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Tunicamycin-resistant *Leishmania mexicana amazonensis*: Expression of virulence associated with an increased activity of *N*-acetylglucosaminyltransferase and amplification of its presumptive gene

(trypanosomatid protozoa/N-glycosylation/drug resistance/macrophages)

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ABSTRACT **Tunicamycin at 10 μg/ml inhibits the growth and infectivity of the parasitic protozoan *Leishmania mexicana amazonensis*. Tunicamycin-resistant variants of this parasite were produced by (*i*) gradual acclimatization of cells to increasing concentrations of the drug up to 80 μg/ml and (*ii*) a single-step selection of ethyl methanesulfonate-pretreated or differentiating leishmanias with the drug at 10 μg/ml. Prolonged exposure to the drug increases stability of drug resistance of those resistant to 10 μg/ml. Tunicamycin-resistant cells contain amplified DNA, which hybridizes in proportion to the cells' degree of drug resistance with Alg 7, a cloned DNA probe apparently encoding yeast *N*-acetylglucosaminyltransferase. This enzyme from all variants remained sensitive to inhibition by tunicamycin, but its specific activity was up to 15-fold higher than that of the wild type. Thus, amplification of the gene encoding this enzyme appears to result in its overproduction in the variants, accounting for their resistance to tunicamycin. The tunicamycin-resistant cells are more virulent to mice than their parental wild type. Thus, leishmanial virulence may be related to amplification or expression of gene(s) encoding enzymes involved in the regulation of N-glycosylation of parasite proteins.**

Molecular mechanism of virulence remains largely unknown in diseases caused by parasitic protozoa. Glycoproteins on the surface of these parasites are often implicated in their evasion of host immunity or invasion of their host cells to establish parasitism. Since glycosylation of proteins can change their properties and thus their biological functions (1), molecular regulation of this event in the parasites may be important in considering their virulence.

Leishmania mexicana amazonensis is a trypanosomatid protozoan that causes human cutaneous leishmaniasis. The parasite exists as extracellular promastigotes in the sandfly gut and as intracellular amastigotes in the mammalian macrophages. The glycoconjugates of this and other species on the cell surface or released as excreted factors appear to be important virulent factors in intracellular parasitism of macrophages (2, 3). One useful approach to study this is to produce leishmanial variants resistant to inhibitors of protein glycosylation.

We report here our success in producing variants of this parasite resistant to tunicamycin (TM), which inhibits *N*-acetylglucosamine-1-phosphotransferase in the dolichol pathway of protein glycosylation (4). The TM-resistant cells possess an increased amount of this enzyme, apparently due to gene amplification. They are more virulent than the parental wild type, pointing to the expression of a gene or genes encoding this and possibly other related enzymes as a molecular basis of leishmanial virulence.

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MATERIALS AND METHODS

Leishmania and Macrophage. *L. mexicana amazonensis* (LV78) and the murine macrophage line J774G8 were obtained and grown as described (5). Promastigotes were cultured at 27°C in medium 199 with 25 mM Hepes, pH 7.4, and 10% heat-inactivated fetal bovine serum. Established procedures were followed for the cultivation and isolation of amastigotes (5, 6).

Treatment of Leishmania with TM. TM (Sigma) was dissolved in 0.01 M NaOH at 1–2 mg/ml. Promastigotes at 5×10^6 cells per ml in the complete medium were treated with TM at 2, 4, 8, and 10 μg/ml. Viable promastigotes were counted in a hemacytometer.

Production of Leishmanial Variants Resistant to TM. Promastigotes were made resistant to TM in one of three ways: (*i*) *Stepwise selection.* Wild-type cells were gradually acclimatized to grow by increasing concentrations of the drug—i.e., 2, 4, 6, 8, 10, 20, 40, 60, and 80 μg/ml. (*ii*) *Single-step selection of cells pretreated with ethyl methanesulfonate.* Wild-type logarithmic-phase promastigotes were treated at 10^7 cells per ml with ethyl methanesulfonate at 9 mg/ml for 4 hr in the complete culture medium and allowed to grow in ethyl methanesulfonate-free medium for 2 days. Serial dilutions of these cells from 10^2 to 10^7 per 100 μl were each plated onto the complete culture medium (25 ml per 9-cm Petri dish) containing 1% agar with and without TM at 10 or 80 μg/ml. Colonies that developed on the agar plates were transferred into liquid medium for further testing their drug sensitivity. (*iii*) *Single-step selection by treating leishmanias during differentiation.* Amastigotes isolated from infected macrophages were treated at 27°C with 10 μg of TM per ml at 10^7 cells per ml in the culture medium under conditions for their differentiation into promastigotes.

