Overproduction of a bifunctional thymidylate synthetase-dihydrofolate reductase and DNA amplification in methotrexate-resistant *Leishmania tropica*

(protozoan parasite/drug resistance/gene amplification)

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ABSTRACT *Leishmania tropica* promastigotes that are highly resistant to methotrexate, a dihydrofolate reductase inhibitor, have been developed. Organisms resistant to 1 mM methotrexate have a 40-fold increase in dihydrofolate reductase which is associated with thymidylate synthetase, and they contain amplified regions of DNA that may be directly visualized on stained gels of restriction digests. The amplified DNA in these organisms is about 56 kilobases in length, has a copy number about 80-fold higher than that of wild-type organisms, and constitutes about 10% of the nuclear DNA. When the methotrexate-resistant *L. tropica* are propagated in drug-free medium, the dihydrofolate reductase-thymidylate synthetase protein and the amplified DNA decrease in a parallel fashion until they are indistinguishable from the levels in wild-type organisms. However, when these apparent revertants are again challenged with 1 mM methotrexate, enzyme overproduction and DNA amplification occur rapidly. As in mammalian cells, it appears that drug resistance in parasitic protozoa may be mediated by gene amplification.

Drug resistance in protozoan parasites was first described early in this century by Paul Ehrlich in his studies on the chemotherapy of trypanosomiasis (1). Since then, numerous instances of drug-resistant *Trypanosoma* (2, 3), *Leishmania* (3), and *Plasmodium* (4) have been documented. There are relatively few drugs that are curative agents for the diseases caused by pathogenic protozoa, and the emergence of drug-resistant organisms is a particularly serious problem. Furthermore, because knowledge of the mechanisms of drug resistance in protozoan parasites is limited, no rational approaches exist for circumventing or avoiding this problem.

We have embarked upon investigations to define the molecular bases of drug resistance in pathogenic protozoa. In this report, we describe the development and properties of methotrexate (MTX)-resistant strains of *Leishmania tropica* promastigotes. We show that resistant organisms overproduce the drug target—a bifunctional dihydrofolate reductase (DHFR)-thymidylate synthetase (TS) enzyme—and amplify certain regions of DNA. These properties are unstable when selective pressure of MTX is removed but rapidly reemerge when organisms are reexposed to normally lethal concentrations of the drug. Last, we show that only a 10-fold increase in gene copy number in these protozoa may be detected by direct visualization of ethidium bromide-stained gels of DNA restriction digests.

MATERIALS AND METHODS

Growth of Organisms. *L. tropica* promastigotes (strain 252; Iran) obtained from S. Meshnik (Rockefeller University) were grown at 26°C in M199 medium (GIBCO) supplemented with

20% fetal calf serum, 25 mM Hepes (pH 7.4), gentamycin at 50 µg/ml, and, when specified, MTX. The doubling time (t_2) for wild-type organisms was 10–12 hr, and maximal cell density was $ca. 2 \times 10^7$ /ml. Cells were maintained in logarithmic phase by seeding at $ca. 5 \times 10^5$ cells per ml when cells approached maximal density; for growth rate determinations, cell number was determined daily until cell growth entered stationary phase. EC_{50} values refer to the concentration of MTX that inhibited the growth rate by 50%; unless otherwise specified, one passage refers to five generations of growth, or a 32-fold expansion in cell number, followed by reseeding in fresh medium. Cells were counted by using a Coulter Counter ZBI.

Selection of MTX-Resistant Cell Lines. MTX-resistant strains of *L. tropica* promastigotes were obtained by using a stepwise selection process. Cells were seeded at approximately 10^6 /ml in medium containing the specified amount of MTX. When the cell density exceeded 10^7 /ml, or if the growth rate began to plateau, the cells were transferred into fresh medium containing the same concentration of MTX. The doubling time stabilized within five passages (five generations each), and cells were seeded into the next higher concentration of MTX after 10 such passages. By using MTX concentrations of 5, 10, 20, 50, 100, 500, 1,000, and 2,000 µM, cell lines resistant to 1,000 and 2,000 µM MTX were established in continuous culture and are designated as strains R1000 and R2000, respectively. Unless otherwise specified, the R1000 strains used here were recently selected and propagated in 1 mM MTX for about 75 generations.

