mine neurons make synaptic contact with epinephrine neurons to modulate epinephrine synthesis and/or release, and NPA acts on the receptors at these synapses. This conclusion could not be made solely from the observations reported here, however. The effects of NPA and other dopamine agonists on hypothalamic epinephrine and corticosterone could be two separate consequences of a "stress" associated with activation of central dopamine receptors, since hypothalamic epinephrine can be depleted by stress [13] and, of course, corticosterone levels are increased by stress. Further studies will be needed to address the mechanism(s) by which dopamine agonists of diverse structural types decrease hypothalamic epinephrine and increase serum corticosterone concentration.

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Formycin B resistance in *Leishmania**

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There are a few curative agents for the diseases caused by pathogenic protozoa, and the emergence of drug-resistant organisms presents a serious problem. Further, since knowledge of how these organisms acquire drug-resistance is limited, there are no rational approaches to avoid or circumvent this problem. One objective of this laboratory has been to define molecular mechanisms of drug resistance in pathogenic protozoa. Our approach is to select strains of tissue culture forms of protozoa which are resistant to drugs having different modes of action and then to elucidate the biochemical basis of resistance.

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† Abbreviations: FoA, Formycin A; FoB, Formycin B; FoA-MP, FoA-DP, FoA-TP, Formycin A 5′-mono-, di- and triphosphates, respectively; FoB-MP, Formycin B 5′-monophosphate; DME, Dulbecco's modified Eagle's medium; TCA, Trichloroacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and HPLC, high performance liquid chromatography.

Recently, we reported the selection of methotrexate-resistant *Leishmania tropica* and demonstrated that the mechanism of resistance involved overproduction of dihydrofolate reductase which was mediated by gene amplification [1]. In the present work, we describe the selection of *L. tropica* promastigotes which are highly resistant towards the nucleoside analog Formycin B (FoB)†. We show that the resistant organisms are impaired in their abilities to transport this drug and to accumulate the nucleotide metabolites believed to be responsible for cytotoxicity.

Materials and methods

Growth of organisms. *L. tropica* promastigotes (strain 252; Iran) obtained from S. Meshnick were grown at 26° in M199 medium (GIBCO, Grand Island, NY) supplemented with 20% fetal calf serum, 25 mM Hepes (pH 7.4), and gentamicin at 50 μg/ml or in Dulbecco's Modified Eagle's medium (GIBCO) supplemented with 10% fetal calf serum and 50 μg/ml gentamicin. For growth rate studies, cells were seeded at 1×10^6 cells/ml and counted daily using a Coulter Counter ZBI until cell growth entered stationary phase. The IC_{50} values refer to the concentration of drug that inhibited the growth rate by 50%.

Selection of FoB-resistant cells. FoB-resistant *L. tropica* promastigotes were obtained by using a stepwise selection

process. Cells were seeded at *ca.* 10^6/ml in medium containing the specified amount of FoB. When the cell density reached 0.5 to 1.0 × 10^7/ml, the cells were transferred to fresh medium containing the next higher concentration of FoB. By using FoB concentrations of 0.2, 0.5, 1, 3, 10, 30 and 100 μM, a cell line resistant to 100 μM FoB was established in continuous culture and is designated as strain FR-100. Unless otherwise specified, the FR-100 cell line was maintained in medium containing 100 μM FoB. To remove exogenous FoB for the procedures described below, FR-100 cells growing in the presence of FoB were pelleted by centrifugation (1000 *g*; 4 min). The cell pellet was washed twice with FoB-free medium and then grown for *ca.* five generations in FoB-free medium.

Enzyme assays. Cultures were harvested by centrifugation when the cell density was 0.8 to 1.0 × 10^7/ml, and the pellet was washed twice with ice-cold phosphate-buffered saline. If not used immediately, cells were frozen in dry ice–acetone and stored at −80°. Cell extracts were prepared by resuspending the cell pellet to a density of about 2 × 10^9 cells/ml in water. The cells were then disrupted by sonication (Bronwill Biosonic, P150; three 5-sec bursts) at 0° and centrifuged at 30,000 *g* for 30 min at 4°. The supernatant fraction was applied to a Sephadex G25 column (5 ml), and the protein in the excluded volume was used immediately for enzyme assays. Protein concentration was determined by the method of Bradford [2].