Variants resistant to TM at 10 μg/ml and produced by methods *i*, *ii*, and *iii* are referred to as "Step 10," "EMS 10," and "Differentiation 10," respectively. Those resistant to 80 μg/ml are similarly referred to as "Step 80," etc.

Assay for *N*-Acetylglucosamine-1-phosphotransferase. *N*-Acetylglucosaminyltransferase was assayed on the basis of incorporation of *N*-acetyl[^{3}H]glucosamine from UDP-*N*-acetyl[^{3}H]glucosamine into dolichol phosphate by an estab-

Abbreviations: EtdBr, ethidium bromide; TM, tunicamycin; Mt 10, leishmania variants resistant to TM at 10 μg/ml (Differentiation 10, EMS 10, and Step 10 refer to variants produced by different methods of selection); Mt 80, etc., leishmania variants resistant to TM at 80 μg/ml.

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lished method (7–9) with modifications. The final assay mixture in a total volume of 200 μl consisted of 50 mM Tris·HCl at pH 7.4, 4 μg of dolichol phosphate (Sigma), 0.25% Triton X-100, 5 mM $MnCl_2$, 0.25 μCi of UDP-*N*-acetyl[^{3}H]glucosamine (New England Nuclear) (20.4 Ci/mmol; 1 Ci = 37 GBq), and 100 μg of microsomal protein. Leishmanial microsomes of wild-type and TM-resistant promastigotes grown in TM-free medium for four or five passages in 2 weeks were isolated as described for trypanosomes (7). Microsomal protein concentrations were determined by the Pierce BCA (bicinchoninic acid) protein assay. Enzyme activity was assayed at 30°C. Reaction was terminated after 10 min by the addition of 5 ml of $CHCl_3/CH_3OH$ (2:1, vol/vol). Samples were processed for lipid extraction exactly as described (7). Incorporation of *N*-acetyl[^{3}H]glucosamine into dolichol phosphate was determined by liquid scintillation counting, and the activity of *N*-acetylglucosaminyltransferase is expressed as cpm/10 min per mg of microsomal protein.

Isolation of DNAs. DNAs were isolated from promastigotes and from *Saccharomyces cerevisiae* spheroplasts (10) supplied by E. E. McKee (Chicago Medical School). Cells were lysed overnight at 37°C in 1% $NaDodSO_4$ containing proteinase K (Sigma) at 75 μg/ml, 80 mM NaCl, and 45 mM EDTA. Samples were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1, vol/vol) and once with chloroform/isoamyl alcohol (24:1, vol/vol). Ethanol precipitates of the aqueous phase were digested at 37°C for 2 hr with ribonuclease A (Sigma) at 50 μg/ml and then for 2 hr with proteinase K at 50 μg/ml in 5 mM EDTA/80 mM NaCl. Subsequent extraction, precipitation, and solubilization of DNA were repeated as described above.

Agarose Gel Electrophoresis of Endonuclease-Digested DNA. DNAs were digested to completion with endonucleases—e.g., *Bam*HI at 5 units/μg of DNA—and electrophoresed at 1 V/cm in 0.3% agarose containing 80 mM Tris, 80 mM boric acid, 1 mM EDTA, and ethidium bromide (EtdBr) at 0.5 μg/ml (pH 7.8).

Dot-Blot and Southern Hybridization. DNA samples were boiled for 5 min and dot-blotted in serial dilutions onto Zetabind (AMF, Meriden, CT) in the presence of 10× SSC (1× = 150 mM NaCl/15 mM sodium citrate, pH 7.4). Endonuclease-digested DNA electrophoresed in agarose gel was treated with 0.25 M HCl (11) and then transferred to Zetabind as described by Southern (12). Filters were hybridized with the plasmid Alg 7 (13). pBR322 with this insert in *Escherichia coli* (DHI) was isolated by the alkaline lysis method (14) and labeled with [α-^{32}P]dATP (New England Nuclear; 3000 Ci/mmol) as described (15). After hybridization under conditions specified by the manufacturer (AMF) for 16 hr, the filters were washed twice at room temperature for 15 min in 2× SSC with 0.1% $NaDodSO_4$ and then at 60°C for 30 min twice in 0.1× SSC with 0.1% $NaDodSO_4$. Filters were exposed to Kodak X-Omat film at room temperature or at −80°C with Cronex intensifying screen.

