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METABOLISM OF FORMYCIN B BY *LEISHMANIA*
AMASTIGOTES IN VITRO

Comparative Metabolism in Infected and Uninfected Human
Macrophages*

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Leishmaniasis is initiated when sandflies inject the extracellular promastigote form of the parasite into the skin. Promastigotes are rapidly phagocytized, after which they transform into the intracellular amastigote form. The multiplication of the latter within macrophages leads to clinical disease. Treatment of leishmaniasis is primarily with pentavalent antimonials, parenteral and potentially toxic drugs with a 10–25% failure rate. The inosine analogue formycin B (FoB) inhibits multiplication of promastigotes (1–3) and is leishmanicidal to both antimony-sensitive and antimony-resistant amastigotes within human macrophages in vitro (4). FoB is also leishmanicidal to *Leishmania donovani* amastigotes in infected hamsters after either intraperitoneal (1) or oral administration (J. D. Berman et al., *Exp. Parasitol.* In press). Biochemical studies of the metabolism of FoB in promastigotes revealed that this compound is initially converted to its 5'-nucleotide, formycin B 5'-monophosphate (FoB-MP), which is subsequently metabolized to adenosine nucleotide analogues of formycin A (FoA) that become incorporated into RNA (2, 3). The FoA nucleotides or FoA incorporation into RNA are believed to be responsible for the cytotoxic effects of FoB towards promastigotes.

The present study was undertaken to determine the metabolism and mechanism of action of FoB in amastigotes within cultured human macrophages, a model that is comparable to the clinical situation. In addition, the metabolism of FoB in uninfected human macrophages and in free amastigotes was investigated.

Materials and Methods

Mono-dispersed *Leishmania* amastigotes of cutaneous strains WR 401 (Iran), WR 227 and WR 420 (Panama), and WR 338 (Dominican Republic) were obtained from foot pads

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of infected BALB/c mice (5). Human macrophages were derived from monocytes of peripheral blood of normal volunteers by 6 d of in vitro cultivation as monolayers in 2-cm² plastic wells and infected as previously described (5). To infect macrophages, $\sim 1.5 \times 10^6$ mono-dispersed amastigotes were added to each culture well, which contained $\sim 10^5$ macrophages. After 4 h at 35°C, cultures were washed twice with 1 ml of Hank's buffered saline solution (HBSS; Gibco Laboratories, Grand Island, NY) to remove nonphagocytized parasites. Specified concentrations of [³H]FoB (1.9 Ci/mmol; ICN Pharmaceuticals) were added in 1 ml of medium consisting of RPMI 1640 (Gibco Laboratories) with 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum. Infected and noninfected cultures were maintained at 35°C in the presence of [³H]FoB for specified times after which the media was aspirated and the monolayer of cells was washed four times with 1 ml HBSS at ambient temperature. Each experiment utilized 11 wells, one of which was used to count the number of organisms and/or macrophages after Wrights/Giemsa staining (5). The other wells in an experimental group were processed in a cold room as follows: The HBSS in one well was aspirated and replaced with 0.6 ml of 0.6 M trichloroacetic acid (TCA) and the macrophages were scraped into the acid. The HBSS in the second well was aspirated, the TCA suspension from the first well was added, and the macrophages were again scraped into the acid. This process was repeated until all macrophages within an experimental group were obtained in a single TCA suspension. To insure complete recovery, the entire process was repeated with 0.1 ml cold TCA which was then combined with the first suspension. The suspension was centrifuged at 10,000 g for 1 min (0°C). The supernatant was neutralized by extraction with tri-*n*-octylamine in Freon 113 (7); the acid-insoluble fraction was washed with three 0.1-ml portions of 0.3 M TCA (0°C) and once with 0.2 ml of 95% EtOH (-20°C). For experiments with mono-dispersed amastigotes, 1.2×10^8 cells (strain 227) were incubated for 16 h at 35°C in 6 ml of culture medium that contained 0.2 µM [³H]FoB. After the cells were harvested by centrifugation and washed with HBSS, acid-soluble and -insoluble fractions were prepared (2). Analyses of the metabolites of FoB and of the incorporation of FoA into RNA were performed as previously described (2).

Results

When human macrophages infected with *Leishmania* strain 401 were incubated for 8 h with 0.2 µM [³H]FoB, high performance liquid chromatography (HPLC) analysis of the acid-soluble extract gave four radioactive peaks in addition to FoB (Fig. 1). These peaks were identified as FoB-MP, FoA-MP, FoA-DP, and FoA-TP, the latter being the predominant metabolite. The same metabolites were found in uninfected macrophage controls, but they were present in significantly lower amounts (Fig. 1).

