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Impaired drug uptake in methotrexate resistant *Crithidia fasciculata* without changes in dihydrofolate reductase activity or gene amplification

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Crithidia fasciculata cells grown in defined medium are sensitive to methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR). When cells are challenged with 2–5 μM MTX, cell division ceases after 3–4 divisions and the cells become rounded and immotile for approximately 60 h, with a 40% decrease in cell viability occurring during this period. The cells then recover normal morphology and cell division resumes. Cells which undergo this treatment can be transferred directly into high levels of the drug (1–2 mM). The resistance phenotype is stable in the absence of the drug. Resistance correlates with impaired uptake of [^{3}H]MTX, which in wild-type cells is taken up by a carrier-mediated process. There is no indication of gene amplification at the DNA level or at the level of DHFR activity, as occurs in the case of MTX-resistant *Leishmania major*. Several lines of MTX-resistant *L. major* which show gene amplification also exhibit impaired uptake of [^{3}H]MTX.

Key words: *Crithidia fasciculata; Leishmania major*; Methotrexate; Drug resistance; Methotrexate transport; Dihydrofolate reductase

Introduction

Trypanosomatid protozoa, the etiological agents of several important animal and human diseases, rapidly develop resistance to drugs used in therapy [1–2] and to several drugs used under experimental conditions [3–5]. In the case of *Leishmania major* promastigotes in culture, resistance to high levels of methotrexate (MTX), an inhibitor of dihydrofolate reductase (DHFR), was reported to be associated with the amplification of two regions of DNA, one of which apparently encodes the bifunctional thymidylate synthetase-dihydrofolate reductase gene (TS-DHFR) [5–7]. The amplified DNA is present initially in the form of 50–100 copies of two distinct extrachromosomal circular DNA molecules of 30 and 85 kb. Both the resistance phenotype and the amplified circular molecules are lost by growth in the absence of the drug. After prolonged culture of resistant cells in MTX, the amplified DNAs appear to integrate into chromosomal DNA, and the resistance phenotype becomes stable in the absence of selective pressure [5,6].

We have studied the development of resistance to MTX by the monogenetic trypanosomatid, *Crithidia fasciculata*. The development of resistance occurs in the majority of the cell population and is associated with a decrease in the rate of transport of the drug into the cell and not with amplification of the DHFR gene, as in the case of *L. major*. Furthermore, we found that several cell lines of MTX-resistant *L. major* which do show gene amplification also show decreased uptake of MTX when compared with sensitive cells. These results indicate that more than one mechanism can be involved in the resistance of trypanosomatids to MTX.

Abbreviations: BHI medium, Brain Heart Infusion medium; DNP, dinitrophenol; kb, kilobase pairs; KD medium, Kidder–Dutta medium; MTX, methotrexate; NEM, *N*-ethylmaleimide; Tes, *N*-[tris(hydroxymethyl)methyl]amino-ethanesulfonic acid; TS-DHFR, thymidylate synthetase-dihydrofolate reductase.

Materials and Methods

Cell culture. C. fasciculata were grown in 2–5 ml

liquid culture in either Brain Heart Infusion (BHI) (Difco laboratories) plus hemin (10 μg ml^{-1}), or in Kidder–Dutta (KD) defined medium [8], in 30 ml screw cap tubes rotated at 6 rpm at 27°C. In both culture media, the doubling time for wild type cells was approximately 4 h, and maximal cell density was approximately 10^8 cells ml^{-1}. Cells were maintained in log phase by reseeding into fresh medium when they approached maximal cell density. Cell number was determined using a hemocytometer.

Cells were plated on 1% agar plates (Difco Bactoagar) prepared with KD medium, and the sealed plates incubated inverted at 27°C for 4 to 7 days.

Aliquots of fresh stock aqueous solutions of MTX (Sigma Chemical Co.) were added to the media used for liquid culture and to agar plates at the concentrations specified below.

Development of MTX-resistant cell lines. MTX-resistant strains of *C. fasciculata* in defined KD medium were initially obtained by a stepwise process as described for *L. major* [5]. Cells were seeded at approximately 10^6 cells ml^{-1} in a medium containing the specified concentration of MTX. When the cell density reached a plateau, the cells were transferred into fresh medium with the same concentration of MTX. When the cell doubling time stabilized, cells were seeded into the next higher concentration of MTX. By using concentrations of 1, 2, 5, 10, 50, 100, 200, 500, 1000 and 2000 μM MTX, cell lines resistant to all these concentrations were established and propagated by continuous subculture in the presence of drug and maintained frozen at −70°C. The resistant lines are labeled R1, R2, ..., R1000. Resistant lines were also obtained by direct adaptation of the 2, 5, 10 or 50 μM resistant cells to 1000 or 2000 μM MTX. These rapidly adapted resistant lines appeared to have the same properties as the stepwise adapted lines.

A clonal strain of *L. major* promastigotes (POJ1) (previously known as *Leishmania tropica*) obtained from D.V. Santi (University of California, San Francisco) was grown in 2 ml M199 liquid medium (Gibco) supplemented with 20% fetal calf serum, 25 mM Hepes (pH 7.4) and 10 μg ml^{-1} hemin in 16 × 125 mm polystyrene culture tubes rotated at 6 rpm at 27°C. Cells resistant to 100 μM of MTX were obtained by the stepwise selection procedure previously described [5]. We noted that, as in the case of *C. fasciculata* described in Results, the *L. major* cells exposed to MTX underwent a transitory period of metabolic stress evidenced by rounding and loss of motility. However, unlike the case with *C. fasciculata*, the *L. major* cells in MTX clumped into huge masses of cells during this initial period of adaptation.