Infection of BALB/c Mice with Leishmanias. BALB/c mice were infected with one of the following: (*i*) wild-type cells as control; (*ii*) wild-type cells treated previously with TM at 10 μg/ml for 12 hr; (*iii*) leishmanial variants resistant to TM at 10 and 80 μg/ml obtained by all methods used and continuously grown in medium with this concentration of the drug; or (*iv*) all TM-resistant variants released from the drug pressure by growing them in drug-free medium for 1–2 weeks. Parasites at 5×10^7 cells per 100 μl were inoculated subcutaneously into the tail base of each mouse. Infection was assessed by measuring the diameter of the lesion. Lesions were also removed for counting the total number of amastigotes in a hemacytometer.

RESULTS

Inhibition of Growth of Promastigotes by TM. TM inhibited the growth of promastigotes in a dose-dependent fashion (not shown). Cells resumed growth when relieved of drug pressure after treatment with TM at 10 μg/ml for about 24 hr. Continuous treatment of cells with TM at 10 μg/ml invariably resulted in their degeneration beyond recovery.

Development of TM-Resistant Leishmanias. Stepwise selection made wild-type cells gradually resistant to TM up to 80 μg/ml in a course of several months. During the period of adaptation, the survival rate of cells varied from 0.01% to 75%.

In the single-step selection, promastigotes with or without prior treatment with ethyl methanesulfonate gave comparable plating efficiencies of $4–6 \times 10^{-5}$ in the agar medium containing TM at 10 μg/ml. However, only the colonies that emerged from ethyl methanesulfonate-pretreated promastigotes grew continuously after transfer to the liquid medium with TM at 10 μg/ml. Plating ethyl methanesulfonate-treated promastigotes on agar medium with TM at 80 μg/ml yielded no colonies.

Treatment of leishmanias during their differentiation from amastigotes to promastigotes with TM at 10 μg/ml resulted in degeneration of most cells, except for a small number that

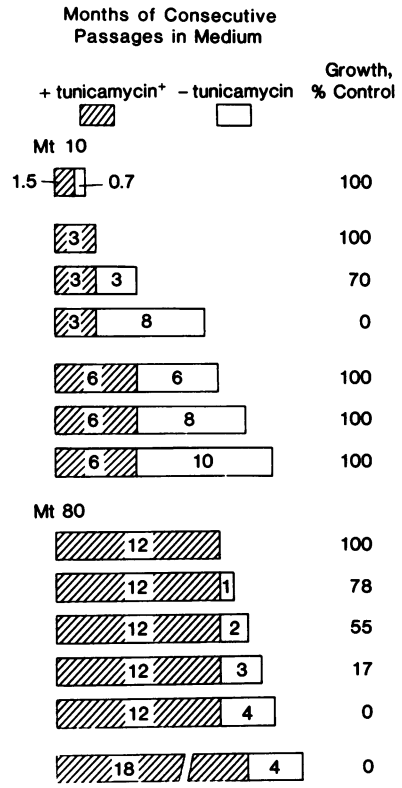

FIG. 1. Stability of TM resistance of drug-resistant *L. mexicana amazonensis*. Promastigotes resistant to TM at 10 μg/ml (Mt 10) and 80 μg/ml (Mt 80) were isolated by stepwise selection. Stability of the drug resistance was determined by testing the sensitivity of cells to the drug after growing them in TM-containing medium and then in TM-free medium for different time periods (months). Cells were subcultured every 4–7 days, during which time the cell density increased from approximately 2×10^6 per ml to $5–10 \times 10^7$ per ml (five to six generations). The densities of cells grown in medium with or without TM were compared. Cell density was estimated by visual inspection of the culture and microscopic evaluation of slide preparations. Growth of cells is expressed as percent of control, determined by the ratio of cell densities in media with and without TM. There is no significant difference among all variants made resistant to the same concentration of TM produced by different methods.