Fig. 2 shows the kinetics of accumulation of FoB metabolites in both infected and uninfected macrophages over a 20-h period of exposure to 0.2 µM [³H]FoB. With infected cells, the nucleotide metabolites accumulated to maximal levels within 4–8 h and showed a slight decrease after 20 h of exposure. Throughout the period, FoA was progressively incorporated into RNA. No incorporation of radioactivity into DNA could be detected. The pattern of FoB metabolism was similar in uninfected cells except for the much lower level of metabolites throughout the period of exposure (Fig. 2B).

Human macrophages infected with a number of *Leishmania* strains were exposed to 0.04 and 0.2 µM [³H]FoB for 16 h. These concentrations correspond to the EC₅₀ and EC₉₀ values for elimination of amastigotes from infected macrophages after 6 d (4). Mono-dispersed amastigotes were also exposed to 0.2 µM [³H]FoB and uninfected macrophages were exposed to 0.2 µM and 10 µM [³H]

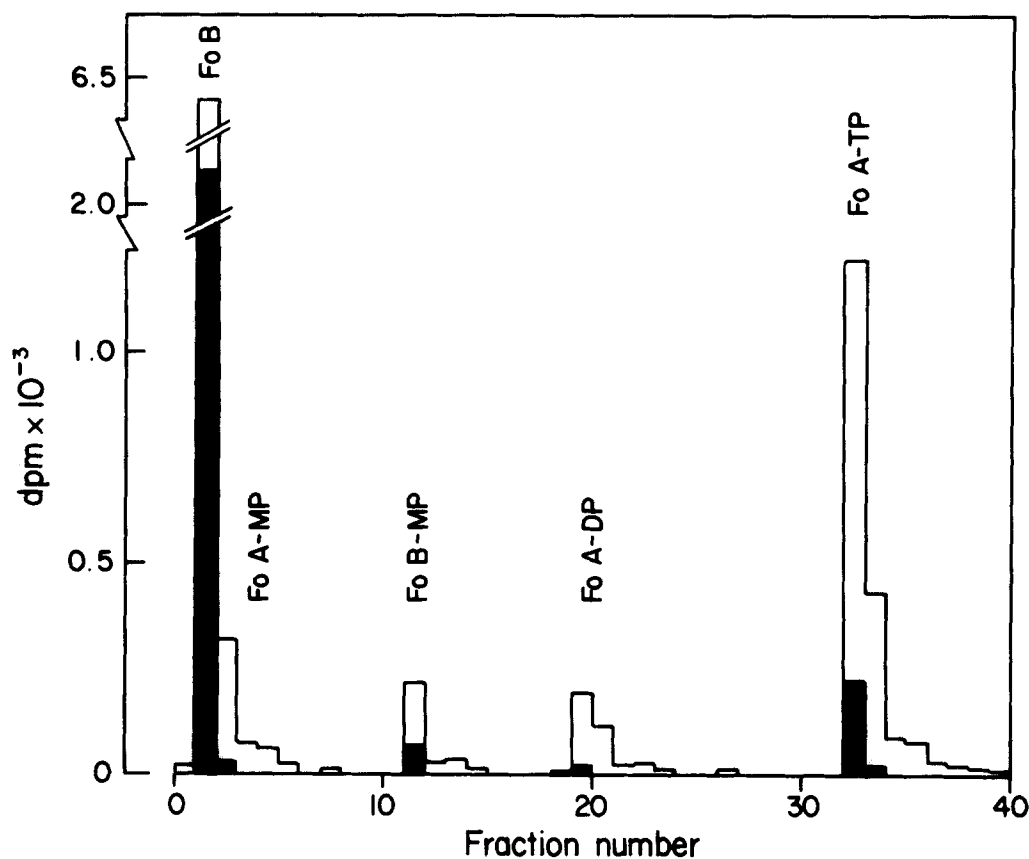


FIGURE 1. HPLC of FoB metabolites in acid-soluble extracts from 1.25×10^5 macrophages infected with $\sim 1.25 \times 10^5$ *Leishmania* amastigotes (strain WR 401) (open bars) and 1.25×10^5 uninfected macrophages (solid bars), after an 8-h exposure to $0.2 \mu\text{M}$ [^3H]-formycin B. The metabolites were separated on a Partisil SAX column (4.6×250 mm) as previously described (2). The histograms indicate the radioactivity in each 3.0-ml fraction.

