

In Vitro Effects of Mycophenolic Acid and Allopurinol Against *Leishmania tropica* in Human Macrophages

JONATHAN D. BERMAN^{1*} AND H. KYLE WEBSTER²

Department of Parasitology, Division of Experimental Therapeutics,¹ and Department of Hematology, Division of Medicine,² Walter Reed Army Institute of Research, Walter Reed Army Medical Center, Washington, D.C. 20012

Received 14 December 1981/Accepted 15 March 1982

The possibility that purine inhibitors or analogs might be effective antileishmanial agents led to the determination of the antileishmanial activity of mycophenolic acid and allopurinol in vitro. The drugs were tested against *Leishmania tropica* amastigotes (mammalian forms) within human macrophages, a model in which achievable serum concentrations of antileishmanial agents currently in use eliminate approximately 90% of the parasites. Mycophenolic acid, an inhibitor of guanosine nucleotide synthesis from inosinic acid, was shown here to inhibit guanosine nucleotide synthesis in *L. tropica* promastigotes (insect forms). When tested against *L. tropica* amastigotes within macrophages, mycophenolic acid eliminated 50% of the parasites at achievable peak human serum levels (20 µg/ml) and 40% of the parasites at trough serum levels (1 to 10 µg/ml). This demonstrates that an inhibitor of guanosine nucleotide synthesis is partially effective against *L. tropica* in vitro. The purine analog allopurinol was also tested and was found to eliminate 50% of *L. tropica* amastigotes in this model. Because mycophenolic acid and allopurinol are partially, but not completely, effective antileishmanial agents in this in vitro model, their in vivo utility remains to be determined by clinical trials.

Leishmaniasis affects perhaps 100 million persons at any one time throughout the tropical and neotropical world (1). The frequent resistance of lesions to standard therapy with pentavalent antimony (16) and their occasional resistance to subsequent therapy with pentamidine or with amphotericin B (16) have prompted a search for new, orally effective antileishmanial agents. Purine analogs and inhibitors are attractive candidates for such study because of the importance of purine nucleotides, such as ATP and GTP, to energy metabolism and nucleic acid synthesis. Marr and colleagues have shown that allopurinol or its derivatives may be effective antileishmanial agents due to parasite-specific incorporation of such compounds into adenosine metabolites (3, 18). Mycophenolic acid prevents the conversion of IMP to GMP in Landschutz ascites cells, calf thymus, and LS cells (7) and has been used experimentally for the oral treatment for psoriasis (8, 11, 15, 17). Here we demonstrate that mycophenolic acid and allopurinol are both partially effective antileishmanial agents in the human macrophage model of leishmaniasis.

MATERIALS AND METHODS

Exposure of *Leishmania*-infected macrophages to antileishmanial agents. Human macrophages, derived from peripheral blood monocytes of normal volunteers by 6 days of in vitro culture, were infected with a 6:1

multiplicity of amastigotes of *Leishmania tropica* 173 at 37°C over 4 h as previously described (4). Infected macrophages (approximately 10⁵ macrophages per culture well) were washed and continued in culture (RPMI 1640 [GIBCO Laboratories, Grand Island, N.Y.], penicillin [50 U/ml], streptomycin [50 µg/ml], 10% heat-inactivated fetal bovine serum [GIBCO]) in the absence (control cultures) or in the presence (experimental cultures) of anti-*Leishmania* agents (4). Cultures were processed in duplicate. Culture medium, containing antileishmanial agents where appropriate, was changed at 3 days. At the end of 6 days of incubation, control and experimental cultures were fixed, and the mean number of amastigotes per 100 macrophages was determined by counting 100 to 200 macrophages in each culture.

Viability of amastigotes after exposure to drugs. The viability of amastigotes was assessed by determining the number that were capable of transforming into promastigotes. Drug-exposed and control macrophages were scraped into Schneider medium revised (GIBCO) containing 30% heat-inactivated fetal bovine serum (10) and left at 24°C for 3 days. Under these circumstances, viable amastigotes transform to promastigotes, and the promastigotes so generated may not have time to multiply significantly. The number of promastigotes in 3-day-old scraped cultures was ascertained by counting in a hemacytometer.

