ELSEVIER

Molecular & Biochemical Parasitology 113 (2001) 199–213

MOLECULAR & BIOCHEMICAL PARASITOLOGY

www.parasitology-online.com.

Pteridine salvage throughout the *Leishmania* infectious cycle: implications for antifolate chemotherapy☆

Mark L. Cunningham, Stephen M. Beverley *

Department of Molecular Microbiology, Washington University School of Medicine, St Louis, MO 63110, USA

Received 10 November 2000; received in revised form 3 January 2001; accepted 5 January 2001

Abstract

Protozoan parasites of the trypanosomatid genus *Leishmania* are pteridine auxotrophs, and have evolved an elaborate and versatile pteridine salvage network capable of accumulating and reducing pteridines. This includes biopterin and folate transporters (BT1 and FT1), pteridine reductase (PTR1), and dihydrofolate reductase–thymidylate synthase (DHFR-TS). Notably, PTR1 is a novel alternative pteridine reductase whose activity is resistant to inhibition by standard antifolates. In cultured promastigote parasites, PTR1 can function as a metabolic by-pass under conditions of DHFR inhibition and thus reduce the efficacy of chemotherapy. To test whether pteridine salvage occurred in the infectious stage of the parasite, we examined several pathogenic species of *Leishmania* and the disease-causing amastigote stage that resides within human macrophages. To accomplish this we developed a new sensitive HPLC-based assay for PTR1 activity. These studies established the existence of the pteridine salvage pathway throughout the infectious cycle of *Leishmania*, including amastigotes. In general, activities were not well correlated with RNA transcript levels, suggesting the occurrence of at least two different modes of post-transcriptional regulation. Thus, pteridine salvage by amastigotes may account for the clinical inefficacy of antifolates against leishmaniasis, and ultimately provide insights into how this may be overcome in the future.

Keywords: Biopterin transporter; Folate transporter; Developmental regulation; Dihydrofolate reductase; Pteridine reductase; HPLC biopterin assay

1. Introduction

Trypanosomatid parasitic protozoa of the genus *Leishmania* are the causative agents of a broad range of human diseases, ranging in severity from self-limiting cutaneous lesions to fatal visceral infections. An estimated 15 million people are infected with a further 350 million at risk in tropical and sub-tropical regions of the world [1]. *Leishmania* are vector-borne parasites transmitted by phlebotomine sand flies. Within the fly, they reside within the alimentary tract where they replicate. Upon entry into stationary phase a proportion of the cell population differentiates from the non-infectious promastigote stage into the highly infectious metacyclic form. After a sand fly bite, metacyclics are deposited into the mammalian host where they are taken up by macrophages. Here, they differentiate into the rapidly dividing, non-flagellated amastigote stage within an acidified phagolysosome. Existing chemotherapy is unsatisfactory, relying upon antiquated pentavalent antimonials such as Pentostam despite considerable host toxicity and some evidence for the emergence of parasite resistance [2]. Moreover, safe vaccines are not available. Consequently, there is considerable interest in the identification and characterization of novel biochemical pathways with the aim of developing new chemotherapies.

Leishmania, in contrast to their mammalian hosts, are pteridine auxotrophs and therefore have an absolute requirement for an exogenous source [3–7]. To

Abbreviations: BT1, biopterin transporter 1; DHFR-TS, dihydrofolate reductase–thymidylate synthase; FT1, folate transporter 1; H_2-biopterin, dihydrobiopterin; H_4-biopterin, tetrahydrobiopterin; H_2-folate, dihydrofolate; H_4-folate, tetrahydrofolate; PTR1, pteridine reductase 1.

☆ *Note:* Nucleotide sequence data reported in this paper are available at the GenBank™ database under the accession number AAB61214

* Corresponding author. Tel.: +1-314-7472630; fax: +1-314-7472634.

E-mail address: beverley@borcim.wustl.edu (S.M. Beverley).