DHFR assay. C. fasciculata cells from resistant lines recently established and propagated for 3–4 passages in the presence of MTX were grown to a density of 8–10 × 10^6 cells ml^{-1} and pelleted by centrifugation (1000 × *g*, 4 min). The cells were washed twice with MTX-free medium and cultivated in drug-free KD medium for different times. Resistant *L. major* cells maintained in 100 μM MTX for 4 months were washed, resuspended and cultivated in MTX-free medium for 24 h. Cells were harvested by centrifugation and the cell pellet was washed with phosphate-buffered saline (200 mM sodium-phosphate, 150 mM NaCl, pH 7.5) at 4°C and resuspended at a density of 1.5 × 10^9 cells ml^{-1} in 50 mM *N*-[tris (hydroxymethyl)methyl]aminoethanesulfonic acid (Tes) (pH 7.4), 1 mM sodium EDTA, 5 mM dithiothreitol, 50 μM phenylmethylsulfonyl fluoride, and 10% (v/v) glycerol at 4°C [5]. Cells were sonicated until no more intact cells were visible. The sonicate was then centrifuged at 15000 × *g* for 30 min at 4°C to yield a cell-free extract with a protein concentration of 4 mg ml^{-1}. Protein concentration was determined by the method of Bradford [9]. DHFR catalytic activity was measured spectrophotometrically at 25°C [10] in 1.0 ml of a mixture containing 100 μM H_2folate, 100 μM NADPH, 50 mM Tes (pH 7.4), 75 mM 2-mercaptoethanol, and 1 mM EDTA. MTX inhibition of DHFR activity in both wild-type and resistant cells was carried out with MTX purified by DEAE-cellulose chromatography [11]. Concentration of purified MTX was determined spectrophotometrically (ϵ_{370}=8100 M^{-1} cm^{-1} in 0.1 N NaOH).

Enzymatic hydrolysis of MTX. Degradation of

MTX by enzymatic hydrolysis in *C. fasciculata* as described by Iwai et al. [12] was measured in cell extracts from wild-type and resistant cells. Cells were grown in absence of MTX for 24 h, washed twice and sonicated in 50 mM Tris-HCl (pH 7.0), centrifuged at 15000 × *g* for 30 min and the enzymatic activity was measured by the method of Levy and Goldman [13].

Nucleic acid analysis. Isolation of total DNA and kinetoplast DNA from *C. fasciculata* and total DNA from *L. major* was performed as described [14]. Restriction endonucleases were obtained from New England Biolabs and Bethesda Research Labs and used as recommended by the suppliers. For direct visualization of amplified regions of DNA, digested nuclear DNA was electrophoresed in 0.7% agarose gels in 90 mM Tris, 90 mM borate and 2.5 mM EDTA (pH 7.9) with 0.5 μg ml^{-1} ethidium bromide and the gels examined by transillumination with 300 nm UV.

Amplified segments of DNA were also detected by the method of DNA renaturation in agarose gels [15] with the following modifications: DNA (35 μg) was digested with Hind III (5 units μg^{-1} DNA), extracted with phenol/chloroform (1:1) and precipitated with ethanol. The precipitate was resuspended in 0.1 mM EDTA, 10.0 mM Tris-HCl (pH 7.5) to 3 μg μl^{-1}. An aliquot (1 μl) of this digest was labeled with [α-^{32}P]dATP using 0.2–0.5 U of Klenow fragment of DNA polymerase I (Bethesda Research Labs). The labeled fragments were extracted with phenol/chloroform and precipitated with ethanol and were used as a tracer in electrophoresis of Hind III fragments, performed as described [15]. After two cycles of denaturation, renaturation and S1 nuclease treatment, the gel was dried and autoradiographed.

MTX transport. C. fasciculata wild-type cells (2×10^7 cells ml^{-1}) were pelleted by centrifugation, washed twice and resuspended in KD medium without adenine and folic acid to the original cell density. Cells from resistant strains maintained for 3 months in drug-containing medium were washed twice with MTX-free medium and grown in the absence of drug for 24 h. Cells were then washed twice with drug-free, adenine and folic acid-free KD medium, pelleted and resuspended in this medium to a density of 2×10^7 cells ml^{-1}.

Prior to transport experiments with *L. major*, cells which have been maintained in drug-containing medium for 3 months were grown in absence of drug for 24 h, washed and resuspended in M199 medium with 25 mM Hepes (pH 7.4) to a density of 10^6 cells ml^{-1}.

Aliquots (1 ml) of these cell suspensions were portioned into Eppendorf tubes and used for transport measurements. [3′, 5′, 7-^{3}H]MTX (Amersham) (purified by DEAE-chromatography [11] and mixed with purified nonradioactive MTX to obtain final concentrations of 9–12.5 μM and specific activities of 1.12–1.51 mCi μmol^{-1}) was added to the cell suspensions at different concentrations, and the transport of the drug into the cell was measured as a function of time and MTX concentration at 27°C and 0°C. [^{3}H]MTX taken up by cells was determined by the following procedure: cells were pelleted in the incubation tube in an Eppendorf Microfuge, the supernate was carefully aspirated and the pellet digested with 0.5 ml of 1 N KOH for 1 h at 80°C. The digested material was mixed with 3 ml of scintillation mixture (PCS, Amersham) and the radioactivity measured in a Beckman scintillation counter. Volumes of intracellular water and extracellular space of *C. fasciculata* were obtained using [^{14}C]inulin (10.9 Ci mol^{-1}) and ^3H_2O (2.5 mCi ml^{-1}) (Amersham) as described [16,17].

Results

Effect of MTX on wild-type cells. Wild-type *C. fasciculata* cells are fairly insensitive to MTX when growing in the undefined BHI medium. In this medium, the growth rate in 100 μM MTX is identical to the growth rate in the absence of the drug. In 1 mM MTX the growth rate is reduced to half of the control value, but the cells still show normal morphology and motility. However, in KD defined medium, wild-type cells are highly sensitive to the drug. As shown in Fig. 1A, the growth rate is halved at 0.22 μM MTX and no growth occurs at MTX concentrations above 5 μM. Similar observations are made when wild-type cells are grown on agar plates containing