Exposure of promastigotes to mycophenolic acid. Promastigotes of *L. tropica* 173 (4) (WRAIR 401) were obtained by allowing amastigotes isolated from infected BALB/c mouse footpads to transform at 24°C over 3 days in Schneider medium revised containing 30%

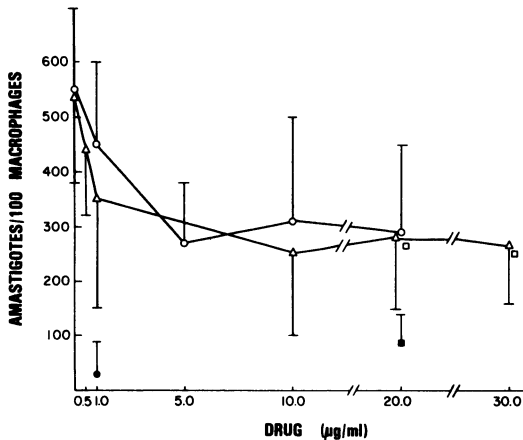


FIG. 1. Elimination of *L. tropica* 173 in human monocyte-derived macrophages by mycophenolic acid and allopurinol. Six-day-old human monocyte-derived macrophages were infected with a 6:1 ratio of *L. tropica* 173 (WRAIR 401) over 4 h. The infected macrophages were then either exposed to media alone (control cultures) or to 0.5 to 30 µg of antileishmanial agent per ml (experimental cultures) for 6 days. The mean number of amastigotes within 100 macrophages at 6 days is plotted versus concentration of antileishmanial agent. Symbols: △, cultures exposed to mycophenolic acid (mean ± standard deviation, $n = 5$ to 8); ○, cultures exposed to allopurinol (mean ± standard deviation, $n = 5$ to 8); □, cultures exposed to 5 µg of allopurinol and either 20 or 30 µg of mycophenolic acid per ml; ●, cultures exposed to amphotericin B (mean ± standard deviation, $n = 5$); ■, cultures exposed to Pentostam (mean ± standard deviation, $n = 4$). For cultures exposed to 5 µg of allopurinol and 20 µg of mycophenolic acid per ml, the range of values was 154 to 310 amastigotes per 100 macrophages ($n = 3$). For cultures exposed to 5 µg of allopurinol and 30 µg of mycophenolic acid per ml, the range was 195 to 427 amastigotes per 100 macrophages ($n = 3$).

heat-inactivated fetal bovine serum. Promastigotes were then diluted to 1×10^6 per ml of modified minimal essential medium (2) containing 10% heat-inactivated fetal bovine serum and allowed to multiply in medium alone (control cultures) or in medium containing mycophenolic acid (30 µg/ml). After 2 to 3 days the promastigotes had multiplied four times and were in log-phase growth in both control and mycophenolic acid-exposed cultures.

Exposure of infected macrophages to radiolabeled bases. Incorporation of radiolabeled purines (hypoxanthine or adenosine) into purines by four groups of 7-day-old macrophages was determined: control macrophages, macrophages infected with *L. tropica* (approximately 500 parasites per 100 macrophages) for 20 h, control macrophages exposed to 40 µg of mycophenolic acid per ml for 16 h, and macrophages infected for 20 h with *L. tropica* and exposed to 40 µg of mycophenolic acid per ml for 16 h. The somewhat high concentration of 40 µg of mycophenolic acid per ml was used in the radiolabeling experiments because the macrophages were only exposed to the drug for a

brief period of time. The macrophages were washed with Hanks balanced salt solution and then incubated in culture medium containing labeled bases and, where appropriate, 40 µg of mycophenolic acid per ml. The radiolabeled purines used were: [G - 3H]hypoxanthine (3.6 Ci/mmol), [8 - ^{14}C]hypoxanthine (46 mCi/mmol), or [8 - ^{14}C]adenosine (15 mCi/mmol) (New England Nuclear Corp., Boston, Mass.). After 3 h (34°C, 5% CO₂) of incubation with label, macrophages were sequentially scraped into 1.0 ml of 1.0 M perchloric acid in phosphate-buffered normal saline (PCA). After washing the wells with 0.25 ml of PCA, the combined 1.25 ml of PCA-extracted macrophages constituting one experimental group was cooled (4°C) for 30 min to allow completion of PCA extraction, neutralized with 10 N KOH, and centrifuged (12,000 × g for 10 min). The clear supernatant was frozen (-70°C) until analysis.