Enzyme Assays. Cells growing in the presence of MTX were grown to a density of $8\text{--}10 \times 10^6$ /ml and pelleted by centrifugation ($1,000 \times g$; 4 min). The cell pellet was washed twice with MTX-free medium and then grown for $ca.$ five generations in MTX-free medium. Cultures were harvested by centrifugation when the cell density was $0.8\text{--}1.5 \times 10^7$ /ml, and the cell pellet was washed with ice-cold phosphate-buffered saline. If not used immediately, cells were stored at -80°C .

Cell extracts were prepared by resuspending the cell pellet to a density of $ca. 1.5 \times 10^9$ cells per ml in ice-cold 50 mM *N*-[tris(hydroxymethyl)methyl]aminoethanesulfonic acid (Tes), pH 7.4/1 mM EDTA/5 mM dithiothreitol/50 µM phenylmethylsulfonyl fluoride/10% (vol/vol) glycerol. Cells were disrupted by sonication (Bronwill Biosonik, PI 50; three 5-sec bursts) at 0°C and then centrifuged at $15,000 \times g$ for 30 min at 4°C to yield an extract containing $ca. 3$ mg of protein per ml. Protein

Abbreviations: MTX, methotrexate; DHFR, dihydrofolate reductase; TS, thymidylate synthetase; H_2 folate, 7,8-dihydrofolate; $CH_2\text{--}H_4$ folate, ($\pm$)-L-5,10-methylenetetrahydrofolate; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; Tes, *N*-[tris(hydroxymethyl)methyl]aminoethanesulfonic acid; t_2 , time required for doubling the number of organisms; kb, kilobase(s).

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concentrations were determined by using the method of Bradford (5).

TS was quantitated as the covalent complex of 5-fluoro-2-deoxy[6-³H]uridine 5'-monophosphate ([6-³H]FdUMP), (±)-L-5,10-methylenetetrahydrofolate (CH₂-H₄folate), and enzyme (6). Cell extracts (0.2 ml) were incubated for *ca.* 10 min at 25°C with 0.1 mM CH₂-H₄folate and 0.1 μM [6-³H]FdUMP (18 Ci/mmol; 1 Ci = 3.7 × 10¹⁰ Bq; Moravsek Biochemicals). The protein-bound [6-³H]FdUMP was isolated by gel filtration on Sephadex G-25 (7) with 20 mM Na₂HPO₄, pH 7.4/10 mM 2-mercaptoethanol as eluant or on nitrocellulose filters as described (8). Likewise, DHFR was quantitated by treatment of extracts with 50 nM [³H]-MTX and 25 μM NADPH and the complex was isolated by gel filtration on Sephadex G-15. The catalytic activity of dTMP synthetase was measured by release of ³H from [2-¹⁴C, 5-³H]-dUMP in the presence of 0.1 mM CH₂-H₄folate as described (9), except that the buffer used was 50 mM Tes, pH 7.4/5 mM dithiothreitol/1 mM EDTA/25 mM MgCl₂. DHFR catalytic activity was measured spectrophotometrically at 25°C (10) in 1.0 ml of a mixture containing 0.1 mM H₂ folate, 0.1 mM NADPH, 50 mM Tes (pH 7.4), 75 mM 2-mercaptoethanol, and 1 mM EDTA. One unit of activity is defined as the amount of enzyme that produces 1 nmol of product per min.

Nucleic Acid Techniques. Isolation of DNA from *Leishmania* promastigotes was performed as described (11); nuclear DNA was separated from kinetoplast DNA by centrifugation in CsCl/ethidium bromide density gradients (12). Restriction endonucleases were obtained from commercial sources and used as described (13). The following procedures were used as described: electrophoresis in agarose gels in Tris/EDTA/acetate buffer (13), preparative isolation of DNA fragments from Sea-Plaque agarose gels (14), labeling of DNA fragments with ³²P by nick-translation (15), molecular cloning of DNA in the plasmid vector pBR327 (13, 16), Southern blot hybridization (17), and quantitative hybridization of radiolabeled DNA to *Leishmania* DNA immobilized on nitrocellulose filters (11).

RESULTS

DHFR and TS Exist as a Bifunctional Protein in *L. tropica*.