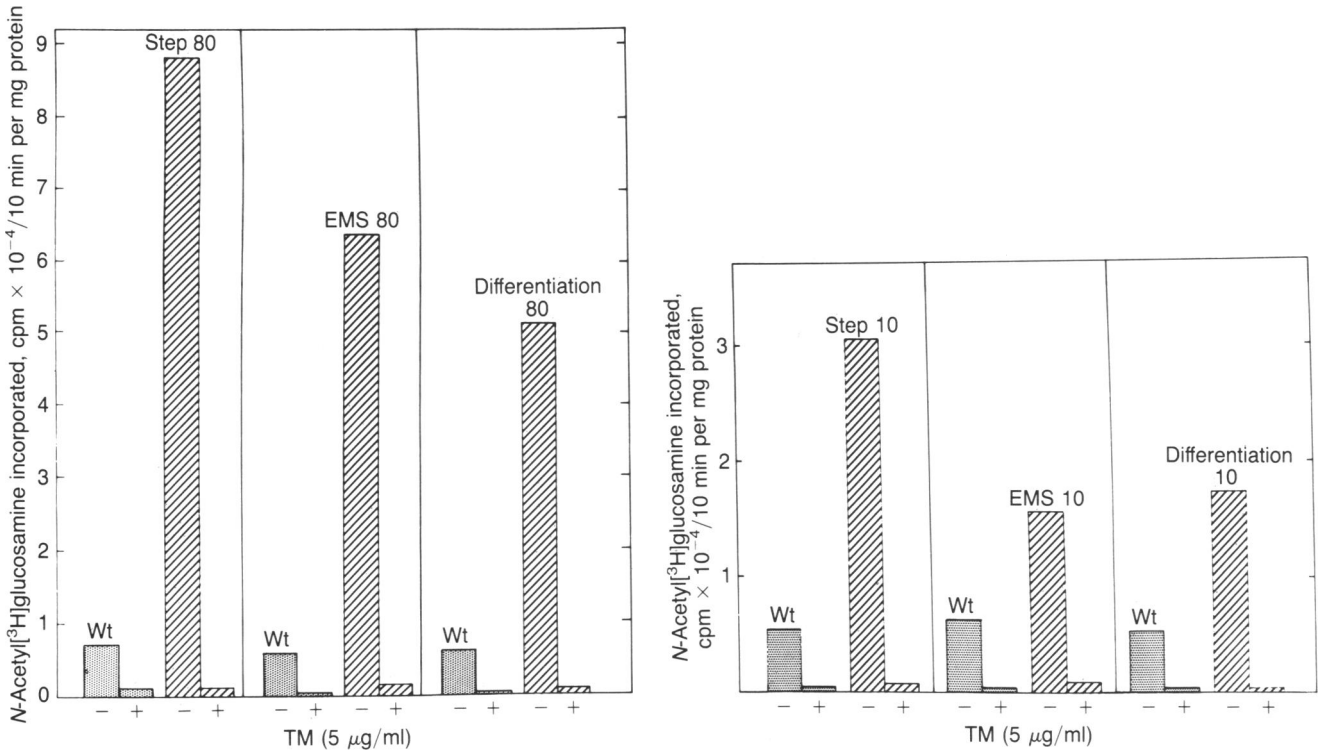

FIG. 2. Specific activity of microsomal *N*-acetylglucosaminyltransferase of wild-type (Wt) and TM-resistant *L. mexicana amazonensis*. + and −, microsomal preparations were incubated with (+) or without (−) TM for 10 min before assay.

emerged after about 2 weeks as variants resistant to this concentration of the drug.

All cell variants made resistant to TM at 10 μg/ml (collectively termed Mt 10) were further adapted to grow by stepwise exposure to media with TM at 20, 40, 60, and 80 μg/ml during a course of several months. Cells resistant to TM at 80 μg/ml (Mt 80) grew continuously in medium with this drug concentration.

Evaluation of TM Resistance of Leishmania Variants. All Mt 10 grown in the presence or absence of the drug were similar to wild type grown in drug-free medium in appearance and growth. Mt 80 grew more slowly than the wild type even in the drug-free medium as long as they retained their drug resistance. All Mt 10 and Mt 80 were about 20- and 100-fold more drug resistant than the wild type, respectively.