FoB. The latter represents the EC_{50} for cytolysis of uninfected macrophages after 6 d (4). Table I shows the total FoA nucleotides present in cell extracts expressed as pmol per 10^6 macrophages or amastigotes; where measured, the amount of FoA incorporated into RNA is also provided. The pertinent results derived from these experiments are as follows: First, when infected macrophages were exposed to $0.2 \mu\text{M}$ FoB there was an excellent linear correlation ($r^2 = 0.977$) between the total number of amastigotes in the macrophage cultures (i.e., parasite burden) and the amount of FoA nucleotides formed. Second, free amastigotes synthesized FoA nucleotides from FoB in a manner similar to infected macrophages. Although the levels of metabolites may be artifactually low because the obligate intracellular organisms were in an unnatural environment, the experimental confirms that the metabolites are indeed formed by the amastigotes. Third, the amount of FoA metabolites in the intracellular amastigotes was correlated with the concentration of FoB to which the infected macrophages were exposed. At $0.2 \mu\text{M}$ FoB, the levels of FoA metabolites in amastigotes were

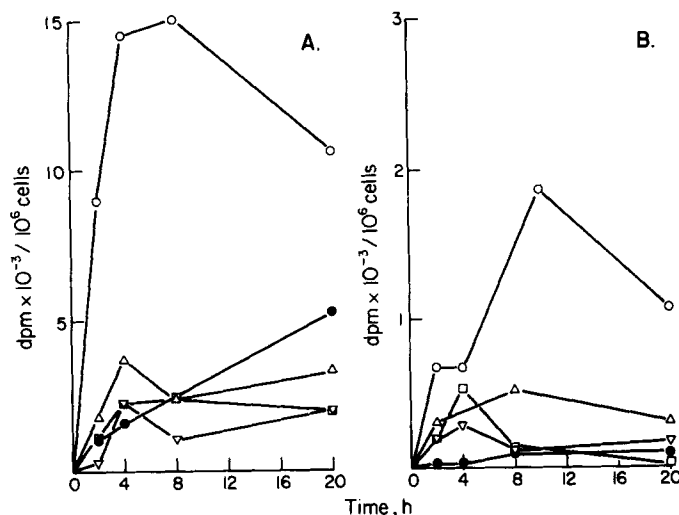


FIGURE 2. Accumulation of formycin metabolites in (A) macrophages infected with *Leishmania* amastigotes (strain WR 401) and (B) uninfected macrophages during treatment with 0.2 μ M [³H]-formycin B. Metabolite levels were determined by HPLC as in Fig. 1. FoA incorporation into RNA was determined as previously described (2). Δ FoB-MP; ∇ , FoA-MP; \square , FoA-DP; \circ , FoA-TP; \bullet , FoA incorporation into RNA.

TABLE I

Formycin A metabolites and incorporation into RNA in infected and uninfected macrophages and in free amastigotes*

Experimental group	FoB concentration μ M	Parasite burden <i>amastigotes per macrophage (average)</i>	FoA nucleotides		RNA incorporation	
			Macrophages <i>pmol/10⁶ cells</i>	Amastigotes <i>pmol/10⁶ cells</i>	Macrophages <i>pmol/10⁶ cells</i>	Amastigotes <i>pmol/10⁶ cells</i>
Macrophages infected with:						
strain 420	0.04	2	4.1	2.1	0.72	0.36
strain 338	0.04	7	5.5	0.8	— [‡]	— [‡]
strain 227	0.2	1	4.1	3.2	1.00	0.95
strain 420	0.2	2	8.9	4.2	2.49	1.22
strain 401	0.2	3	25.6	8.2	— [‡]	— [‡]
strain 227	0.2	5	36.7	7.2	— [‡]	— [‡]
strain 338	0.2	7	62.5	8.8	— [‡]	— [‡]
Free amastigotes	0.2	—	—	1.4	— [‡]	— [‡]
Uninfected macrophages	0.2	—	0.9 [§]	—	0.05 [‡]	—
Uninfected macrophages	10.0	—	22.0 [‡]	—	1.10 [‡]	—

* Cells were exposed to [³H]FoB for 16 h. Parasite burden was determined by counting the number of amastigotes in 100 macrophages and dividing by 100. The levels of radiolabeled metabolites and incorporation into RNA were determined as described in Materials and Methods. FoA nucleotides refers to the sum of FoA-MP, FoA-DP and FoA-TP. Levels of metabolites in amastigotes were determined by subtracting metabolite levels in uninfected controls from the levels in infected macrophages and dividing by the parasite burden.

[‡] Not determined.

[§] Average of four determinations.