Nucleoside phosphotransferase activity was determined by modification of reported procedures [3, 4]. A solution (50 μl) containing 15–20 μg protein, 100 mM NaOAc (pH 5.3), 20 mM GMP and 2.5 mM [^{3}H]FoB (0.2 mCi/mmole; Moravek Biochemicals, Brea, CA) was incubated for 1 hr at 25°. Kinase activity was measured as described by Nelson *et al.* [4] except that the ATP regenerating system was omitted. Reactions were quenched with 50 μl of 0.6 M TCA and neutralized by extraction with 150 μl of 0.5 M tri-*n*-octylamine in Freon 113 [5]. An aliquot (50 μl) of the solution was placed on a small DEAE-cellulose column (0.5 ml) which had been equilibrated previously with 5 mM ammonium formate (pH 4.5). The column was washed with 2.5 ml of the equilibration buffer to elute [^{3}H]FoB and then 2.5 ml of 100 mM ammonium formate (pH 4.5) to elute [^{3}H]FoB-MP, each of which was counted in Aquasol II.

Enzymic hydrolysis of FoB-MP to FoB was determined by incubation of a 50-μl solution containing 25–30 μg protein, 1 mM FoB-MP and 100 μM sodium acetate (pH 5.3) at 25° for 1 hr. After quenching and neutralization as described above, FoB and FoB-MP were separated by HPLC on a Lichrosorb RP-18 column (4.6 × 250 mM) using a 100-ml linear gradient of 0–50% MeOH/H_2O with a flow rate of 2 ml/min. Retention volumes of FoB and FoB-MP were 20 and 3.5 ml respectively.

Metabolism and uptake studies. The metabolites formed upon treatment of *L. tropica* cells with [^{3}H]FoB were identified and quantitated as previously described [5]. Uptake and transport studies were performed by centrifugation (15,000 *g*) of cells treated with [^{3}H]FoB through a layer of 400 μl inert silicone oil and into 200 μl of 0.6 M TCA as described by Cybulski *et al.* [6]. The acid soluble extract was neutralized by extraction with two 150-μl portions of 0.5 M tri-*n*-octylamine in Freon 113 and the [^{3}H]FoB was separated from phosphorylated metabolites by DEAE-cellulose chromatography as described above or by HPLC [5].

Results and discussion

FoB inhibits the growth rate of wild-type *L. tropica* promastigotes with an apparent IC_{50} of about 0.1 μM and is leishmanicidal at 10 μM [5]. As with the related drug, allopurinol riboside [4], the metabolism of FoB in *Leishmania* involves formation of its 5′-monophosphate and subsequent animation to 5′-nucleotides of the cytotoxic FoA, which is incorporated into the RNA [5, 7, 8]. Although the exact mechanism of cytotoxicity of FoB is unresolved, most workers believe that it is due to one of the nucleotide metabolites of FoA or to FoA incorporation into RNA [5, 7–9].

When *L. tropica* promastigote cells were treated with FoB, beginning at 0.2 μM and followed by stepwise increases after organisms grew at lower concentrations of the drug, strains capable of growing in FoB at concentrations up to 100 μM were established. During the initial stages of selection, when cells were transferred into higher concentrations of FoB there was a period of cell arrest lasting for 2–3 days, followed by an outgrowth of resistant organisms. Once cells became resistant to 10 μM FoB, however, there was no lag phase upon transfer to higher levels of the drug but the growth rate was still somewhat slower than wild-type organisms. After 4 weeks in medium containing 100 μM FoB, the growth rate of the FR-100 strain became essentially identical to that of the wild-type organisms in drug-free medium. When the FR-100 line was propagated in drug-free medium for at least 60 generations (about 1 month) and then reseeded in medium containing 100 μM FoB, there was no inhibition of growth. These results suggest that the biochemical properties which confer FoB resistance in the FR-100 line are stable and do not inhibit normal growth.