Stability of TM Resistance of Leishmania Variants. Cells selected by all three methods to become resistant to TM at 10 μg/ml and continuously exposed to the drug for 6 months retained their drug resistance much longer than those preexposed to the drug for only 3 months (Fig. 1). All those made resistant to 80 μg/ml were much less stable: continuous exposure of these cells to TM for as long as 18 months allowed them to retain their drug resistance for no more than 3 months under drug-free conditions (Fig. 1). The loss of drug resistance from these cells appeared to be complete, as the revertants were as sensitive to TM as the wild type.

TM resistance was retained by leishmanias after one cycle of promastigote–amastigote–promastigote differentiation (the amastigote stage being under drug-free conditions) through cultured macrophages for up to 14 days.

***N*-Acetylglucosamine-1-phosphotransferase Activity of Wild-Type and TM-Resistant Variants.** The enzyme activity was linear with the amount of microsomal protein from 50 to 200 μg and with reaction times of 0, 5, and 10 min. The microsomal enzymes from both the wild type and all TM-resistant cells were almost completely inhibited by TM at 5 μg/ml, but the uninhibited enzyme activity was about 3- to 5-fold and 10- to 15-fold higher in Mt 10 and Mt 80 than in the wild-type cells (Fig. 2).

DNA Amplification in TM-Resistant Leishmania Variants. *Bam*HI digests of DNA gave four amplified EtdBr-staining fragments, 12, 14, 15, and 22 kilobase pairs (kbp) long, which were most prominent in Mt 80, less obvious in Mt 10, and unnoticeable in the wild type (Fig. 3). Southern blot analysis showed that these *Bam*HI fragments of the amplified DNA hybridized to Alg 7 plasmid (Fig. 4*A*). TM-resistant cells after ethyl methanesulfonate treatment gave a slightly different

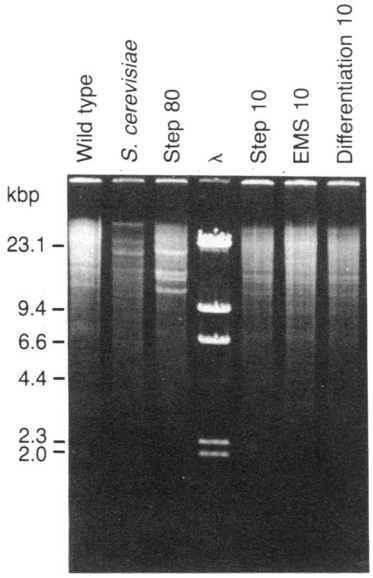

FIG. 3. *Bam*HI digests of amplified DNA in TM-resistant *L. mexicana amazonensis*. DNA was isolated from promastigotes and yeast spheroplasts (*S. cerevisiae*). *Bam*HI digests were electrophoresed in 0.3% agarose and stained with EtdBr. A *Hin*dIII digest of phage λ DNA was used as a standard.