0166-6851/01/$ - see front matter
PII: S0166-6851(01)00213-4

overcome this deficiency, *Leishmania* has evolved a complex and versatile pteridine salvage network capable of scavenging a wide array of conjugated and unconjugated pteridines, notably folate and biopterin, respectively (see Fig. 1). Two distinct plasma membrane transporters mediate the active accumulation of pteridines: folate uptake occurs predominantly via the folate transporter (FT1) (Moore JB, Beverley SM, manuscript in preparation), whereas biopterin transport occurs exclusively via biopterin transporter 1 (BT1) [8–10] (Moore & Beverley, manuscript in preparation). Whilst the active uptake of folates by transporters is well known, BT1 is the only known example of an unconjugated pteridine transporter (for reviews see [11,12]).

Folate and biopterin serve as cofactors only in their fully reduced tetrahydro-forms, H_4-folate and H_4-biopterin, respectively. In *Leishmania* and mammalian cells, H_4-folate is generated from folate and H_2-folate by the NADPH-dependent enzyme dihydrofolate reductase (DHFR) [13]. In *Leishmania* and other protozoans, DHFR occurs as a bifunctional enzyme, joined to thymidylate synthase (DHFR-TS) [14–16]. The principal role of H_4-folate is as an essential co-factor in the de novo synthesis of thymidylate in *Leishmania* [16]; correspondingly, genetic deletion of DHFR-TS is lethal in the absence of thymidine [17] and *DHFR-TS* knockout parasites are not able to establish infections in mice [18].

In mammalian cells H_4-biopterin is synthesized de novo, or salvaged through DHFR-mediated reduction of H_2-biopterin [19]. In contrast, in *Leishmania* the de novo biopterin synthetic pathway is absent [3–7] and DHFR-TS shows no activity with biopterin or H_2-biopterin [20]. Instead, reduced biopterin is generated through the action of the novel enzyme Pteridine Reductase 1 (PTR1), which sequentially reduces oxidized biopterin to dihydro- (H_2-biopterin) and then H_4-biopterin [20–23]. This NADPH-dependent enzyme is structurally unrelated to DHFR and belongs to the short-chain dehydrogenase family [22–26]. PTR1 exhibits a broad specificity for pteridine substrate and will also reduce folate to the H_2- and H_4-forms [20–22]. Deletion of the *PTR1* gene is lethal to the insect stage promastigotes but can be offset by provision of reduced pterins but not folates, indicating an essential role for unconjugated pteridines [20–22]. While H_4-biopterin is an essential cofactor in many reactions including ether lipid cleavage, aromatic amino acid hydroxylations, molybdopterin synthesis and nitric oxide synthesis in higher eukaryotes [27–30], the role(s) of H_4-biopterin in *Leishmania* has not been clearly established, although involvement in oxidant resistance has been proposed (Nare et al., manuscript in preparation).

Despite reduced folate and biopterin being essential for growth, anti-pteridines have not shown much