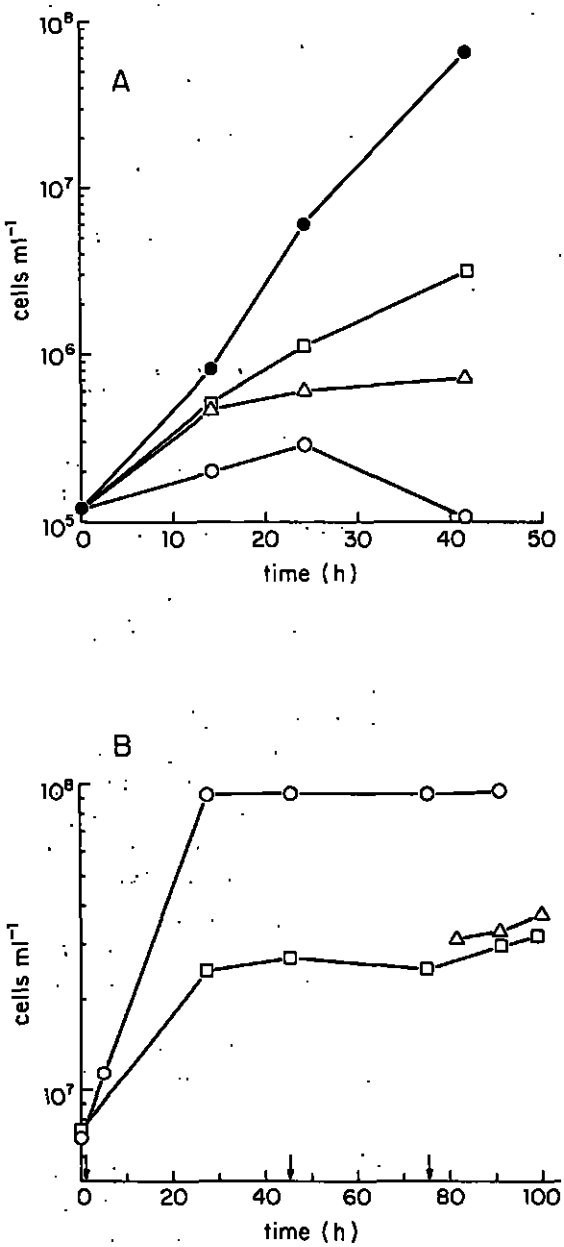

Fig. 1. Effect of MTX on growth of wild-type *C. fasciculata* in KD medium. (A) Growth curves of cells in increasing MTX concentrations. No MTX (•), 0.3 μM (□), 0.5 μM (△) and 1.0 μM MTX (○). (B) Growth curves of cells in KD medium in absence (○) and in presence (□) of 2 μM MTX. At the times indicated by arrows, cells were washed with drug-free medium and used for determination of plating efficiency on 1% agar. After 80 h of incubation in 2 μM MTX, an aliquot of the cell culture was washed and resuspended in fresh KD medium with 2 μM MTX at the original cell density (△).

MTX. In the absence of drug, wild-type cells form colonies 2–3 mm in diameter after four days of growth with a plating efficiency of approximately 30% [4]. In the presence of 2 μM MTX, only very small colonies appear four days after plating.

As shown in Fig. 1B, in KD liquid medium with 2 μM MTX, wild-type cells initially go through 3–4 cell divisions and then cease dividing. At this point, the cells exhibit an abnormal morphology, the major features of which are a rounded, swollen shape, intense cytoplasmic granulation and short, immotile flagella (Fig. 2). This state persists until approximately 80 h after transfer of the wild-type cells into drug-containing medium. At this stage, the cells begin to recover morpholog-

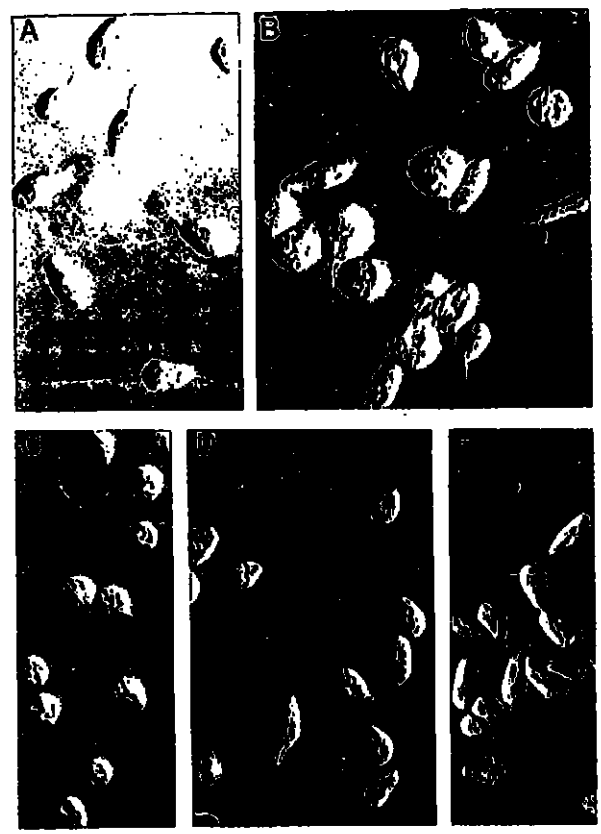

Fig. 2. Morphological changes in wild-type *C. fasciculata* during incubation in KD medium containing 2 μM MTX (see Fig. 1B for growth curve). (A) Cells in log phase just before transfer into MTX-containing medium; (B) 27 h; (C) 72 h; (D) 96 h; (E) 120 h. Photomicrographs of living cells taken with Normarski optics. Magnification, × 1000.

ically, resume motility and reinitiate cell division (Fig. 2D). In the following 250 h, even with frequent medium change, the cells divide at a growth rate which is 20% of the value for wild-type cells growing in the absence of drug. Thereafter, cells divide at the wild-type rate. The number of cell divisions which the cells undergo before reaching the arrested stage is independent of the initial cell density in the medium (data not shown). Nor is the time course of the morphological transition and the temporary arrest of cell division affected by transferring the cells into fresh medium (Fig. 1B); this implies that the inhibition of cell division is not due to depletion of nutrients in the medium. The relative plating efficiency of the cells on drug-free KD agar decreases to 60% of non drug-treated control cells at the beginning of the plateau period (t=30 h) and remains constant thereafter. The colonies obtained from cells plated during the plateau period were heterogeneous in size after four days of growth. The absence of a dramatic decrease in relative plating efficiency and microscopic observations (data not shown) that no more then 40% of the cells die during the initial drug treatment (up to 300 h) and that the cells return to normal shape and begin dividing after 80 h all imply that the appearance of the resistance phenotype is not due to a low frequency of spontaneous resistant mutants taking over the culture. Rather it is an adaptation of most of the cells in the original population to higher drug levels.