It is well established that, in most mammalian and bacterial cells, DHFR exists as a monomer of *M_r* ≈ 18,000 (18) and TS exists as a dimer of identical subunits with *M_r* ≈ 35,000 each (19). Recently, it was shown that, upon gel filtration, both enzymes from *Crithidia fasciculata* and *Plasmodium berghei* coelute with *M_r*

≈ 150,000 (20); the two enzyme activities in *C. fasciculata* have been purified and demonstrated to exist as a bifunctional protein in the native state. The DHFR and TS activities from *L. tropica* also coeluted from Sephacryl S-200 with *M_r* ≈ 150,000 (Fig. 1). Furthermore, the enzymes copurified to apparent homogeneity upon sequential chromatography on DEAE-cellulose and MTX-agarose (unpublished data), and we conclude that they exist as a bifunctional protein in their native state. This is further supported by results described below in which we show that organisms treated with MTX, a specific inhibitor of DHFR, respond by coordinate increases in both enzymes.

Development and Properties of MTX-Resistant *L. tropica*.

MTX inhibits the growth rate of wild-type *L. tropica* with an apparent EC₅₀ of 0.5 μM. When cells were treated with MTX, beginning at 5 μM and followed by stepwise increases after organisms adapted to lower concentrations of the drug, strains capable of growing in MTX at concentrations up to 2 mM were established. When wild-type organisms were first exposed to 5 μM MTX, the apparent growth rate initially decreased to 25% of normal but increased to near-normal rates (*t*₂ ≈ 12 hr) after four passages and remained constant thereafter. As cells were sequentially exposed to MTX concentrations of up to 100 μM, the initial growth rate was half that of wild-type cells but stabilized to near-normal rates after three to five passages. As the MTX was increased to 500 μM or greater, the growth rate of organisms remained half that of wild type and did not change upon subsequent passages in medium containing the same or higher concentrations of MTX. Throughout the selection procedure, most organisms remained motile as viewed microscopically. When wild-type organisms were placed directly in medium containing 1 mM MTX, they degenerated and died.

As viewed under ×400 magnification, *L. tropica* promastigotes growing in MTX-containing medium acquire unusual forms. Whereas some retained the characteristic slender flagellated form of wild-type organisms, many adopted a spherical shape, often with shortened flagella, and usually were less motile. Furthermore, the rosettes that are normally observed with these organisms in culture appeared to involve more organisms and to form at lower cell density than in wild-type cells grown in drug-free medium. When such cells were placed in MTX-free medium, they rapidly reverted to the normal morphology.

When cells resistant to 500 and 1,000 μM MTX were seeded in drug-free medium, the growth rate increased to that of wild-type organisms within the first passage. We have also observed

FIG. 1. Sephacryl S-200 gel filtration of TS and DHFR from *L. tropica*. A crude protein extract (0.2 ml containing 1.2 mg of protein) from the *L. tropica* R2000 cell line was chromatographed on a 1.5 × 45 cm Sephacryl S-200 column equilibrated and eluted, at 12 ml/hr, with 50 mM Tes, pH 7.4/1 mM EDTA/5 mM dithiothreitol/10% glycerol; 1.35-ml fractions were collected and assayed for TS and DHFR activity.

that MTX-resistant organisms grown in drug-free medium retain their ability to survive when reseeded in medium containing MTX; however, the resumption of "normal" growth was dependent on how long the adapted organisms were allowed to grow in the absence of drug. The R1000 line was grown in the absence of MTX for 75, 200, and 300 generations and then was reseeded in 1 mM MTX. Growth of organisms withdrawn from MTX for 75 generations was similar to the initial selection process; that is, growth was slow ($t_2 \approx 52$ hr) during the initial passage but showed no lag period, and the rate stabilized to $t_2 \approx 20$ hr during the second passage. In contrast, cells that had been withdrawn from MTX for 200 and 300 generations showed a lag phase of about 6 days before growth resumed; during this latent period, most organisms (70% at 5 days) remained motile as viewed microscopically. Growth initially resumed with $t_2 \approx 100$ hr until the cell number increased 10-fold, and then growth stabilized to $t_2 \approx 20$ hr after two subsequent passages.

Overproduction of DHFR and TS in MTX-Resistant *L. tropica*. Wild-type *L. tropica* promastigotes possess TS and DHFR values of 3 pmol/mg and 20 units/mg, respectively. Concomitant with the emergence of MTX-resistance there is an increase in the DHFR-TS bifunctional enzyme. Cells adapted to MTX at 20 μ M and 500 μ M had approximately 5- and 20-fold higher levels, respectively. The R1000 line used here has TS and DHFR values of 120 pmol/mg and 830 units/mg, respectively, an approximate 40-fold increase in the bifunctional enzyme compared to wild-type organisms. These values may be underestimates because enzyme levels could have decreased over the five generations of growth in MTX-free medium required to remove residual drug prior to enzyme assays (see below).