When the FR-100 cells were treated with 3 μM [^{3}H]FoB, intracellular radioactivity was associated only with FoB, FoB-MP, FoA-MP, FoA-DP, FoA-TP, and FoA incorporated into RNA. Although this is the same pattern of metabolism as occurs in wild-type organisms [5, 8], the concentrations of the metabolites in resistant cells were reduced 50- to 100-fold (Fig. 1). Thus, in the most general sense, the mechanism of resistance in the FR-100 cell line may be ascribed to a reduction in the metabolites of FoB which are presumed to be responsible for its cytotoxicity [5–8]. The most likely specific mechanisms would involve drug catabolism, a defect in one or more of the enzymes involved in the metabolism of FoB, or altered transport.

We were unable to detect any catabolism of FoB by the FR-100 line of *L. tropica*. When cells were treated with 3 μM [^{3}H]FoB for 8 hr, HPLC of the deproteinized medium demonstrated that all of the radioactivity co-migrated with authentic FoB. Interestingly, we did observe that extracts from the FR-100 cell line degraded FoB-MP to FoB at a rate of 280 nmoles · mg^{-1} · hr^{-1}. However, since this degradation of FoB-MP was similar in extracts from wild-type organisms (238 nmoles · mg^{-1} · hr^{-1}), this activity, a nucleotidase or phosphatase, cannot account for the FoB resistance in the FR-100 strain.

In the FR-100 cells, there was no apparent accumulation of any of the nucleotide intermediates which would be expected if one of the enzymes involved in the conversion of FoB-MP to FoA-TP were defective or deficient (Fig. 1). Thus, if the resistance is due to a defect in metabolism, the most likely candidate would be the enzyme which converts FoB to FoB-MP. As previously observed with allopurinol riboside [4], ATP did not serve as a phosphate donor to FoB using crude cell extracts of *L. tropica*. This is not surprising since it appears that in *Leishmania* inosine analogs are converted to their 5′-monophosphates by a nucleoside phosphotransferase and not an ATP-dependent kinase [3, 4]. The activity of nucleoside phosphotransferase in cell extracts was determined by measuring the conversion of [^{3}H]FoB to [^{3}H]FoB-MP using GMP as the phosphate donor, which was linear over a period of at least 1 hr. Extracts from the wild-type cells showed an activity of 2.2 ± 0.4 nmoles · mg^{-1} · hr^{-1}, and the resistant strain showed an activity of 2.4 ± 1.5 nmoles · mg^{-1} · hr^{-1}. These values are about 60-fold less than reported for *L. donovani* using allopurinol riboside as a substrate [4] and could be artifactually low because of the concomitant conversion of

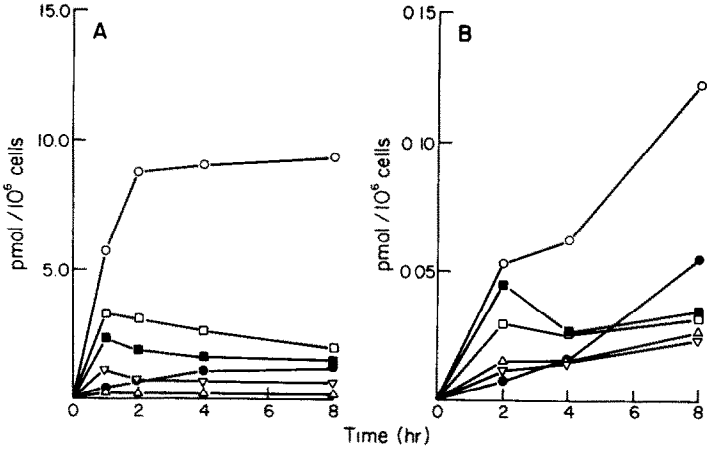

Fig. 1. Accumulation of Formycin B and its metabolites in (A) wild-type and (B) FR-100 *L. tropica* promastigotes treated with 3.0 μM[^{3}H]FoB. Preparation of cell extracts and analytical techniques were as previously described [5]. Key: (■) FoB; (□) FoB-MP; (△) FoA-MP; (▽) FoA-DP; (○) FoA-TP; (●) FoA incorporation into RNA.

FoB-MP to FoB by the phosphatase/nucleotidase activity previously described. Nevertheless, the data thus far available do not indicate any substantial difference in the nucleoside phosphotransferase activities from wild-type and FoB-resistant *L. tropica*.