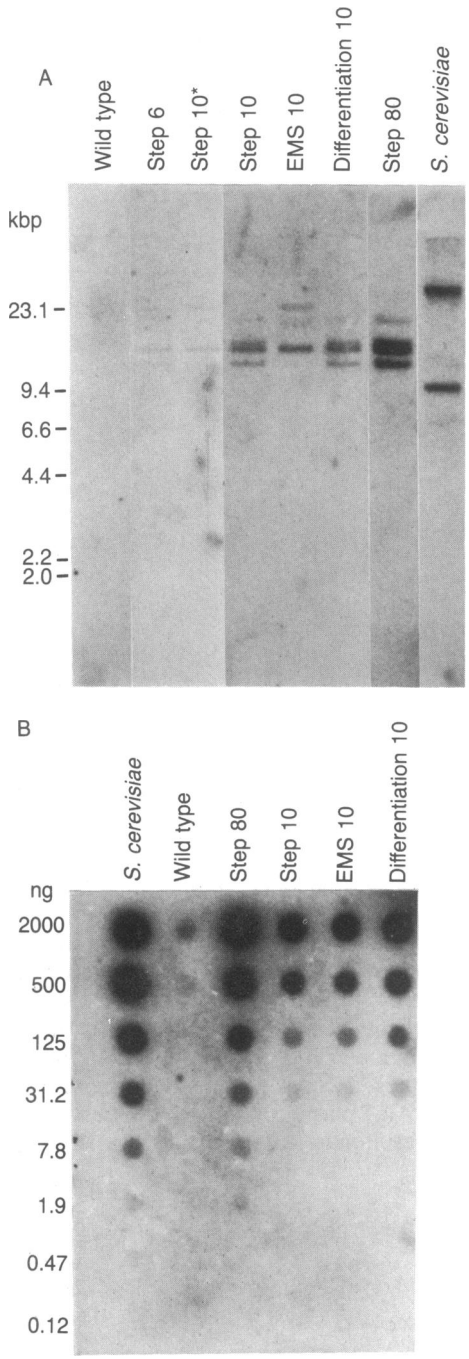

FIG. 4. DNA amplification of TM-resistant *L. mexicana amazonensis* determined by Southern and dot-blot hybridization with Alg 7 plasmid. DNAs were completely digested with *Bam*HI and then electrophoresed through an agarose gel or dotted for Southern (*A*) and dot-blot (*B*) hybridization with Alg 7. The DNA samples of leishmanias are named as described in the text, and Step 6, Step 10*, and Step 10 are from cells made resistant to TM by stepwise increases of drug concentrations to 6 μg/ml, 10 μg/ml in 1 month, and 10 μg/ml for longer than 6 months, respectively.

hybridization pattern of amplified bands (EMS 10 in Fig. 4*A*). However, the same hybridization banding pattern was seen for all Mt 80, including the one produced from EMS 10. Alg 7 hybridized strongly with yeast DNA as expected (Fig. 4*A*).

Estimation by dot-blot hybridization using the same probe gave about 30-fold and 100-fold amplifications of the DNA in Mt 10 and Mt 80, respectively, as compared to wild-type cell DNA (Fig. 4*B*). The amplified DNA was lost from the drug-sensitive revertants that emerged from TM-resistant cells cultured in the absence of drug (not shown).

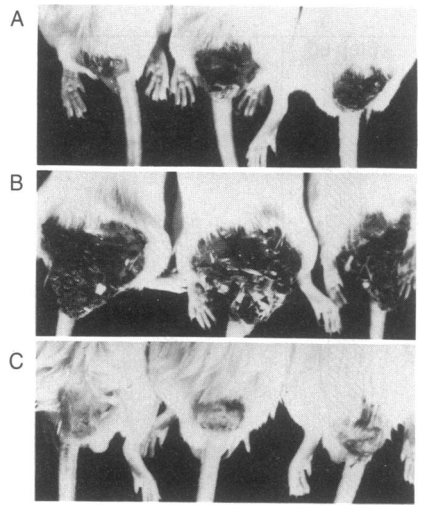

FIG. 5. Lesions in mice infected with wild-type and TM-resistant promastigotes of *L. mexicana amazonensis*. Promastigotes grown in media with or without the drug were inoculated into the tail base of male BALB/c mice. Four series of independent experiments were carried out and four or five mice were used for each leishmania inoculum. The lesions were photographed 159 days after infection. (*A*) Wild type; (*B*) cells selected stepwise to become resistant to TM at 10 μg/ml; (*C*) wild-type cells pretreated with TM at 10 μg/ml. Differentiation 10, EMS 10, and Step 10 all gave comparable results.

Virulence of TM-Resistant Leishmanias to Mice. Results from four independent experiments showed that all Mt 10 produced lesions larger than did the parental wild type. Fig. 5 shows the lesions in mice after infection for 159 days from a representative series of such experiments. The diameters of the lesions and the total numbers of amastigotes (mean ± SD) therein produced by Mt 10 were 18.2 ± 1.9 mm and 3.17 ± 0.2 × 10^9 cells, respectively, whereas those produced by the wild type were 10.5 ± 1.0 mm and 1.4 ± 0.1 × 10^9 cells, respectively. Early ulceration and metastasis of the lesions were also associated with the TM-resistant parasites. All Mt 80 were less virulent than Mt 10, but were more so than the parental wild type (not shown). The development of the lesion was considerably delayed in mice infected with the wild type pretreated with TM at 10 μg/ml for 12 hr (Fig. 5*C*). TM-sensitive revertants were similar to the parental wild type in their virulence to mice (not shown).