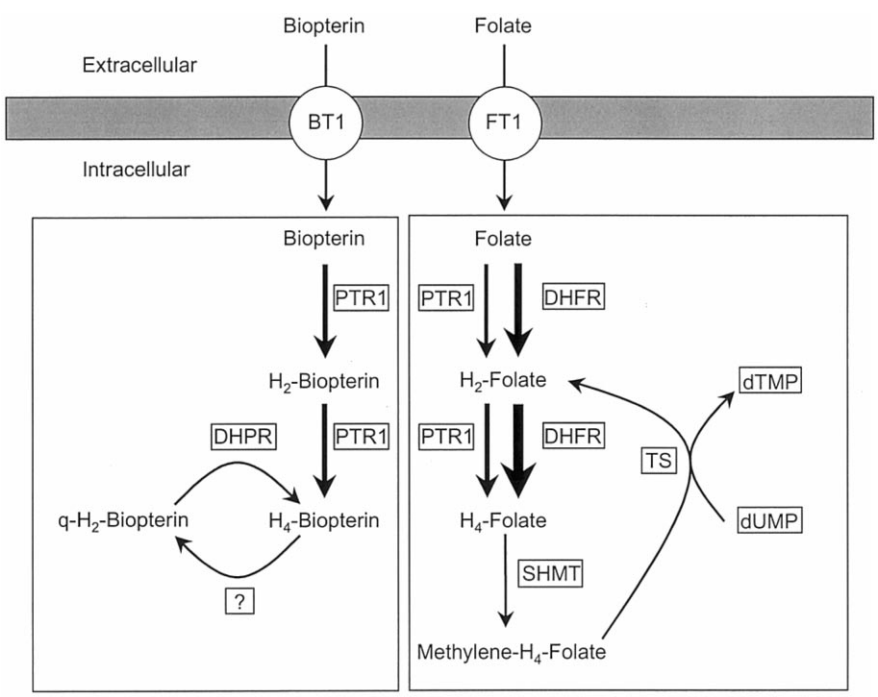

Fig. 1. Pteridine salvage in *Leishmania*. The diagram shows the transporters responsible for pteridine accumulation, and their subsequent reduction and utilization within *Leishmania* metabolism. BT1, biopterin transporter 1; FT1, folate transporter 1; PTR1, pteridine reductase 1; DHFR-TS, bifunctional dihydrofolate reductase–thymidylate synthase (the activities are shown separately in the figure); SHMT; serine *trans*-hydroxymethyl transferase; DHPR, dihydropteridine reductase; q-H_2-biopterin, quinonoid dihydrobiopterin, and '?', unknown enzyme(s) utilizing H_4-biopterin. The widths of the arrows indicate the relative contributions of each enzyme in steps where more than one enzyme is implicated [20]. Please see the text for literature references.

promise clinically against *Leishmania* in contrast to other protozoal infections [5,31–36]. Our current understanding of pteridine metabolism is almost entirely based on studies of the promastigote (insect) stage of the parasite, as this is readily cultivated in quantities sufficient for biochemical studies. From these studies, we have proposed that enzymes of the pteridine salvage pathway may be responsible for the poor efficacy of anti-pteridines against *Leishmania* promastigotes [20,21,37]. In this work, we have extended these studies to pteridine salvage within the infectious cycle of three different *Leishmania* species, including ones responsible for cutaneous (*L. major*, *L. mexicana*) and fatal visceral disease (*L. donovani*). We made use of the unique properties of each species in culture to gain access to two key developmental stages. With *L. major*, we were able to study the infectious metacyclic stage, which arises within the sand fly prior to transmission to vertebrates. With *L. mexicana*, we were able to make use of its ability to differentiate in vitro to an amastigote-like form that closely resembles amastigotes recovered from infected macrophages, the ultimate target of prospective chemotherapy. These studies establish the importance of pteridine salvage pathways throughout the infectious cycle of this deadly parasite and provide a clearer understanding of the requirements for successful anti-folate based chemotherapy.

2. Materials and methods

2.1. Reagents

[3′,5′,7,9-^{3}H]-Folate, [3′,5′,7-^{3}H]-methotrexate and [^{3}H(G)]-biopterin (randomly labeled) were purchased from Moravek. Dihydrobiopterin (H_2-biopterin) was purchased from Schircks Laboratories. Folate-deficient medium (fdM199) was custom-manufactured by Gibco-BRL and comprises of M199 with Hanks salts and lacking both folate and thymidine [20]. Fetal calf serum was purchased from Bio-Whittaker, and embryonic bovine fluid was from Sigma. Trypticase was purchased from Becton Dickinson. All other reagents were of analytical grade.