Exposure of promastigotes to radiolabeled bases. Incorporation of radiolabeled purine into control and mycophenolic acid-exposed promastigotes was assessed by incubating both groups of promastigotes with label for 3 or 6 h. After incubation, the promastigotes were pelleted by centrifugation (2,000 × g for 20 min) and were resuspended in 1.0 ml of incubation medium, to which was added 1.0 ml of PCA. This PCA extract was then processed in the manner described above.

Assessment of incorporation of label by macrophages and by promastigotes. Assay of purine nucleotides was by simultaneous UV radioactivity-high-performance liquid chromatography. This method detects all major purines and has been described in detail (20). The method allows the simultaneous quantitation of both UV absorbance (concentration) and radioactivity for each separated component (peak). Briefly, nucleotides were separated by anion-exchange gradient high-performance liquid chromatography. Concentrations were determined by an external standard method, using purine standards of known purity. Radioactivity was recorded by peak integration and reported as radioactivity per chromatography peak.

RESULTS

Effect of drugs on *L. tropica* in human macrophages. Mycophenolic acid at 10 to 30 µg/ml eliminated approximately 50% of *L. tropica* from human macrophages, compared with simultaneously cultivated controls (Fig. 1). The peak achievable serum levels of this drug are 10 to 20 µg/ml after a single oral dose of 400 to 1,000 mg (6, 14). Thirty micrograms per milliliter was used as an approximation of the serum level that might be obtained if larger doses were administered. Approximately 40% of the parasites were eliminated by 1 µg of the drug per ml (Fig. 1); this concentration corresponds to the trough serum level (1 to 10 µg/ml) 6 h after a single oral dose (6, 14).

Allopurinol eliminated about 50% of the parasites at a concentration of 5 µg/ml (Fig. 1). The peak achievable serum level of allopurinol after standard 300-mg oral doses is 1 to 2 µg/ml (9); 3 to 6 µg/ml might reasonably be expected to be

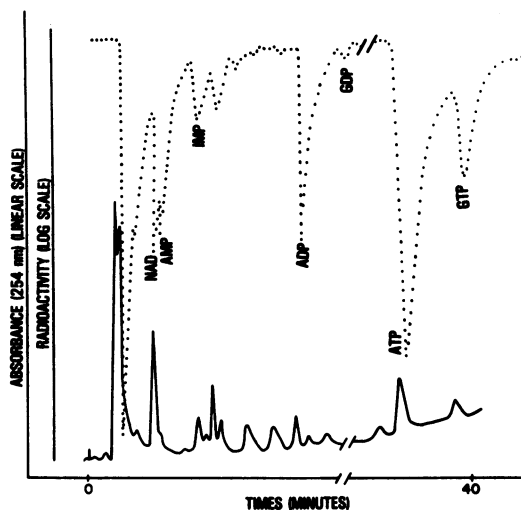


FIG. 2. Chromatography elution profile for separation of purine nucleotides in PCA extract of macrophage monolayers. Monolayers of control macrophages (approximately 1×10^5 cells per well) were extracted sequentially (well to well, total of 11 wells) with 1.25 ml of PCA-phosphate-buffered saline. The neutralized extract was then analyzed by the described high-performance liquid chromatography method, which permits simultaneous detection of both absorbance (254 nm) and radioactivity of a separated component (peaks). The figure shows the output as traced by the strip-chart recorder for UV absorbance (solid line, linear scale) and radioactivity (broken line, log scale) detectors. Nucleotide concentrations were determined by an external standard method involving integration of UV peak areas. The area under the radioactivity peak was measured by machine integration and is reported as radioactivity per chromatography peak. Note that the nucleotide concentrations constitute nucleotide pools, whereas nucleotide radioactivity counts constitute newly synthesized compound within the pool.

achieved with the 900-mg doses given to patients with malignancies.

Incubation of *L. tropica*-infected macrophages with a combination of mycophenolic acid (20 or 30 $\mu\text{g/ml}$) and allopurinol (5 $\mu\text{g/ml}$) resulted in no greater elimination of parasites than was achieved by the drugs individually (Fig. 1).