Currently available data suggest that the DHFR in MTX-resistant cells is indeed increased in amount and is not a structurally altered protein with increased catalytic efficiency or decreased binding affinity for MTX. First, use of [3 H]FdUMP and [3 H]MTX as active site titrants for TS and DHFR demonstrated the increased levels of the DHFR-TS protein in the R1000 line. Second, as described above and shown in Fig. 2, the DHFR activity was coordinate with the molar amount of bifunctional enzyme, which demonstrates that its catalytic efficiency is not altered. Third, when crude extracts from wild-type and R1000 cells possessing 1 unit of DHFR were preincubated with various concentrations of MTX for 10 min, 50%

inhibition of the initial velocities of the enzymes from both sources occurred at 2 nM MTX.

The high levels of DHFR-TS in MTX-resistant *L. tropica* decreased when cells were grown in the absence of MTX for extended periods (Fig. 2). When the R1000 line was grown in MTX-free medium for five passages (25 generations) the DHFR-TS had decreased by 80%; by 100 generations and thereafter, enzyme levels were comparable to those in wild-type organisms. Interestingly, when R1000 cells which were grown for 300 generations in MTX-free medium were reseeded in 1 mM MTX, the dTMP synthetase levels increased 25-fold within the time required for the number of organisms to increase 10-fold.

Amplified DNA in MTX-Resistant *L. tropica*. A common mechanism for MTX resistance in mammalian cells involves amplification of the DHFR gene (21, 22). Not unexpectedly, attempts at hybridization of mouse DHFR cDNA (23) with MTX-resistant *L. tropica* were unsuccessful. However, when DNAs from wild-type and R1000 *L. tropica* were digested with various restriction endonucleases, electrophoresed in agarose gels, and stained with ethidium bromide, we found specific fragments from the R1000 DNA that were not apparent in the DNA from wild-type cells (Fig. 3). The amplified DNA fragments were contained in nuclear DNA because prior removal of the kinetoplast DNA did not alter the patterns of amplified DNA fragments from those shown in Fig. 3. Thus, the R1000 strain of MTX-resistant *L. tropica* contains one or more regions of DNA that is present in greatly increased amounts relative to wild-type *L. tropica*. To demonstrate that selection of the R1000 MTX-resistant line was not a unique event, we have subsequently established five separate lines of MTX-resistant *L. tropica* by starting in each case from the parent line; each of these exhibited a major fraction of the amplified DNA fragments visualized in the R1000 line used here.

Two of the amplified R1000 DNA fragments, the 2-kilobase (kb) *Eco*RI and the 1.9-kb *Hind*III fragments (Fig. 3), were isolated and inserted into the plasmid vector pBR327 by molecular cloning techniques (13, 16). Recombinants containing inserts of the expected molecular weight were labeled with 32 P and hybridized to Southern blots of appropriate restriction endonuclease digests of wild-type and R1000 DNA; those recombinants that exhibited intense hybridization to R1000 DNA and weak hybridization to wild-type DNA were selected. Two recombinant plasmids having amplified DNA fragments were used

FIG. 2. Reversion of MTX-resistant *L. tropica* grown in MTX-free medium. The *L. tropica* R1000 cell line was grown in the absence of MTX for >200 generations. Gene copy number was quantitated by hybridization of 32 P-labeled pLR-R1 (○) or pLR-HM3 (●) to genomic *Leishmania* DNA immobilized on nitrocellulose filters.

FIG. 3. DNA amplification in MTX-resistant *L. tropica*. *Leishmania* DNA was isolated from wild-type (S), MTX-resistant (R1000), and revertant R1000 (Rev.) grown 205 generations in the absence of MTX and cleaved with the indicated restriction endonucleases. Electrophoresis was in 0.8% agarose; size standards were obtained by using a *Hind*III digest of λ DNA.

in further experiments: pLR-R1 containing the 2-kb *Eco*RI fragment, and pLR-HM3 containing the 1.9-kb *Hind*III fragment. Each of these fragments in the wild-type and R1000 DNA was quantitated by hybridization of excess 32 P-labeled pLR-R1 or pLR-HM3 DNA to total *Leishmania* DNA immobilized on nitrocellulose filters (11).