Establishment of MTX-resistant lines by stepwise selection. Cells which underwent the initial 2 μM MTX treatment and resumed normal growth (after approximately 300 h in 2 μM MTX) can survive in higher concentrations of MTX. Following serial passages of the parental cells in stepwise increases of concentrations of MTX, cell lines resistant up to 2 mM MTX were established. This drug concentration is approximately 9000-fold higher than is necessary to cause 50% inhibition of the growth of wild-type cells. Cells transferred from 2 μM into higher MTX concentrations initially showed slower growth, but stabilized at normal growth rates after three or four passages (4–5 generations each). At concentrations as high as 500 μM the stabilized growth rate was near to normal, while at 1000 μM the rate remained at approximately half of the rate observed with wild-type cells in absence of drug. At this MTX concentration the maximum cell density was also reduced to 10% of the normal value. Cell lines (R2, R5, R10, ..., R1000) resistant to MTX in the range from 2 to 1000 μM were maintained by continuous subculture at the respective MTX concentration.

Establishment of MTX-resistant lines by direct exposure of R2 cells to high MTX concentrations. To test the necessity of stepwise increase of MTX concentration for the development of cells with high levels of resistance, cells from the resistant lines, R2, R5 and R50, were transferred directly into 1000 μM MTX-containing liquid medium. The R2 cells did not divide immediately in 1000 μM MTX, but established a normal growth rate within 5 days. However, no morphological changes occurred during this lag period as described above for wild-type cells in 2 μM MTX. The R5 cells resumed growth after 48 h in 1000 μM MTX, while the R50 cells remained dividing at the original rate, unaffected by the higher concentration of the drug. Similar observations were made by streaking R2, R10 and R100 cells on 1% agar drug-gradient plates [18], containing MTX in the range of concentration from 0 to 1000 μM. The R10 and R100 cells grew normally on these plates, as well as the R1000 cells used as control, while the R2 cells grew more slowly, as observed from the diameters of the colonies at 4 and 7 days after plating (data not shown).

Stability of the resistance phenotype in absence of selection. To address the question of the stability of resistance to MTX in the absence of selective pressure, R100, R200, R500 and R1000 cells were grown in drug-free KD medium for approximately 200 generations. When the cells were transferred back into KD media containing the original MTX concentrations, all strains showed a retention of the resistance phenotype (Fig. 3). Initially the growth rates were less than in drug-free medium but normal growth rates were established after 2–3 passages in drug-containing medium. Established R1000 cells, which were main-

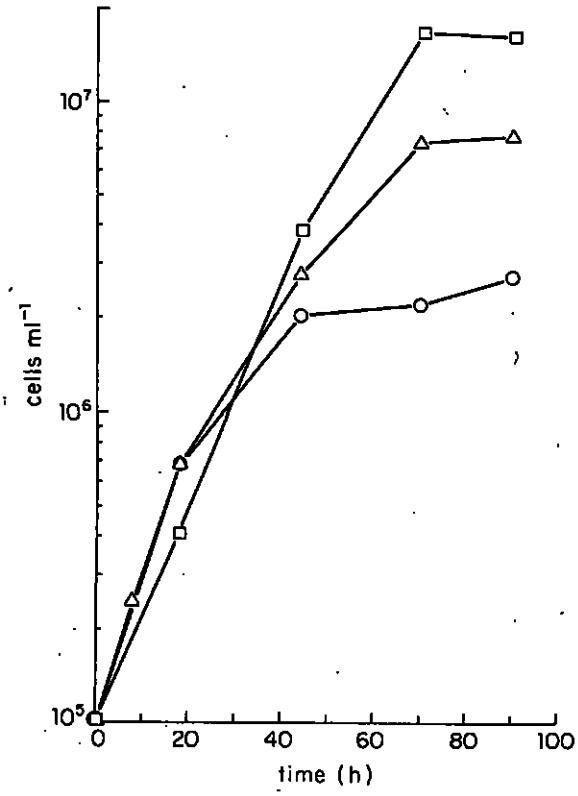

Fig. 3. Stability of MTX resistance phenotype. Cell lines resistant to different concentrations of MTX (R200, R500 and R1000) were grown for approximately 200 generations in drug-free KD medium and were then inoculated into MTX-containing KD medium. R200 in 200 μM MTX (□); R500 in 500 μM MTX (△); R1000 in 1000 μM MTX (○).

tained in drug-free medium for 4 months (700 generations) and then tested for MTX resistance by streaking on drug gradient plates (0–1000 μM MTX), grew normally at all drug concentrations. These results suggest that the resistant phenotype is stable in the absence of selective pressure.

Absence of cross resistance to other drugs. To test for the specificity of the resistance developed against MTX, resistant cell lines R2, R10, R100 and R1000 were streaked on gradient plates containing drugs which are structurally unrelated to MTX. After seven days no growth was observed in the presence of 5-fluorouracil (0–20 μg ml^{-1}), 6-azauridine (0–10 μg ml^{-1}), actinomycin D (0–10 μg ml^{-1}) and tubercidin (0–10 μg ml^{-1}), implying that there is no cross-resistance to these drugs.

Amplified DNA in wild-type and MTX-resistant cells. Because MTX resistance in *L. major* has been correlated with amplification of the DHFR gene as well as of other regions of DNA of unknown functions [5–7], the possible amplification of specific regions of DNA in MTX-resistant *C. fasciculata* was examined by two methods. Coderre et al. [5] showed that amplified genomic DNA sequences in *L. major* can be directly visualized in ethidium bromide-stained gels of restriction digests due to the low genomic complexity of these cells. Fig. 4A shows a comparison of the restriction fragment profiles obtained by digestion of total DNA derived from wild-type and R1000 *C. fasciculata* cells. In both cell lines, identical patterns of restriction fragments were observed, with no indication of specific amplified DNA sequences in resistant cells which are not present in wild-type cells. Identical observations were made using DNA from the cell lines R5, R50, R100 and R500 (data not shown). The technique of DNA renaturation in agarose gels [15] was also used to detect and compare amplified DNA sequences in wild-type and R1000 MTX-resistant cells (Fig. 4B). Again, no new amplified sequences were detected in the R1000 line genomic DNA.

As a control of our ability to visualize amplified regions of trypanosomatid DNA by the direct ethidium bromide staining procedure, 12 independent lines of *L. major* cells resistant to 100 μM MTX were obtained by the stepwise adaptation method, and the DNA from each cell line was analyzed. All 12 lines of *L. major* developed resistance to MTX which was associated with gene amplification as described by Coderre et al. [5] (Fig. 4C).