Achievable serum levels of Pentostam (20 $\mu\text{g/ml}$) or of amphotericin B (1 $\mu\text{g/ml}$) were employed as a positive drug control in these experiments (5). These drugs eliminated a mean of 83 and 94% of parasites, respectively, compared with the controls (Fig. 1).

The viability of amastigotes present after drug exposure was ascertained in experiments in which control and drug-exposed infected macrophage cultures were scraped so that viable amastigotes would transform into promastigotes. In a typical experiment, the number of amastigotes

in allopurinol (5 $\mu\text{g/ml}$)-exposed cultures was 50% of controls, and the number of promastigotes derived from allopurinol-exposed macrophages was 23% of the number of promastigotes derived from control macrophages. For mycophenolic acid (20 $\mu\text{g/ml}$), the amastigote percentage was 60% of controls, and the promastigote percentage was 23% of controls. These comparisons indicate that many of the amastigotes visible after drug exposure were viable in the sense of being capable of transforming into promastigotes.

Effect of mycophenolic acid on metabolism of control macrophages and of *L. tropica*-infected macrophages. ATP and GTP are formed from hypoxanthine as follows. First, hypoxanthine is phosphoribosylated to form IMP. Second, IMP is converted to either AMP, ADP, and ATP; or to GMP, GDP, and GTP. In these experiments, both uninfected and infected macrophages incorporated labeled hypoxanthine into adenosine and guanosine nucleotides via IMP. The chromatographic elution profile for labeled control macrophages (Fig. 2) demonstrates that the synthesis of these nucleotides could be clearly quantitated. Nucleotide synthesis was also reproducible: in one experiment in which the synthesis of ATP by 10^6 uninfected macrophages was determined in duplicate, the values were 11,990 dpm and 11,568 dpm. Uninfected and infected macrophages typically contained 10 to 20 nM ATP per 10^6 macrophages and synthesized ATP and ADP in a ratio of about 5:1 (Table 1).

Uninfected and infected macrophages exposed to 40 μg of mycophenolic acid per ml showed decreased incorporation of hypoxanthine into total guanosine nucleotides. In three experiments, the mean decrease of total guanosine nucleotides in mycophenolic acid-treated control macrophages was 46%; the mean decrease of total guanosine nucleotides in mycophenolic acid-treated infected macrophages was 43% (Table 2). Mycophenolic acid did not inhibit adenosine nucleotide synthesis by either uninfected or infected macrophages (Table 1).

Effect of mycophenolic acid on metabolism of *L. tropica* promastigotes. Exposure of *L. tropica* promastigotes in cell-free media to 30 μg of mycophenolic acid per ml resulted in a 51% mean decrease in the synthesis of guanosine nucleotides compared with simultaneously cultivated control promastigotes (Table 2). There was less than a 5% decrease in adenosine nucleotide synthesis in the experimental group compared with controls.

DISCUSSION

Achievable serum concentrations of mycophenolic acid, an agent used experimentally to

TABLE 1. Adenosine nucleotides in *L. tropica*-infected, mycophenolic acid-treated, and control macrophages

Treatment ^a	ADP		ATP		ATP/ADP
	nmol ^b	dpm ^c	nmol	dpm	
Uninfected, untreated	1.0	623	11.8	3,516	5.6
Uninfected, mycophenolic acid treated	1.0	2,037	13.9	7,980	3.9
Infected, untreated	0.9	677	18.4	4,068	6.0
Infected, mycophenolic acid treated	1.3	793	14.4	5,376	6.8

^a Infected and uninfected macrophages cultured in 16-mm-diameter wells with and without mycophenolic acid (40 µg/ml) were incubated with [³H]hypoxanthine (specific activity, 3.6 Ci/mmol) for 3 h at 34°C, extracted, and analyzed as described in the text.

^b Nanomoles per 10⁶ macrophages. Data represent ADP or ATP pool within macrophages.

^c Disintegrations per minute, radioactivity per chromatography peak (value is for integrated peak area). Data represent newly synthesized ADP or ATP by macrophage.

treat human psoriasis, eliminated about 50% of *L. tropica* parasites from human macrophages in vitro, compared with simultaneously cultivated controls. Probable achievable serum concentrations of allopurinol also eliminated about 50% of the parasites.