With pLR-R1 as the hybridization probe, the R1000 DNA exhibited approximately 80-fold higher binding than did DNA from wild-type organisms. An increase of 85-fold was observed when pLR-HM3 was used as the hybridization probe; these values represent the mean of three separate determinations. The size of the amplified region(s) of R1000 DNA was estimated by summation of the molecular weights of the amplified fragments detected after restriction endonuclease digests. As shown in Table 1, the data obtained by using eight different restriction enzymes indicate that the amplified DNA in R1000 *L. tropica* has a size of approximately 56 kb (range, 46–74 kb). We note that a given restriction enzyme may provide an underestimate of the size as a result of the presence of fragments of identical size or inability to detect fragments in certain regions of the gels or an overestimate as a result of heterogeneity or polymorphism in the location of restriction enzyme sites. The total amount of DNA amplified in the R1000 *L. tropica* may be cal-

Table 1. Amplified DNA fragments in restriction endonuclease-cleaved DNA from MTX-resistant *L. tropica*

Restriction enzyme	Fragment sizes, kb	Sum
<i>Hind</i> III	17.7, 10.5, 9.2, 8.1, 7.3, 5.2, 4.7, 4.4, 4.0, 1.9, 0.8	74
<i>Eco</i> RI	25, 21, 2.0	48
<i>Bam</i> HI	21, 13, 10.5, 2.1, 1.8	48
<i>Kpn</i> I	21, 10.9, 9.5, 7.2, 3.5	52
<i>Bgl</i> II	20, 13, 7.1, 4.5, 4.0, 3.5	52
<i>Sst</i> I	9.6, 8.4, 7.0, 6.6, 5.8, 4.4, 3.2, 2.8, 2.2, 1.9, 1.7, 1.6, 1.5, 1.0, 0.9, 0.8, 0.7	60
<i>Sal</i> I	6.6, 5.7, 4.9, 4.4, 3.8, 3.6, 2.9, 2.5, 2.4, 2.2, 1.9, 1.7, 1.2, 0.9, 0.8	46
<i>Xba</i> I	24, 21, 17, 5	67

culated as follows: 46–74 kb $\times$ 80- to 85-fold amplification $\approx$ 3,700–6,300 kb. Because the total nuclear DNA content of *Leishmania* is reported to be about 5×10^4 kb (24), we estimate that approximately 7–12% of the nuclear DNA in the R1000 *L. tropica* is amplified DNA.

As with DHFR-TS, the amplified DNA of MTX-resistant *L. tropica* was unstable in the absence of MTX, decreasing in parallel to the loss of DHFR-TS (Fig. 2). When the R1000 line was grown in MTX-free medium for 100 or more generations, the copy number of amplified DNA reverted to levels indistinguishable from those in the parent wild-type organisms. Furthermore, as with DHFR-TS, when revertants were reseeded in 1 mM MTX the same amplified regions appeared in the emerging resistant organisms.

The ability to detect restriction fragments of amplified DNA in *Leishmania* by direct visualization of ethidium bromide-stained gels led us to investigate the sensitivity of this technique. Equal amounts of DNA from R1000 and wild-type *L. tropica* were digested with *Xba* I; serial dilutions of the restricted R1000 DNA were made by using the wild-type restricted DNA as diluent, and then equal amounts of total DNA were subjected to electrophoresis and staining. The 5-kb fragment of amplified DNA was still evident when present at an effective copy number of 5–10 over the wild-type (Fig. 4). This simple experiment demonstrates that DNA amplifications of about 10-fold may be detected in *Leishmania* by ethidium bromide staining of appropriate restriction digests. The ability to detect amplified DNA in this manner is largely due to the low complexity of *Leishmania* DNA and the resultant low background staining of gels in which restriction fragments have been separated. The sensitivity was greatest when enzymes that cleaved *Leishmania* DNA infrequently were used, such as *Xba* I, *Eco*RI, and *Bam*HI. With enzymes that cleaved *Leishmania* DNA frequently, such as *Sal* I, *Sst* I, *Hpa* II, *Msp* I, and *Sau* 3A, the sensitivity was considerably decreased (Fig. 3).