2.2. Leishmania culture

The strains of *Leishmania* used were: *L. major* Friedlin V1 (MHOM/JL/80/Friedlin), *L. major* CC-1 [38], a null mutant of CC-1 lacking PTR1 (*ptr1*$^-$) or over-expressing PTR1 [21], *L. donovani* Sudanese strain 1S2D (MHOM/SD/00/1S-2D) and *L. mexicana* (MNYC/BZ/62/M379). In this work, all species and/or strains of *Leishmania* have been shown in previous studies to be fully virulent. Promastigotes were maintained by serial passage in M199 medium supplemented 10% heat-inactivated fetal calf serum at 26°C as described [38]. This medium was further supplemented with 2 μg ml^{-1} H_2-biopterin for growth of *L. major ptr1*$^-$ and geneticin (G418; 10 μg ml^{-1}) for growth of the PTR1-over-expressing line. *L. mexicana* axenic amastigotes were grown at 34°C in 5% CO_2 in a simplified version of JH-3 medium [39] developed by David Russell (Washington University, St Louis). The axenic amastigote medium consists of M199 supplemented with 0.25% glucose, 0.5% trypticase, 0.1 mM adenine, 0.0005% hemin, 0.075% L-glutamine, 0.0001% biotin, 10% heat-inactivated fetal calf serum and 10% heat inactivated embryonic bovine fluid. Axenic amastigotes were obtained by diluting late log-phase *L. mexicana* promastigotes sixfold into axenic amastigote medium, and incubating at 34° C in the presence of 5% CO_2 for 5 days, by which time differentiation was complete. Axenic amastigotes were maintained by serial passage every 4 days. Logarithmic, early stationary and 48 h stationary phase promastigotes were taken when cells reached $3–6 \times 10^6$ ml^{-1}, maximal density (typically 2.5×10^7) and 48 h after reaching maximal density, respectively. Amastigotes were analyzed in either logarithmic ($1–3 \times 10^6$ cells ml^{-1}) or 48 h after entry into stationary phase (typically 2.5×10^7cells ml^{-1}). Metacyclic promastigotes were isolated from 48 h stationary phase cultures of *L. major* by virtue of their inability to react with peanut agglutinin as described [40].

2.3. Purification of radiolabeled ligands

[^{3}H]-Folate and [^{3}H]-methotrexate were purified by HPLC as described previously [41] on an octadecylsilane column using a mobile phase of 10 mM trifluroacetic acid with a 0–20% gradient of acetonitrile, 8 mM trifluroacetic acid. [^{3}H]-Biopterin was purified by HPLC on an octadecylsilane column using an isocratic mobile phase of 10% methanol. Purified isotopes were aliquoted and stored at −80°C for up to 6 months, without significant breakdown.

2.4. Preparation of cell lysates

Cells were recovered by centrifugation, washed twice with phosphate buffered saline (PBS: 138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4 and 1.8 mM KH_2PO_4) and resuspended at 2×10^9 cells ml^{-1} in 10 mM Tris–Cl pH 7.0 supplemented with protease inhibitors as described [42]. Cells were lysed by three rounds of freeze–thaw and sonication. Extracts were clarified by centrifugation at $15\,000 \times g$ for 30 min at 4°C. Low-molecular-mass components were removed by ultrafiltration using Centricon-3 concentrators (3000 MW cut-off) with three washes with lysis buffer. Protein concentrations were determined using the Pierce Micro BCA dye-binding assay.

2.5. Transport assays

Accumulation of [^{3}H]-biopterin and [^{3}H]-folate was monitored as described previously [41,43] (Moore et al., and Moore and Beverley, manuscripts in preparation). Cells were harvested by centrifugation, washed twice with transport medium (fdM199 medium lacking serum and pteridines [20]), and resuspended at 5×10^8 cell ml^{-1} in transport medium. Then 100 μl of cell suspension was mixed rapidly with 100 μl of substrate and layered over a 100 μl cushion of either dibutylphthalate (density 1.043 g ml^{-1}; for promastigotes) or dibutylphthalate/mineral oil mix (for axenic amastigotes; density 1.023 g ml^{-1}). The final concentration of biopterin was 200 μM (typical specific activity 10 μCi μmol^{-1}) whereas the final concentration of folate was 10 μM (typical specific activity 150 μCi μmol^{-1}). Triplicate samples were incubated for various times, at either 23 or 4°C, and the reaction terminated by centrifugation through the oil layer at $16\,000 \times g$ for 30 s. The aqueous layer was aspirated and the oil layer washed twice with PBS. The cell pellet was recovered, lysed with 100 μl of Triton X-100 and counted in 5 ml of Scintiverse II scintillation fluid. Net uptake due to active transport at 23°C was determined by subtracting values obtained at 4°C.