DHFR activity in wild-type and resistant C. fasciculata. Because MTX resistance in mammalian cells [19,21–27], insects [28] and *Leishmania* [5] has been correlated with increased levels of DHFR activity, the activity of this enzyme was measured in both wild-type and MTX-resistant *C. fasciculata* cell lines. Since MTX interferes with the DHFR assay, resistant cells growing in MTX were transferred into MTX-free KD medium and maintained in this medium for different times before being used in the assay. As shown in Fig. 5,

within 8 h of growth in drug-free medium the DHFR activities from several lines of resistant cells reached a plateau, which was identical within experimental error to the level of enzyme activity in wild-type cells. We conclude that there is no increase in DHFR activity in the MTX-resistant cell lines as compared to the wild-type cell line. This result is consistent with the absence of ob-

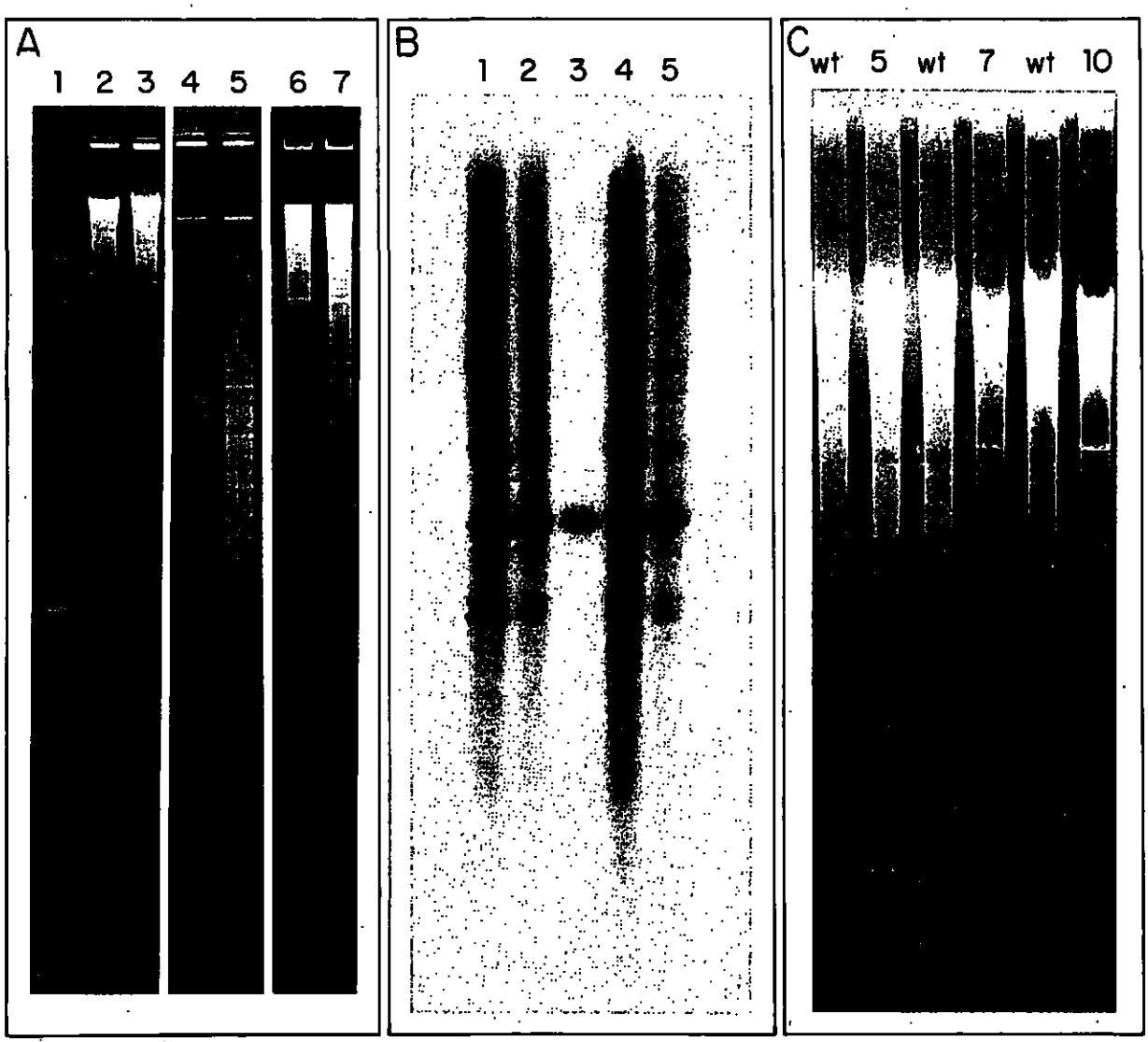

Fig. 4. Detection of amplified DNA in wild-type and MTX-resistant *C. fasciculata* and *L. major*. (A) Agarose (0.7%) gel electrophoresis of total DNA extracted from wild-type and MTX-resistant *C. fasciculata* cells, respectively, digested with Bam HI (lanes 2 and 3), Pst (lanes 4 and 5) and Hind III (lanes 6 and 7). Lane 1 contains Hind III fragments of Lambda phage DNA. The gel was stained with ethidium bromide. (B) Agarose (1%) gel electrophoresis of DNA extracted from wild-type and MTX-resistant *C. fasciculata* cells digested with Hind III and mixed with labeled tracer DNA followed by 'in gel' DNA denaturation, renaturation and S1 nuclease digestion (see Materials and Methods). Tracer DNA, labeled with ^{32}P with Klenow fragment of DNA polymerase I, was mixed with excess driver DNA; duplex DNA was detected by autoradiography after S1 digestion. Lane 1: driver=wild-type, tracer=wild-type; lane 2: driver=R1000, tracer=R1000; lane 3: driver=kinetoplast DNA from wild-type, tracer=kinetoplast DNA from wild-type; lane 4: driver=R1000, tracer=wild-type; lane 5: driver=wild-type, tracer=R1000.(C) Agarose (0.8%) gel electrophoresis of total DNA extracted from wild-type (wt) and three cell lines of *L. major* resistant to 100 μM MTX (cell lines 5,7 and 10) digested with Xba I.