The biochemical mechanism of the antileishmanial effect of mycophenolic acid in this model was investigated by assessing the purine metabolism of amastigote-infected macrophages and

of promastigotes in axenic culture. Mycophenolic acid inhibits synthesis of GMP from IMP and was shown here to inhibit guanosine nucleotide synthesis by promastigotes. It was not possible to demonstrate a similar metabolic block in macrophage-contained amastigotes because the decrease in guanosine nucleotide synthesis in control macrophages was insignificantly different from the decrease in infected macrophages. This similarity was probably due to the small volume of amastigotes compared with their macrophage hosts (estimated to be 3%). The mechanism of action of mycophenolic acid against amastigotes therefore is inferred to be inhibition of IMP-to-GMP conversion.

Because *Leishmania* are obligate intramacrophage parasites, parasite nutrition must be in large part supplied by metabolic pools of the host. Metabolism in the model host should therefore be vigorous and comparable with that found in cells in general. Because mycophenolic acid inhibits purine metabolism and allopurinol is a purine analog, the purine metabolism of the 7-day-old human monocyte-derived macrophages used in these experiments was investigated. Control, infected, and mycophenolic acid-exposed macrophages all contained at least 10 nmol of ATP per 10⁶ macrophages and synthesized ATP and ADP in a ratio of about 5:1. Substantial ATP pools with synthesis of the adenosine nucleotides in a similar ratio are findings comparable with those of other cell types, such as lymphoblasts (19), and are indicative of cellular vitality as regards purine metabolism.

These considerations and the fact that this model is the only one reported in which achievable serum levels of the antileishmanial agents currently in use eliminate 90 to 100% of the

TABLE 2. Effect of mycophenolic acid on total guanosine nucleotide synthesis in various cell types^a

Expt no.	Cell type	Total guanosine nucleotide (dpm) ^b in:		
		Control cells	Mycophenolic acid-treated cells ^c	% Decrease due to mycophenolic acid
1 ^d	Uninfected Mφ	82,809	41,178	50
	Infected Mφ	59,099	24,844	58
2 ^e	Uninfected Mφ	3,016	1,321	56
	Infected Mφ	1,689	1,134	32
3 ^e	Uninfected Mφ	1,792	1,235	31
	Infected Mφ	2,013	1,131	44
4 ^f	Promastigotes	8,018	4,168	48
5 ^e	Promastigotes	10,480	4,840	54

^a Uninfected and infected macrophages (Mφ) and promastigotes growing in cell-free culture.

^b Total guanosine nucleotides = GDP + GTP. Nucleotides were separated by high-performance liquid chromatography. The two nucleotide peaks were collected as individual fractions, which were counted by conventional liquid scintillation spectrometry.

^c Mycophenolic acid was added at a final concentration of 30 µg/ml.

^d Experiment performed with [³H]hypoxanthine (3.6 Ci/mmol).

^e Experiment performed with [¹⁴C]hypoxanthine (46 mCi/mmol).

^f Experiment performed with [¹⁴C]adenosine (58 mCi/mmol).

parasites (5) suggest that the effect of these drugs in this model may be qualitatively predictive of *in vivo* efficacy. In preliminary human trials with allopurinol for *L. donovani*-infected patients, three of six patients who failed to respond completely to pentavalent antimony were cured with allopurinol (12). None of four patients who were treated with allopurinol alone was cured (12). The demonstration that allopurinol has partial but not complete activity, even at high concentrations, in this *in vitro* model is in accord with this preliminary data, which together suggest only partial *in vivo* effectiveness for this drug. However, allopurinol eliminated virtually all amastigotes in a tumor macrophage *in vitro* model (3), and the precise value of allopurinol as an adjunctive agent in leishmaniasis remains to be determined by further trials.

This *in vitro* study also suggests that mycophenolic acid might have use as an adjunctive agent for leishmaniasis, although as for allopurinol it is difficult to interpret what only partial *in vitro* efficacy implies for *in vivo* activity. The toxicity of mycophenolic acid for humans has been variously reported. Several studies showed only mild gastrointestinal distress and none-to-mild hematological disturbances (6, 8, 11, 13, 17), but one study showed significant hematological toxicity (15). The further implication of these experiments is that inhibitors of guanosine nucleotide synthesis may be attractive candidates for antileishmanial agents.

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