2.6. Assay of DHFR-TS and PTR1

Levels of DHFR-TS in cell lysates were measured by the quantitative binding of [^{3}H]-methotrexate as described [44,45]. PTR1 activity was determined by two methods under optimal conditions of 10 μM H_2-biopterin as substrate and 100 μM NADPH as cofactor in 20 mM sodium acetate pH 4.7 [20]. First, time-dependent oxidation of NADPH was monitored spectrophotometrically as described [20,21]. Second, an HPLC-based method was developed, which follows H_4-biopterin formation by virtue of its differential sensitivity to alkaline treatment relative to the H_2-biopterin [46]. Typically, enzyme preparations were incubated with H_2-biopterin and NADPH in a total volume of 500 μl. At intervals (2–20 min) following initiation of the reaction, 100 μl aliquots were removed and the reaction terminated by addition of 10 μl of 0.1 M KI/I_2, plus either of 5 μl of 1 M HCl or 5 μl of 1 M NaOH. Samples were vortexed briefly and incubated for 1 h at 25°C in the dark; then, 10 μl of 1 M HCl was added to the alkaline samples and the precipitated proteins removed by centrifugation at $16\,000 \times g$ for 2 min. To the supernatants, 20 μl of 0.1 M ascorbate was added followed by neopterin as a loading control (2 μM final concentration) and the final volume adjusted to 200 μl with H_2O. Samples were separated by HPLC using an octadecylsilane column with 5% methanol as a mobile phase with a flow rate of 1 ml min^{-1}. Neopterin, biopterin and pterin were detected by fluorescence using a Waters 474 Scanning Fluorescence detector, excitation and emission wavelengths of 350 and 440 nm, respectively. Products were quantitated against standards whose concentrations were determined from published extinction coefficients: biopterin, $\varepsilon_{362\ nm} = 8.3 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1 M NaOH; H_2-biopterin, $\varepsilon_{330\ nm} = 6.2 \times 10^3\ M^{-1}\ cm^{-1}$ at pH 6.8; pterin, $\varepsilon_{358\ nm} = 7.3 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1 M NaOH; neopterin, $\varepsilon_{362\ nm} = 8.3 \times 10^3\ M^{-1}\ cm^{-1}$ in 0.1M NaOH [27,46]. Following alkaline treatment, H_4-biopterin and H_2-biopterin were detected as pterin and biopterin, respectively whereas following acid treatment both H_4- and H_2-biopterin were oxidized to biopterin [46]. Comparisons of total biopterin obtained after acid treatment agreed closely with the total pterin plus biopterin levels obtained after alkaline treatment, indicating that the recovery was quantitative.

2.7. Molecular biological methods

Genomic DNA was isolated from late logarithmic phase promastigotes by the LiCl method [47]. Southern blot analysis using 0.7% agarose gels and transfer to nylon membranes was performed as described [48]. Blots were hydridized overnight in Church buffer (1% BSA fraction V, 7% SDS, 1mM EDTA, 260 mM sodium phosphate pH 7.2) plus 100 μg ml^{-1} sheared salmon sperm DNA at 65°C as described [49]. All blots were washed twice with 2× SSC (1× SSC is 0.15 M sodium chloride, 15 mM sodium citrate pH 7.0), 0.1% SDS at room temperature for 5 min, twice at 65°C for 15 min in the same solution and, finally twice with 0.2× SSC, 0.1% SDS at 65°C for 15 min. DNA hybridization probes were made by random-priming using [α-^{32}P]-dCTP as described [50].