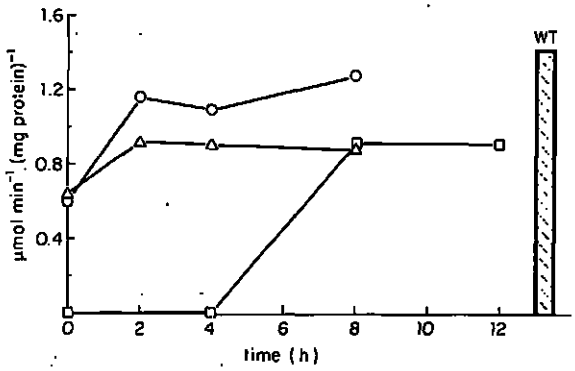

Fig. 5. DHFR activity in wild-type (WT) and MTX-resistant *C. fasciculata*. Resistant cells were transferred into drug-free KD medium and assayed for DHFR activity at different times after transfer. R10 (○), R100 (△) and R1000 (□).

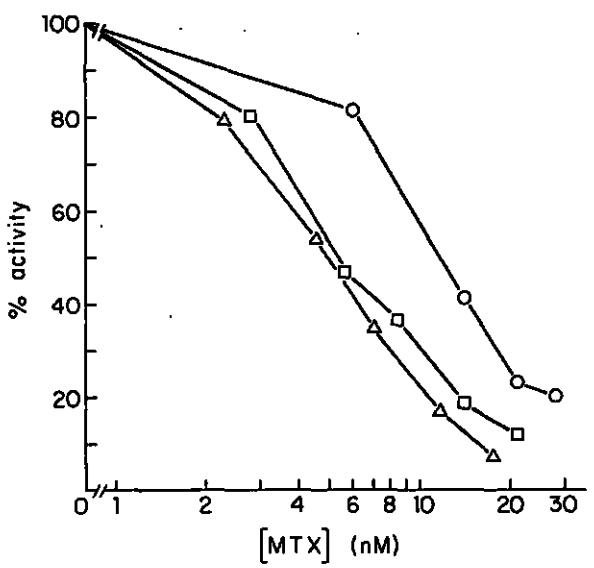

Fig. 6. MTX inhibition of DHFR activity in wild-type and MTX-resistant *C. fasciculata*. Cellular extracts from wild-type (□) and R1000 (△) were assayed for DHFR activity in presence of variable amounts of MTX. Results are expressed as the enzymatic activity with MTX present relative to the activity in the absence of inhibitor. Purified bovine DHFR (Sigma) (○) was used as a control.

servable DNA amplification in the R1000 cells.

MTX-sensitivity of DHFR activity in wild-type and resistant cells was also measured. In both cell lines the inhibition of this enzyme by MTX was identical (Fig. 6), indicating that the resistance phenotype is not due to appearance of an enzyme with lowered affinity for MTX.

Enzymatic hydrolysis of MTX in wild-type and resistant cells. In *C. fasciculata*, MTX is metabolized by a folate-hydrolyzing enzyme into glutamate and 4-amino-4-deoxy-10-methylpteroic acid [12]. To test whether the resistance to MTX could be related to an increase in the degradation of the drug, the activity of this enzyme was measured in triplicate samples from extracts of both wild-type and resistant cells. Similar activities were found in both cell lines, namely 29 nmol h^{-1} (mg protein)$^{-1}$ in wild-type and 36 nmol h^{-1} (mg protein)$^{-1}$ in resistant cells.

This enzymatic activity is also present in *L. major* (D.V. Santi, personal communication). We found no significant difference in activity between wild-type cells and one line of cells resistant to 100 µM MTX (cell line 11).

MTX transport in C. fasciculata. Because changes in drug transport have been frequently observed in MTX-resistant cell lines from several species [17,29–32], rates of net transport of MTX into both wild-type and resistant *C. fasciculata* cells were compared. As shown in Fig. 7A, there is a dramatic decrease in the rate of accumulation of [^{3}H]MTX in the R1000 resistant cells as compared to the wild-type cells. Because the efflux of labeled intracellular MTX following removal of extracellular MTX in wild-type *C. fasciculata* is very rapid ($t_{0.5}$=2 min) and because incubation of R1000 resistant cells with up to 1 µM [^{3}H]MTX did not result in accumulation of any observable intracellular drug (results not shown), we conclude that the low level of intracellular drug in resistant cells is due to a decreased rate of uptake rather than to an increase in the rate of drug exit, due, for example, to an increased catabolism of the drug and diffusion through the membrane.

Resistant cells retain the characteristic low rate of MTX uptake even when maintained in drug-free medium for a long period of time. R1000 cells maintained for 700 generations in absence of drug presented significant reduced uptake of [^{3}H]MTX when compared with wild-type cells (results not shown). Identically decreased drug uptake was also apparent in the cell lines R10 and R100 (results not shown).

The uptake of [^{3}H]MTX in wild-type cells follows saturation kinetics, suggesting the involve-

ment of a carrier-mediated type of membrane transport (Fig. 7B). The apparent K_t and V_{max} are 251.43 nM and 4.39 pmol min^{-1} mg^{-1}, respectively. An additional indication for the involvement of a carrier in the uptake of MTX in wild-type cells is given by the suppression of uptake by the sulfhydryl reagent, *N*-ethylmaleimide (NEM) [33] (Fig. 8); sulfhydryl reagents are known to cause inhibition of carrier-mediated transport of folate compounds in mammalian cells [34].

From the experiment shown in Fig. 7A and from the value for intracellular volume of 2.09 μl (mg protein)$^{-1}$ (obtained by the use of [^{14}C]inulin and ^3H_2O) the internal MTX concentration in wild-type cells was calculated to be approximately 1.4 μM at the saturation point when cells were incubated with 0.1 μM MTX. The suppression of uptake by azide [35] and dinitrophenol [36] (Fig. 8) and the significantly lower uptake at 0°C than at 27°C all indicate that the carrier-mediated transport of MTX into wild-type cells is energy-dependent, as is the case for other cell types [37–39].