DISCUSSION

L. tropica promastigotes possess DHFR and TS as a bifunctional protein, a property previously reported for the enzymes

FIG. 4. Limits of detectability of amplified DNA in *L. tropica*. Equal concentrations of DNA from wild-type and R1000 *L. tropica* were digested to completion with *Xba* I. A 1:2 serial dilution of the digested R1000 DNA was made by using the digested wild-type DNA as diluent, yielding progressively decreasing effective copy numbers of amplified DNA. Then, 1- μ g samples of the digested DNAs, corresponding to 1 (wild-type DNA), 2.5, 5, 10, 20, 40, and 80 (undiluted R1000 DNA) copies of amplified DNA, were subjected to electrophoresis in a 0.7% agarose gel, and the DNA was visualized by staining with ethidium bromide.

from *C. fasciculata* and *P. berghei* (20). In response to stepwise selection with MTX, a specific inhibitor of DHFR, *L. tropica* promastigotes were obtained that are resistant to high concentrations of the drug. Organisms have been established in continuous culture in medium containing MTX at 4,000-fold higher concentrations than its apparent EC_{50} value for the parent strain. Concomitant with the development of MTX-resistance, there is a coordinate increase in the levels of both DHFR and TS. The R1000 line (resistant to 1,000 μ M MTX) used for most studies here has *ca.* 40-fold higher levels of the bifunctional protein than does the parent line. The DHFR from R1000 and wild-type cells have similar catalytic efficiencies and are inhibited by similar concentrations of MTX. Thus, the overproduced enzyme has no obvious structural alterations that might account for MTX resistance in *L. tropica*.

Associated with MTX resistance and enzyme overproduction in *L. tropica* is the amplification of certain sequences of nuclear DNA. Analysis of gels obtained from the digests of eight restriction enzymes indicates that the size of the amplified DNA region(s) is between 46 and 74 kb. Two fragments of the amplified sequences have been cloned and, when used as hybridization probes, indicate the copy number of complementary sequences in the R1000 line to be 80- to 85-fold higher than in the parent wild-type organism. From the size and copy number of the amplified DNA in the R1000 line, it appears that the amplified DNA constitutes approximately 10% of the total nuclear DNA.

When MTX-resistant *L. tropica* were transferred to drug-free medium, many of their properties reverted to those characteristic of the wild-type organisms. Initially, there was a rapid reappearance of the morphology and growth rate of wild-type organisms. With increasing passages in MTX-free medium there was a gradual decline in both the DHFR-TS protein and amplified DNA, which followed a pattern similar to that reported for MTX-resistant mouse cells (11). After 25 generations in MTX-free medium, the level of the DHFR-TS bifunctional protein decreased to *ca.* 20% of that observed in the R1000 cell line, and by 100 generations the enzyme levels and the copy number of amplified DNA sequences were indistinguishable from those found in the wild-type organisms. Interestingly, when the R1000 revertants were rechallenged with 1 mM MTX, they did not die as do wild-type organisms. Instead, they rapidly reacquired resistance, increased levels of DHFR-TS protein, and amplified regions of DNA.

Many of the characteristics of MTX-resistant *L. tropica* described here are strikingly similar to those reported for some lines of MTX-resistant mammalian cells (21, 22). In the latter, drug resistance is mediated by overproduction of DHFR and DNA amplification, both of which are frequently unstable in the absence of MTX (25, 26). The amplified DNA includes the coding sequence for DHFR, and enzyme overproduction is mediated by a gene dosage effect. From the results described here, and by analogy to MTX-resistance in mammalian cells, it appears that a major factor in the development of MTX resistance in *L. tropica* is overproduction of an apparently normal bifunctional DHFR-TS enzyme. It is reasonable to conclude that the amplified DNA in MTX-resistant *Leishmania* contains the coding sequence(s) for the bifunctional protein and that enzyme overproduction is mediated by a gene dosage effect. Indeed, preliminary results indicate that MTX-resistant *L. tropica* do have increased levels of an RNA transcript(s) that hybridizes with both pLR-R1 and pLR-HM3 cloned DNAs.

As a final point, it is of interest that DNA amplifications in *Leishmania* of only 10-fold may be detected by direct visualization of ethidium bromide-stained gels of separated restriction fragments. By comparison, nearly 1,000-fold amplification of mammalian DNA is required before direct visualization of

restriction fragments is possible (27, 28). This property of *Leishmania* is due in part to the lower complexity of its genomic DNA and to the lower frequency of cleavage sites on the DNA for many restriction enzymes. An important aspect of this finding is that hybridization probes may not be necessary to detect DNA amplification in *Leishmania*, and the method should be applicable to other protozoans as well. The targets of most drugs used for treatment of pathogenic protozoans are not known, making the preparation of hybridization probes unfeasible, and the frequency of emergence of drug-resistant strains is quite high. We suggest that direct visualization of properly restricted DNA might be used to detect gene amplification in such organisms and provide insight into the mechanism(s) of drug resistance.

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