Total RNA was isolated using the phenol/guanidine isothyocyanate reagent TRIzol® (Gibco BRL) according to the manufacturers instructions. Typically RNA from 2×10^8 cells was extracted using 1 ml of reagent. RNA was separated using a 1% agarose/formaldehyde gel and transferred to nylon as described [48]. Blots were hydridized overnight in Church buffer at 65°C [49] and then washed twice with 2× SSC, 0.1% SDS at room temperature for 5 min followed by two washes at 65°C for 15 min in the same solution. *PTR1* and *DHFR-TS*-probed blots were subsequently washed twice with 0.2× SSC, 0.1% SDS at 65°C for 15 min. Relative intensities of hybridizing transcripts were determined by densitometry.

2.8. Cloning of the L. major Friedlin V1 BT1 gene

A *L. major* Friedlin V1 cosmid library made in the vector cLHYG [51] was probed with the *L. donovani BT1* gene and six overlapping cosmids were isolated.

From two *BT1*-containing cosmids (5.1.1 and 6.1.1, lab. strain numbers B4032 and B4033, respectively) the *L. major* BT1 gene was identified and sequenced.

3. Results

3.1. An HPLC-based assay for measurement of PTR1 activity in cell lysates

PTR1 but not DHFR-TS is able to reduce H_2-biopterin, potentially allowing the measurement of PTR1 and DHFR separately in crude parasite extracts [20]. However, the low levels of PTR1 activity in total cell extracts rendered the standard NADPH-based spectrophotometric method inadequate [20,21]. We developed a sensitive alternative assay, where the time-dependent formation of H_4-biopterin was monitored by HPLC. Following enzymatic synthesis, the product H_4-biopterin is converted to pterin under conditions of alkaline oxidation, whereas the substrate H_2-biopterin is oxidized to biopterin (Fig. 2A; Ref. [46]). These two forms are readily separated by HPLC and quantitated by fluorescence (Fig. 2B).

The HPLC assay was validated with purified *L. major* PTR1 [20]. With H_2-biopterin as substrate, purified PTR1 showed a time-dependent increase in pterin formation (derived from the product H_4-biopterin) and concomitant decrease in the amount of biopterin (derived from the substrate H_2-biopterin; Fig. 2B and C). Pterin formation was not observed following alkaline treatment if PTR1 was omitted (data not shown). Acid treatment of the samples, which yields biopterin from both oxidized and reduced biopterins, showed that the total biopterin levels were quantitatively equal to the sum of the pterin and biopterin under alkaline conditions (data not shown). Thus, the HPLC based method provided a faithful readout of H_4-biopterin levels. Significantly, the rates of pterin (H_4-biopterin) formation and biopterin (H_2-biopterin) loss were equivalent, as expected, and linear with time