Data obtained from numerous experiments involving HPLC analysis of metabolites of FoB in wild-type *Leishmania* suggest that the drug is concentrated in the cells. Even after extensive washing of cells, which should remove readily permeable molecules, concentrations of FoB are found in extracts which are higher than that of FoB in the medium. For example, when wild-type *L. tropica* cells were treated with 3 μM [^{3}H]FoB, and washed well, and the acid soluble extracts were analyzed by HPLC, FOB was present at a level that corresponded to 2.5 pmoles/10^6 cells within 1 hr (Fig. 1). Using the reported value that 10^{10} *Leishmania* promastigotes occupy a volume of 1.0 ml [4], we calculate that the corresponding intracellular concentration of FoB in wild-type organisms would be at least 25 μM, almost 10-fold higher than the extracellular concentration of the drug. In contrast, acid soluble extracts of the FR-100 line treated with 3 μM [^{3}H]FoB over a period of 8 hr possessed no more than 0.05 pmoles FoB/10^6 cells, or an intracellular concentration of 0.5 μM. These results suggested to us that the resistant cells might be deficient in their ability to transport or concentrate FoB. We therefore measured the rates of FoB uptake over shorter periods of time utilizing a technique which involves rapid quenching of transport and metabolism by centrifuging cells through an oil layer into TCA [6]. As shown in Fig. 2, uptake of FoB in wild-type *L. tropica* occurred at a rate of about 0.25 pmole · (10^6 cells)$^{-1}$ · min^{-1}, whereas resistant cells showed no measurable uptake in 2 min. Further, since the majority of intracellular radioactivity in the wild-type cells was [^{3}H]FoB, it may be concluded that the FR-100 line is deficient in its ability to transport the nucleoside.

In summary, by exposing *L. tropica* promastigotes to stepwise increases in the concentration of FoB, organisms were obtained which were highly resistant to the drug. The resistance was probably of genetic origin since it was retained after organisms were grown in the absence of FoB for numerous generations. Compared to the parent strain, FoB-resistant *L. tropica* had a greatly reduced capacity to accumulate the nucleotide metabolites that are believed to

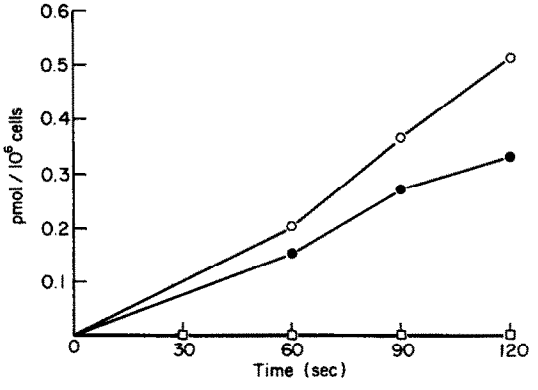

Fig. 2. Uptake of Formycin B by *L. tropica* promastigotes. Ten million cells were incubated in 400 μl DME containing 3.0 μM [^{3}H]FoB. Transport and metabolism were rapidly quenched by centrifuging cells through oil into TCA as described in Methods. Total uptake was determined for wild-type (○) and FR-100 (□) strains. FoB levels in wild-type cells (●) were determined after separation from the nucleotide metabolites as described in Materials and Methods.

be responsible for cytotoxicity. Our experiments have thus far failed to implicate catabolism or a defect in metabolism as causes of the reduced levels of these metabolites. Rather, the transport of FoB was greatly impaired in the resistant cells and, unlike wild-type cells, the resistant cells did not appear to concentrate the drug. It may be concluded that at least one of the mechanisms of resistance involves structural alteration or depletion of membrane components involved in the transport of FoB. Studies are in progress to further define the nature of this block and whether transport of other nucleosides are affected in the FoB-resistant cell line.

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The formation of the acidic and alcoholic metabolites of MD 780236

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The compound 3-{4[(3-chlorophenyl)-methoxy]phenyl}-5-(methylamino)methyl)-2-oxazolidinone methane sulphonate (MD 780236) has been shown to act as both a substrate and an inhibitor of monoamine oxidase-B and mainly as a substrate for monoamine oxidase-A [1–3]. The immediate product of the monoamine oxidase-catalysed oxidation of MD 780236 will be the corresponding aldehyde [2] and studies of the metabolic fate of this compound have shown the corresponding acid and alcohol metabolites to be formed *in vivo* and *in vitro* [2]. These results suggest that, like the aldehyde produced by the oxidation of the biogenic amines, the immediate oxidation product of MD 780236 is a substrate for aldehyde reductase and dehydrogenase actvities and that the relative rates of formation of the two products will depend on the kinetic properties of these two enzymes [4, 5].