MTX-transport in L. major. To answer the question whether gene amplification and decreased drug uptake are mutually exclusive mechanisms of resistance to MTX in trypanosomatids, we measured drug internalization in several independently derived lines of MTX-resistant *L. major* which showed DNA amplification. Table I shows that all resistant cell lines tested exhibited decreased net transport of MTX as compared with the parental sensitive cell line. This low-uptake phenotype was maintained for 60 generations in the absence of drug (Table I). However, as shown previously [5], the DNA amplification in these lines in the absence of selective pressure de-

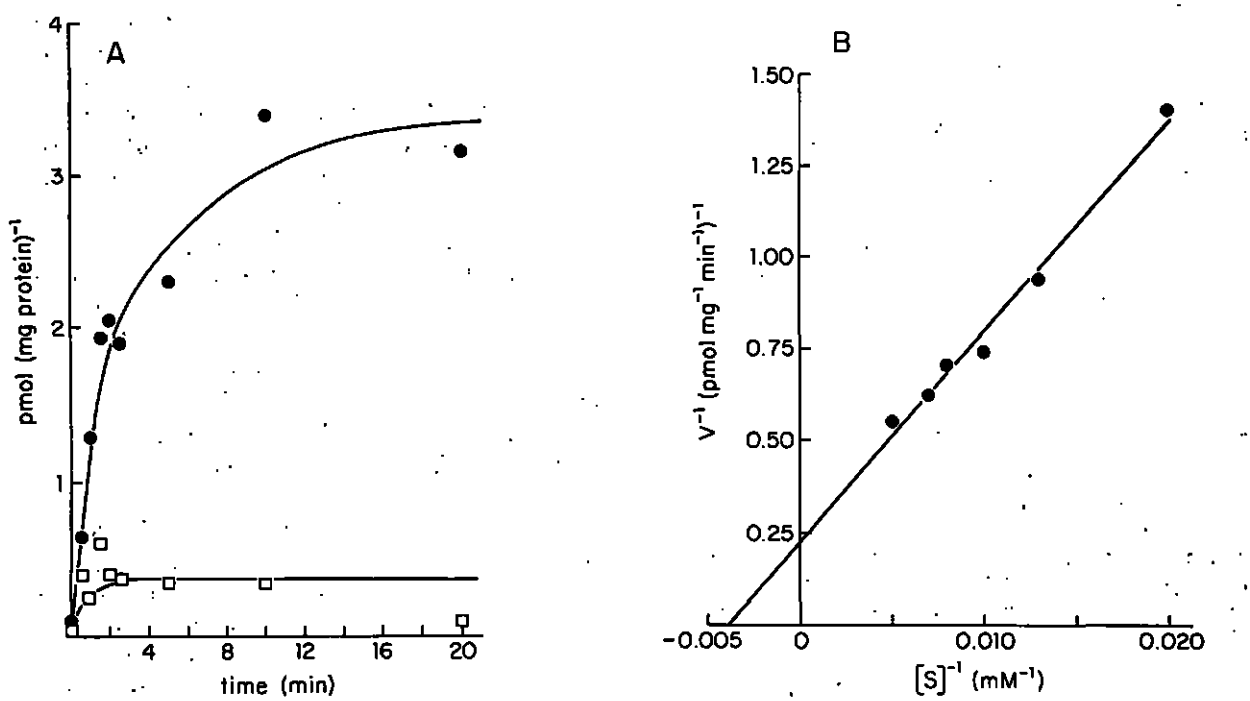

Fig. 7. [^{3}H]MTX uptake by wild-type and MTX-resistant R1000 *C. fasciculata*. Cells were washed twice and ressuspended in KD medium without folic acid and adenine and equilibrated at 27°C and 0°C. (A) Assays were initiated by addition of [^{3}H]MTX to a final concentration of 0.1 μM. Each point represents the difference between uptake at 27°C and 0°C and is the mean of 6 (wild-type (●)) or 3 (R1000 (□)) independent determinations. Values for t=0 were derived from volumes of extracellular space (wild-type, 1.01 μl mg^{-1}; R1000, 0.41 μl mg^{-1}) calculated using tritiated water and [^{14}C]inulin. (B) Lineweaver–Burk plot of [^{3}H]MTX uptake by wild-type cells (measured as in A). Each point represents the mean of 3 measurements. The calculated values for K_t and V_{max} in wild-type cells were 251.43 nM and 4.39 pmol min^{-1} mg^{-1}, respectively. No significant increase in uptake as a function of MTX concentration was observed in R1000 resistant cells.

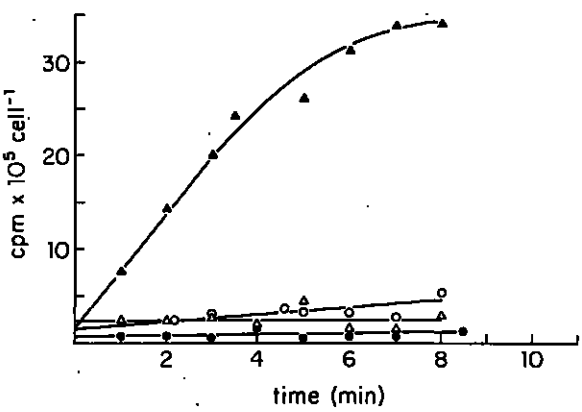

Fig. 8. Effect of dinitrophenol, azide and NEM on uptake of [^{3}H]MTX by wild-type *C. fasciculata*. Cells were incubated in KD medium containing 9 nM [^{3}H]MTX at 27°C. Inhibitors were added to the cell suspension 10 min before addition of [^{3}H]MTX. After incubation cells were pelleted in an Eppendorf Microfuge and washed twice with 200 mM sodium-phosphate (pH 7.4) at 4°C, and digested with 0.5 ml 30% H_2O_2 at 68°C for 8–12 h. Radioactivity was determined as described in Material and Methods; no additions (▲), 1.5 mM DNP (○), 10 mM azide (△), 1 mM NEM (●).

creased to wild-type levels (data not shown).

In order to test whether occurrence of an altered DHFR enzyme in MTX-resistant *L. major* could account for the decreased intracellular accumulation of drug, we compared the MTX sensitivity of DHFR in wild-type and in two lines of cells resistant to 100 μM MTX (cell lines 5 and 10). As has been described previously for *L. major* resistant to 1000 μM MTX [5], the DHFR activities from wild-type and resistant cells show identical drug sensitivity. In both cell lines 50% inhibition of enzymatic activity occurred at 5 nM MTX. Therefore, it seems that in these lines of MTX-resistant *L. major*, the decreased accumulation of MTX is due to an altered drug uptake as well.