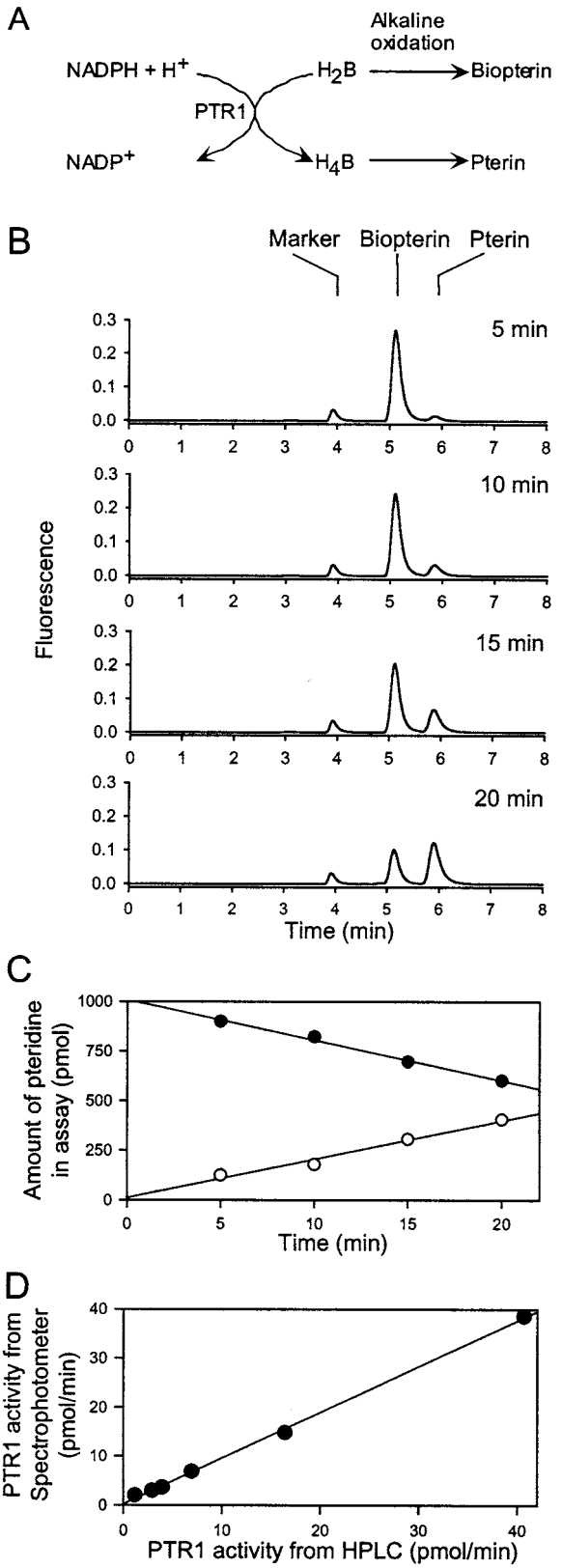

Fig. 2. (*Continued*)

Fig. 2. An HPLC method for measurement of PTR1 activity. (A) Scheme for the differential alkaline oxidation of H_2-biopterin (H_2B) and H_4-biopterin (H_4B), yielding biopterin or pterin respectively. (B) PTR1-dependent formation of pterin. Aliquots were removed from a PTR1 assay every 5 min, subjected to alkaline oxidation and analyzed by HPLC as described in Section 2. Bona fide standards were used to identify and quantitate the pteridines. (C) Time course of PTR1 activity. The decrease in substrate H_2-biopterin (detected as biopterin; ●) and the concomitant increase in the product H_4-biopterin (detected as pterin; ○) at various times following addition of PTR1 is shown. (D) Comparison of PTR1 activities measured by either the HPLC (*x*-axis) or spectrophotometric (*y*-axis) methods. In parts B–D, assays contained 100 μM NADPH, 10 μM H_2-biopterin, 20 mM sodium acetate pH 4.7, and purified *L. major* PTR1 (0.5 μg in parts B and C; 0.05–1 μg in part D), in a total volume of 500 μl.

(Fig. 2C) and added PTR1 (up to saturating values; data not shown).

These data suggested that the HPLC method was suitable for quantifying PTR1 activity. We compared the results of the HPLC assay with that of the spectrophotometric assay, using serial dilutions of purified *L. major* PTR1 (Fig. 2D). The two assays were in good agreement over the range of product formation of 1–40 pmol min^{-1}. Outside of this range the activities determined using the HPLC method deviates significantly from linearity (not shown). At the low end, the amounts of pterin formed approach the limits of detection and spontaneous oxidation of the H_4-biopterin becomes proportionately significant. At the high end, substrate depletion becomes a factor; in these circumstances, product formation can be measured over a shorter period of time.

