contains the amplitude and the apparent rate constant values associated to each of the two components. While the sum ($A_1 + A_2 + C$) reflecting total DNM fluorescence signal was indistinguishable in WT and resistant membranes ($P > 0.05$), individual analysis of A_1 , A_2 and C reflected significant differences ($P < 0.05$)

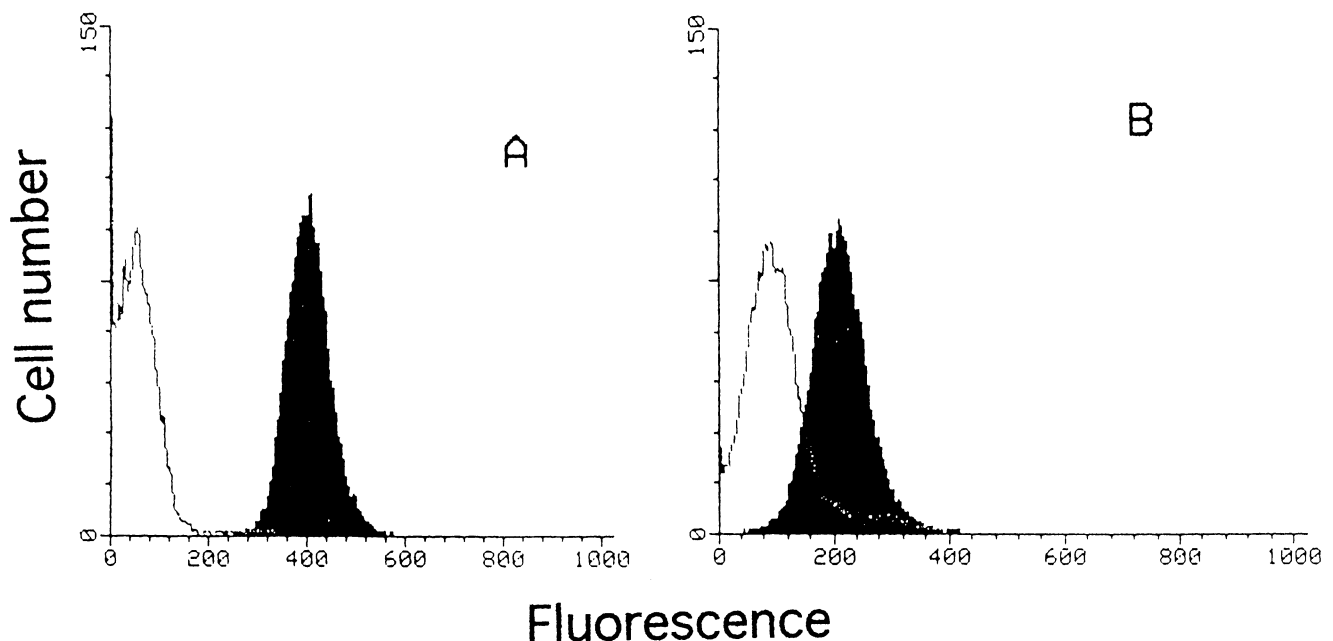


FIG. 4. Cellular accumulation of DNM in *L. tropica* lines. Fluorescence intensity histograms were obtained by flow cytometry after incubation of WT (A) and DNM-R150 (B) parasites with 8 μ M DNM for 1 hr at 28° (Black profile). White profile corresponds to a fluorescence histogram of parasites incubated without any drug. A total of 5000 cells were counted for each histogram. Experiments were repeated 3 times and gave essentially the same profiles as the ones shown here.

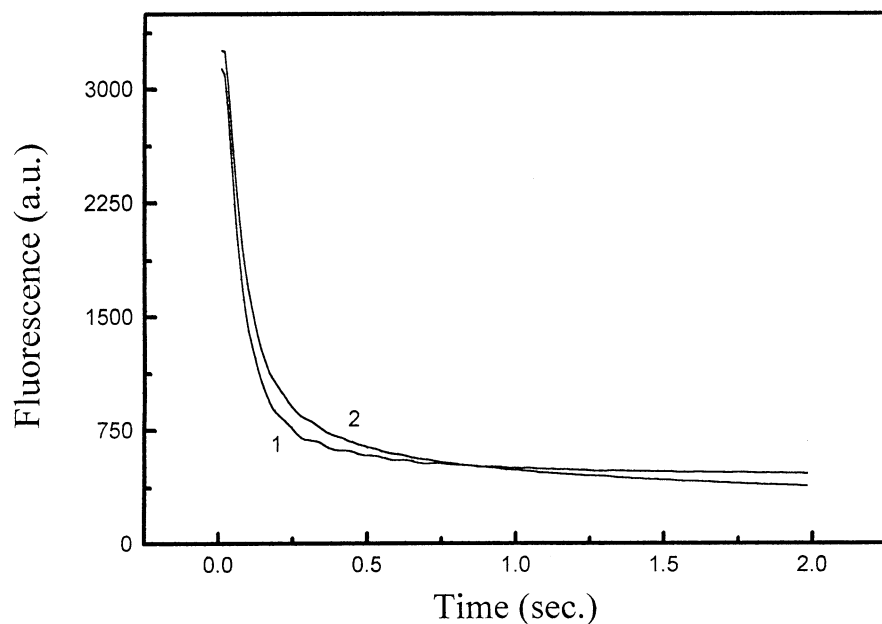


FIG. 5. Stopped-flow recordings corresponding to the fluorescence decay of DNM upon mixing WT (1) or DNM-R150 (2) membranes at 25°. Final concentrations were: 5 μ M DNM and 0.5 mg protein/mL for membrane samples. Units at the y axis are expressed as fluorescence arbitrary units (a.u.).