The formation of these metabolites may be important to the monoamine oxidase inhibitory potency of MD 780236 *in vivo* since the alcohol metabolite has been shown to be a potent inhibitor of the B-form of monoamine oxidase whereas the acidic metabolite is considerably less potent [1, 6]. This paper reports the action of aldehyde-metabolizing enzymes on MD 240233, the aldehyde derived from the oxidation of MD 780236.

Materials and methods

The high-K_m aldehyde reductase was purified from ox brain by the method of Rivett *et al.* [7] and the low-K_m enzyme was purified from the same source by a modification of the method of Daly and Mantle [8]. Both enzyme preparations were homogeneous by the criteria of polyacrylamide electrophoresis in the presence and absence of sodium dodecyl sulphate. Aldehyde dehydrogenase was purified from ox liver by a modification [9] of the method of Deitrich *et al.* [10]. Crystalline horse liver alcohol dehydrogenase and coenzymes were obtained from the Boehringer Corporation Ltd., London.

MD 780236 and MD 240233, its hydrated aldehyde derivative, were synthesized by the Department of Chemistry, Centre de Recherche Delalande, Paris. Stock solutions of MD 780236 were prepared in water whereas those of MD 240233 were dissolved in 10% dimethyl sulphoxide. At the concentrations used in the enzyme assays dimethyl sulphoxide had no effect on the activities of aldehyde dehydrogenase or reductase.

All enzyme assays were carried out spectrophotometrically at 30° by following the increase or decrease in absorbance at 340 nm. Aldehyde dehydrogenase activity was determined in a reaction mixture containing, in a total volume of 2.0 ml, 200 μM NAD^+, the appropriate buffer, aldehyde and the enzyme preparation. Alcohol dehydrogenase was assayed in a reaction mixture containing, in a total volume of 2.0 ml, 100 mM potassium phosphate buffer, pH 7.2 250 μM NAD^+, aldehyde and enzyme. The activity of aldehyde reductase was determined in a mixture containing, in a total volume of 3.2 ml, 100 mM potassium phosphate buffer, pH 7.2, 125 μM NADPH and the appropriate concentrations of aldehyde and enzyme. Protein concentration was determined by the method of Markwell *et al.* [11].

Results and discussion

Two major forms of aldehyde reductase have been detected in the livers and brains from several animal species. The more active form has been termed the high-K_m aldehyde reductase [12] and it has been suggested that this enzyme may be identical with L-hexonate dehydrogenase [13] whereas the other form, which has been termed the low-K_m aldehyde reductase [12], may be identical with aldose reductase [13]. Studies with the high-K_m aldehyde reductase from ox brain indicated that it had no significant activity towards MD 240233 at concentrations of up to 50 μM. At this concentration MD 240233 had no inhibitory effect on the reduction of 500 μM pyridine-3-aldehyde by this enzyme. In contrast the low-K_m aldehyde reductase was active towards this substrate with a K_m value of 2.8 ± 0.3 μM and a maximum velocity of 102 nmol · min^{-1} mg^{-1} (Fig. 1). This maximum velocity was 28 ± 3% of that determined with pyridine-3-aldehyde, a good substrate for this enzyme [14]. The activity towards a mixture of MD 240233 and pyridine-3-aldehyde, each at a concentration of 10 times its K_m value, was 50.4 ± 3.2% of the sum of the activities obtained when the two substrates were assayed separately indicating that the same enzyme was responsible for the metabolism of these two aldehydes [15].

Another enzyme that might catalyse the reduction of aldehydes is alcohol dehydrogenase. However, no significant activity of this enzyme towards MD 240233 at concentrations of up to 100 μM could be detected. The compound was, however, an inhibitor of the reduction of acetaldehyde, a concentration of 25 μM MD 240233 resulting in 90% inhibition of the activity towards 900 μM acetaldehyde.