[‡] Average of two determinations.

three- to fivefold higher than when 0.04 μM FoB was used. Likewise, the amount of FoA metabolites in uninfected macrophages was dose-related: the levels of metabolites in cells treated with 10 μM FoB were ~ 25 -fold higher than in macrophages treated with 0.2 μM FoB. Last, and perhaps most important, the amount of FoA metabolites that were formed in uninfected macrophages was far less than that formed in infected macrophages, or even in individual amastigotes.

Discussion

The results described here demonstrate that *Leishmania* amastigotes within cultured human macrophages metabolize FoB in the same manner as do extracellular promastigote forms (2, 3). The nucleoside is converted to FoB-MP, FoA-MP, FoA-DP, and FoA-TP, the latter representing $\sim 70\%$ of the total metabolites; further, FoA is incorporated into RNA. No other metabolites were observed nor was incorporation into DNA detectable. There was an excellent correlation between the parasite burden and the amount of FoA metabolites formed, indicating that the intracellular parasites are responsible for the metabolic conversions. In promastigotes, the anti-leishmanial activity of FoB has been associated with its conversion to cytotoxic nucleotides of FoA or incorporation of FoA into RNA or both (2, 3). The experiments described here correlate the killing of amastigotes within macrophages with the synthesis of FoA nucleotides. That is, the levels of FoA nucleotides formed in amastigotes and the efficacy of FoB in eliminating the organisms within macrophages are both related to the concentration of the drug used.

It has been reported that FoB is not phosphorylated in mammalian cells (8) and is remarkably nontoxic in animals (9). We show here that uninfected macrophages do in fact metabolize FoB in a manner qualitatively similar to that of *Leishmania*. However, at levels of FoB necessary to eliminate amastigotes from infected macrophages, the amount of metabolites formed in uninfected macrophages is low, and the drug has no visibly apparent deleterious effects on the macrophages. When concentrations of FoB (10 μM) were used that were 50-fold higher than the EC_{90} for elimination of amastigotes, significant levels of FoA metabolites were formed in uninfected macrophages; at this concentration, $\sim 50\%$ of the macrophages undergo cytolysis after a 6-d exposure to the drug. Although we have not yet determined the extent to which other cells are affected by FoB, these results with infected human macrophages suggest that a sufficient therapeutic index exists to warrant serious consideration of FoB as an anti-leishmanial agent in humans.

Summary

Formycin B is metabolized by cutaneous *Leishmania* amastigotes within cultured human macrophages to give formycin B 5'-monophosphate and formycin A 5'-mono-, di-, and triphosphates. Formycin A is also incorporated into RNA. The activity of formycin B against amastigotes was correlated with the levels of formycin A metabolites formed in the parasites. Uninfected macrophages also convert formycin B into the same products, but the levels are markedly lower

than those seen in infected macrophages. The results suggest that a sufficient therapeutic index exists to warrant consideration of formycin B as an anti-leishmanial drug in humans.

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References

1. Carson, D. A., and K. P. Chang. 1981. Phosphorylation and anti-leishmanial activity of formycin B. *Biochem. Biophys. Res. Commun.* 100:1377.
2. Rainey, P., and D. V. Santi. 1983. Metabolism and mechanism of action of formycin B in *Leishmania*. *Proc. Natl. Acad. Sci. USA.* 80:288.
3. Nelson, D. J., S. W. LaFon, T. E. Jones, T. Spector, R. L. Berens, and J. J. Marr. 1982. The metabolism of formycin B in *Leishmania donovani*. *Biochem. Biophys. Res. Commun.* 108:349.
4. Berman, J. D. 1982. *In vitro* susceptibility of antimony-resistant *Leishmania* to alternative drugs. *J. Infect. Dis.* 145:279.
5. Berman, J. D., D. M. Dwyer, and D. J. Wyler. 1979. Multiplication of *Leishmania* in human macrophages *in vitro*. *Infect. Immun.* 26:375.
6. Berman, J. D., and D. J. Wyler. 1980. An *in vitro* model for investigation of chemotherapeutic agents in leishmaniasis. *J. Infect. Dis.* 142:83.
7. Khym, J. X. 1975. An analytical system for rapid separation of tissue nucleotides at low pressures on conventional anion exchangers. *Clin. Chem.* 21:1245.
8. Umezawa, H., T. Sawa, Y. Fukagawa, I. Homma, M. Ishizuka, and T. Takeuchi. 1967. Studies on formycin and formycin B in cells of Ehrlich carcinoma and *E. coli*. *J. Antibiot. (Tokyo).* 20:308.
9. Ishizuka, M., T. Sawa, S. Hori, H. Takayama, T. Takeuchi, and H. Umezawa. 1968. Biological studies on formycin and formycin B. *J. Antibiot. (Tokyo).* 21:5.