Discussion

Development of resistance to MTX, an inhibitor of DHFR, has been extensively studied in mammalian tumor cells because of the widespread use of MTX in cancer chemotherapy. These studies have identified several mechanisms associated with resistance to MTX: defects in drug uptake [29–32], appearance of DHFR with reduced affinity for MTX [29,40–42], diminished formation of cytotoxic MTX-polyglutamates [19], activation of the deoxynucleoside salvage pathway [20] and increased levels of DHFR [21–28]. This last type of mechanism has been found in a wide variety of animal cell lines and has been associated with the amplification of DHFR genes (see ref. 43 for review).

Unlike the situation with mammalian cells, the establishment of resistance to MTX in *C. fasciculata* by stepwise adaptation appears to involve a large fraction of the cell population and not an outgrowth of rare resistant mutants: at least 60% of the population of wild-type cells transferred into a medium containing a low concentration of

TABLE I

Accumulation of [^{3}H]MTX by *L. major* promastigotes

Time of incubation (min)	pmol 10^{-8} cells^{-1} [a]				
	wild type	line 5[b]	line 7	line 10	line 11
1	4.57 ± 1.21	0.22 ± 0.01	0.23 ± 0.34	—	0.74 ± 0.33
	3.46 ± 0.37	0.81 ± 0.04[c]	—	1.29 ± 0.08[c]	1.27 ± 0.21[c]
10	30.20 ± 2.89	0.75 ± 0.13	2.58 ± 0.60	—	3.29 ± 0.53
	25.82 ± 0.89	6.26 ± 0.46[c]	—	9.29 ± 0.51[c]	4.94 ± 0.68[c]

[a] Cells were incubated in serum-free M199 culture medium containing 25 mM Hepes (pH 7.4) with 0.1 μM [^{3}H]MTX (1.51 mCi μmol^{-1}) at 27°C and 0°C for the time indicated. Intracellular radioactivity was measured as described in Material and Methods. Numbers express the difference between values obtained at the two temperatures using the same cell culture and represent the mean and standard deviation of triplicate samples.

[b] Lines 5, 7, 10 and 11 represent four lines of *L. major* resistant to 100 μM MTX.

[c] Cells grown in absence of MTX for 60 generations.

MTX can acquire resistance to the drug without cell division. During adaptation to the drug, the cells apparently undergo metabolic stress, evident in the transitory abnormal cellular morphology, which normalizes when cell division resumes. In addition, cells which were adapted initially to low concentrations of the drug can be transferred into much higher drug concentrations without apparent deleterious effects and these cells resume normal growth after a short period of adaptation.

The adaptation of *L. major* cells in culture to MTX appears to follow the same process in which the entire cell population develops resistance after a transitory period of stress. However, this could not be examined quantitatively since in the presence of MTX the cells agglutinated into large masses during the period of adaptation and only separated from the masses after the return of normal cell shape and motility.

Establishment of resistance to MTX in *C. fasciculata* by these methods is associated with low intracellular drug concentration most likely caused by impaired uptake of drug through the plasma membrane and is not correlated with overproduction of DHFR caused by specific gene amplification, as reported for *L. major* [5–7].

In trypanosomatid protozoa, enzymes involved in synthesis of purines are apparently absent [8,44,45], and therefore the major, if not sole intracellular target of MTX is probably the bifunctional TS-DHFR enzyme. Because this enzyme in both wild-type and resistant cells showed similar MTX sensitivity and specific activity, we conclude that the lack of drug accumulation in resistant cells is not a consequence of reduction of intracellular MTX binding capacity but could be due to an inactivated inward transport of the drug.

It is apparent that MTX transport in *C. fasciculata* is energy-dependent and proceeds via a carrier, with a K_t value which is intermediate between the values found in bacteria and in mammalian cells [37]. In these organisms such carriers are normally involved in transport of natural folates [37–39], and this could be the case in trypanosomatids as well.

There are, however, significant differences in the mechanism by which these three types of cells control the activity of this carrier. In bacteria, folate transport is regulated by a repression mechanism which causes changes in the level of carrier in the membrane in response to changes in concentration of folate in the medium, whereas in mammalian cells the cyclic nucleotide system is used for this purpose [35,37]. *C. fasciculata* behave differently from bacteria by not showing a decrease in MTX uptake after growing for two passages in 4 mM folic acid (results not shown). In addition, *C. fasciculata* behave distinctly from mammalian cells in that MTX uptake is inhibited by azide; in mammalian cells azide causes a decrease in the intracellular concentration of cAMP, which results in a higher intracellular concentration of MTX [35].

A striking property which distinguishes the mechanism of development of resistance to MTX in *C. fasciculata* from the metabolic adaptational processes in bacteria and mammalian cells is that drug resistance and low rate of MTX uptake are maintained indefinitely in the absence of selective pressure. However, in the absence of a traditional genetic approach, definitive proof that the MTX-resistance phenotype in *C. fasciculata* is an inherited genetic change awaits the development of a selectable DNA-mediated transformation system. Nevertheless, the induction of an apparently stable change in the transport properties of an entire cell population by a drug would represent a novel phenomenon in cell biology.

We do not know the molecular mechanism which generates the control of drug entry in resistant cells. The lack of cross-resistance to several other drugs might imply that such a mechanism has some degree of specificity. If the control occurs at the level of the plasma membrane it could be exerted on the activity of folate carriers. Consistent with this interpretation is the result of one experiment we performed in which R1000 *C. fasciculata* cells showed a 40% decrease in uptake of [^{3}H]folic acid as compared with wild-type cells (results not shown).

From the results obtained with *L. major* we conclude that changes in drug transport also play a role in the expression of resistance by these cells, which in addition exhibit amplification of the DHFR gene; however, we do not know the relative contribution of each mechanism in the expression of the resistance phenotype in *L. ma-*

jor. Furthermore, the possibility of cellular heterogeneity within the cell lines studied has not been examined experimentally.

The existence of changes in drug transport in two trypanosomatid species which have become resistant to MTX implies that this phenomenon may be of general importance in this group of parasitic protozoa in terms of the mechanism of development of drug resistance.

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