between WT and DNM-R150 membranes. In spite of the apparent close value of the rate constants (k_1 and k_2) between both types of membranes, they were also significantly different ($P < 0.05$). As is also indicated in Table 2, the kinetic parameters associated to the interaction of DNM with the Rv₁ membranes fall between those of WT and DNM-R150 membranes.

DISCUSSION

A main objective in this work has been to study whether modifications other than overexpression of a Pgp can contribute to the resistant phenotype in an *L. tropica* line resistant to DNM. The line shows an MDR-like phenotype similar to that described in mammalian cells. It is charac-

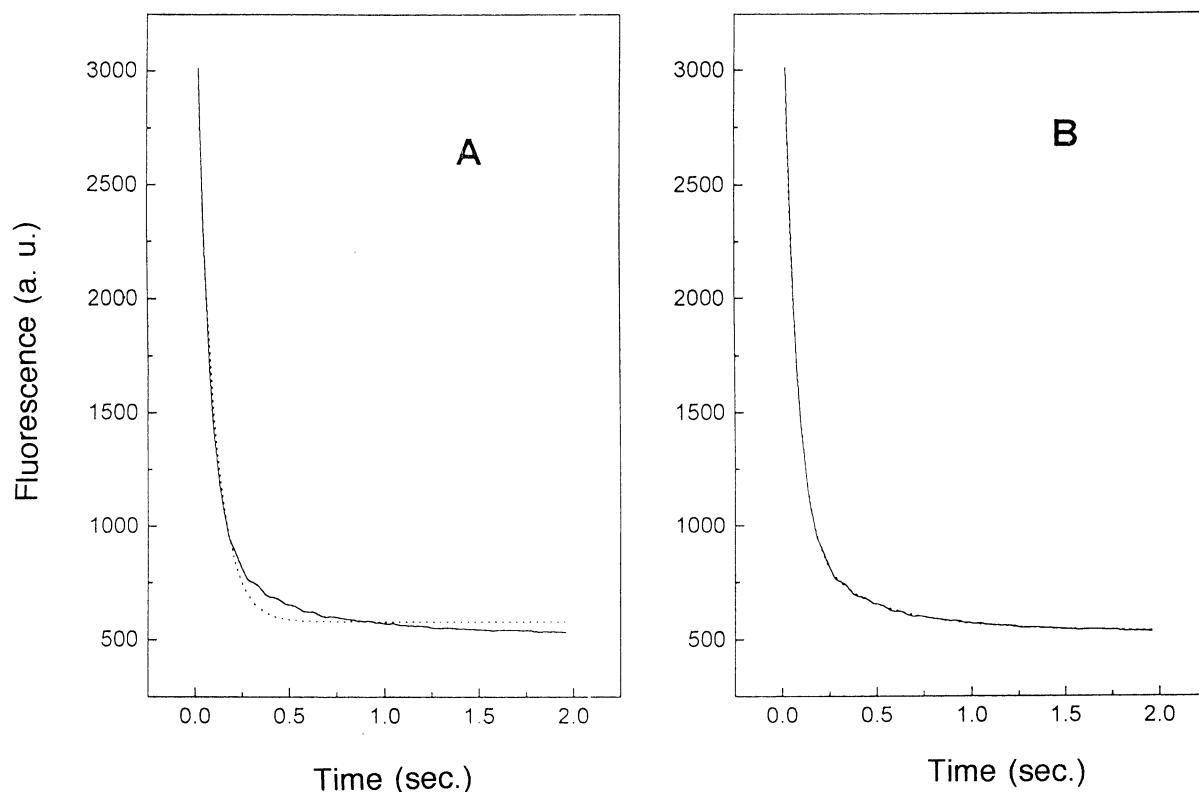


FIG. 6. Experimental trace (solid line) and representative fitting (dashed line) corresponding to the interaction of DNM with WT membranes to equation A described in "Materials and Methods." A, trace and fit corresponding to equation A containing one exponential component. B, trace and fit corresponding to equation A containing the sum of two exponential components.