DISCUSSION

We show here that a protozoan parasite is more virulent than the parental wild type when made resistant to TM, a known inhibitor of *N*-acetylglucosamine-1-phosphotransferase in the dolichol pathway for glycosylation of proteins (1). All isolated TM-resistant variants have an increased activity of the glycosyltransferase and amplified DNA apparently containing the gene encoding this enzyme. Thus, expression of the glycosyltransferase gene may be implicated as the molecular basis of virulence in protozoal infections. The TM-resistant variants are of value as another model to study the mechanism of drug-induced DNA amplification. The availability of these variants may also facilitate the isolation and characterization of the enzyme and its gene.

TM was found to inhibit the growth and infectivity of *L. mexicana amazonensis*, as reported earlier for *Leishmania braziliensis* (16) and *Leishmania donovani* (17). The importance of N-glycosylation of proteins in the cellular functions has been shown in eukaryotic cells (18, 19).

We produced TM-resistant leishmanias to facilitate analysis of the functional importance of their protein glycosylation. All TM-resistant variants were found to have the

following properties: (*i*) The activity of the glycosyltransferase is increased with the level of drug resistance but remains sensitive to inhibition by TM. (*ii*) There is DNA amplification, the degree of which increases with that of the drug resistance. (*iii*) The amplified DNA hybridizes with Alg 7 plasmid, which apparently contains yeast *N*-acetylglucosaminyltransferase gene (13). (*iv*) TM resistance is initially unstable, but prolonged exposure of cells to appropriate concentrations of the drug improves the stability. (*v*) Revertants derived from TM-resistant cells after prolonged culture in drug-free medium lose both the amplification of DNA and that of the enzyme activity. These properties of TM-resistant leishmanias are consistent with those described for methotrexate-resistant mammalian cells (20–22) and leishmanias (23, 24) with amplified dihydrofolate reductase gene. Accordingly, the mechanism of TM resistance developed in *L. mexicana amazonensis* appears to be an amplification of the gene encoding the glycosyltransferase, which leads to an overproduction of the enzyme, enabling cells to overcome the inhibitory effect of TM. DNA amplification associated with TM-resistant cells has not been reported previously to our knowledge, although this was suggested on the basis of finding an increased activity of the enzyme in CHO cells (25). Our interpretation can be strengthened further by determining the quantity of the enzyme at the protein level, the structure of its gene, and its expression at the mRNA level.

Southern analysis of leishmania DNAs with Alg 7 plasmid provides some information on the structure of the amplified sequences in TM-resistant cells. Alg 7 in the probe used, originally cloned from yeast DNA, is no more than 4 kbp in size (13). Transformation of yeasts with plasmids containing DNA complementary to Alg 7 confers TM resistance and increased glycosyltransferase (26). Hybridization of Alg 7 to the amplified leishmania DNA is specific, as it does not hybridize to leishmania highly repetitive tubulin and ribosomal RNA genes, which exist normally in the wild-type cells. DNA from the wild-type leishmanias hybridizes poorly with Alg 7, suggestive of low sequence homology or single (or low) copy number of the gene in these cells. With increasing degree of TM resistance developed in leishmanias, hybridization of Alg 7 with the amplified *Bam*HI fragments of 12, 14, 15, and 22 kbp increased in intensity. Since all variants resistant to TM at 80 μg/ml obtained from several independent experiments ultimately give an identical pattern of DNA amplification and hybridization with Alg 7, there is probably a single structural form of the amplified DNA in these cells. The four amplified *Bam*HI fragments as shown in Figs. 3 and 4*A* (Step 80) have been cloned in pBR322; they are linked and share no sequence homology (unpublished data). The possible explanation for the disparity between Alg 7 and the amplified leishmania DNA in size is not yet known.

We found the virulent phenotype associated with all TM-resistant leishmanias produced. DNA amplification with an increase in the activity of the glycosyltransferase found simultaneously in these cells implicates these events in the mechanism of leishmanial virulence. There is a simultaneous loss of virulence and DNA amplification in all lines of drug-sensitive revertants derived from TM-resistant cells. This suggests strongly that leishmania virulence is linked to the amplified DNA and not to mutation of an unknown gene or genes elsewhere. An increase in the enzyme activity may effect quantitative or qualitative changes in leishmanial glycosylation of proteins, which play important roles in the ability of the parasites to infect macrophages (2). Gene amplification associated with the virulence of leishmanias is also reminiscent of oncogene amplification found to accompany the expression of neoplastic phenotypes in some cancer cells (27–29). Thus, the association of gene amplification with virulence of cells causing diseases of the invasive types may be more common than previously known.

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