TABLE 2. Kinetic parameters corresponding to the initial interaction of DNM with plasma membrane vesicles from WT, DNM-R150 and Rv₁ parasites

| Membranes | A ₁ | k ₁ | A ₂ | k ₂ | C |
|-----------------|----------------|----------------|----------------|----------------|----------|
| WT* | 2858 ± 15 | 16.6 ± 0.2 | 413 ± 16 | 2.70 ± 0.07 | 539 ± 2 |
| DNM-R150* | 3411 ± 33 | 12.2 ± 0.3 | 682 ± 28 | 1.60 ± 0.10 | 414 ± 12 |
| Rv ₁ | 3333 ± 26 | 14.2 ± 0.2 | 635 ± 25 | 1.85 ± 0.08 | 538 ± 6 |

The kinetic parameters were obtained from the fitting of experimental traces describing the interaction of DNM with plasma membranes from WT, DNM-R150 and Rv₁ parasites to equation A (see "Materials and Methods") containing the sum of two exponential terms. Values expressed as the mean ± SE of triplicate determinations are given as fluorescence arbitrary units (A₁, A₂, C) and s⁻¹ (k₁, k₂), respectively.

* All values were significantly different ($P < 0.05$) between WT and DNM-R150 membranes.

terized by the amplification of the *ltrmdr1* gene as an extrachromosomal element (DN circle) and the overexpression of a Pgp of 150 kDa. The resistance of parasites to DNM is unstable without drug pressure, as evidenced by the rapid fall of the IC₅₀ in the revertant lines, a result which is also supported by the Northern and Western blot analyses. The resistance to DNM appears to be due in part to a failure to accumulate the drug, an event associated with a drug efflux process mediated by Pgp. Drug efflux in mammalian cells can be overcome by treatment with different chemosensitizers [15]. As previously reported for *L. donovani* [13], the reversion of the cross-resistance could not be achieved in our DNM-R150 parasites by the use of chemosensitizers such as verapamil and cyclosporin A. Transfection experiments using the *mdr* gene from *L. donovani* and *L. enriettii* [13, 14] have demonstrated that this gene overexpression is responsible for the MDR phenotype observed in the resistant parasites; but the levels of resistance found in the transfectant parasites were significantly lower than in the selected parasites. Differences in RNA processing signals in the transfected construct, the existence of regulating elements, or additional mechanisms could explain the low resistance levels.

Alterations in the balance between influx and efflux of cytotoxic agents have a great influence on the intracellular steady-state level reached by the drugs [32, 33]. Reduced influx in MDR cells is widely reported in the literature [34–36], and this has been the rationale behind the exploration of the permeability of DNM across isolated plasma membranes from WT and resistant parasites. In the vesicular system used here, the absence of either drug binding to intracellular components or active drug efflux, along with the experimental design promoting unidirectional drug movement from the aqueous phase to the lipid bilayers, makes the evaluation of the net influx (inward diffusion) of DNM across the vesicles easier. Our results reveal the occurrence of two kinetic components in the interaction between DNM and the vesicles, resembling the behavior previously reported with tumor cells [6]. According to that model, the fast component (defined by A₁ and k₁) indicates an initial rapid interaction of DNM with membrane surface components, likely involving electrostatic interactions between the protonated form of DNM

with the abundant negatively charged lipophosphoglycan [37]. Also by analogy with the observations in tumor cells, the slow component would reflect inward diffusion of DNM. While A₂ would be associated with the amount of drug traversing the membrane from the outer to the inner leaflet of the bilayer, k₂ would represent the rate at which the diffusion occurs. Consequently, if the influx of anthracyclines in *L. tropica* parasites takes place by passive diffusion as in cancer cells [38], the results reported here would be consistent with a facilitated entrance of DNM into the WT parasites with respect to that in drug-resistant parasites. The simplest explanation for the data associated to the Rv₁ membranes suggests that some of the changes associated with resistance have not fully reversed in the Rv₁ parasites, an observation also supported by the higher IC₅₀ value of the revertant cells as compared with that of the WT parasites. Although the kinetic parameters in Rv₁ are close to those in the resistant membranes, the differences in k₂, reflecting changes in membrane permeability, do not appear sufficient to explain the huge difference in the level of resistance between both cell types.

It has been suggested that Pgp-mediated drug extrusion may directly occur from the lipid phase of the cell membranes [39, 40]. Recently, Eytan et al. have reported that the rate at which drugs traverse the membranes could be a determinant for their efficient export by Pgp [5]. In this context, the observed restricted permeability of the membranes from resistant parasites to DNM could be envisaged as a cellular alteration aimed at facilitating the interaction of the drug with Pgp or Pgp-like pumps inside the membranes. Changes in the diffusion of anthracyclines in several cell systems have been associated with alterations in the lipid composition and/or lipid organization of the membranes [36, 41, 42]. In our opinion, these changes could constitute an additional mechanism to other more efficient drug detoxification processes present in drug-resistant cells.

In conclusion, we propose that the mechanism of DNM resistance in *L. tropica* might involve at least two complementary pathways: one regulating drug efflux, mediated by the overexpression of an *mdr*-like gene termed *ltrmdr1*, and a second independent phenomenon characterized by a restricted membrane permeability to cytotoxic drugs.

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