We tested the performance of the HPLC assay with *Leishmania* crude extracts. Previously we reported the creation of null mutant parasites lacking PTR1 [20,21]. As expected, cell extracts (5 mg protein ml^{-1}) from the *L. major ptr1*$^{-}$ parasites showed no measurable H_2-biopterin reductase activity in the HPLC assay (not shown). We then added purified PTR1 in increasing concentrations to the *ptr1*$^{-}$ extract; H_2-biopterin reductase activity was observed and was quantitatively identical to that obtained with purified PTR1 alone indicating that the assay was unaffected by the presence of the cell extract (data not shown).

In previous studies, the spectrophotometric assay of PTR1 activity in crude lysates of wild-type, *ptr1*$^{-}$ and *PTR1*-overexpressing parasites yielded relative activities of 1 : 0.2 : 3.2 [21], while western blot analysis with anti-PTR1 antisera suggested a relative abundance of 1:0:100 [20]. In contrast, with the HPLC assay we determined PTR1 activity with these three lines to be 21, <2 (below the limit of detection) and 1480 pmol min^{-1} mg^{-1} soluble protein, or 1 : <0.1 : 70 (wild-type:*ptr1*$^{-}$:*PTR1*-overexpressor), in good agreement with the western blot values. Thus, the HPLC-based assay provides a sensitive and robust method for determining PTR1 activities in crude *Leishmania* extracts, without interference from other activities. It is now the preferred method and was employed in all following studies.

3.2. Growth-phase and developmental regulation of *PTR1*

In promastigotes from all species, the highest PTR1 activity was seen in logarithmically growing cells (10–180 pmol min^{-1} mg^{-1} soluble protein; Fig. 3A). The levels decreased somewhat in stationary phase cells, to approximately 70% of log phase values (Fig. 3A). Similar changes in protein levels were seen in western blot studies with *L. donovani* and *L. major* (data not shown; studies with *L. mexicana* were not feasible due to poor cross-reactivity of the anti-*L. major* PTR1 antibody with *L. mexicana* PTR1). We were able to obtain sufficient quantities of purified infective *L. major* metacyclic promastigote to permit PTR1 activity measurements, which showed values similar to total stationary phase cells (7.2 and 8.6 pmol min^{-1} mg^{-1}). Promastigote levels of PTR1 in *L. major* were 10 to 17-fold lower than observed in *L. donovani* and *L. mexicana*, respectively.

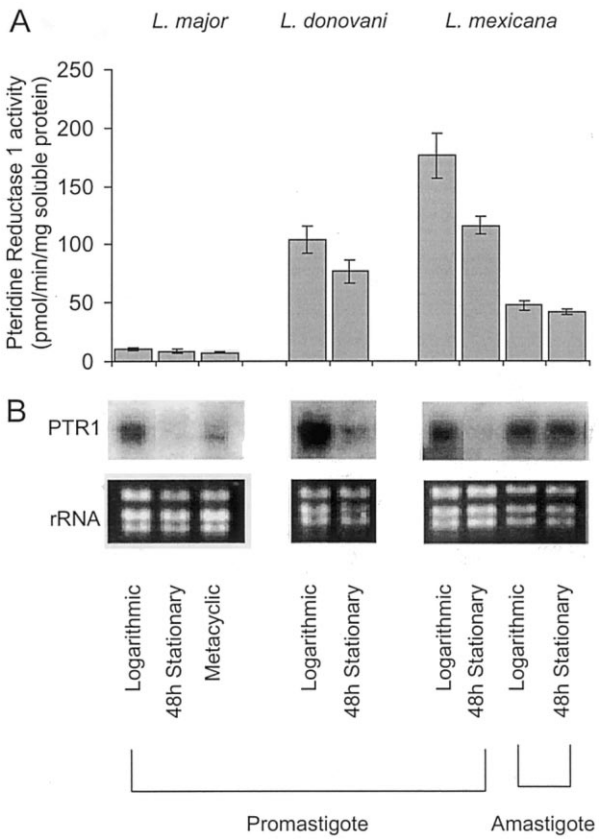

Fig. 3. Expression of PTR1 activity and mRNA during growth and development. (A) PTR1 activity was measured using the HPLC method. For each species and growth stage (indicated at the bottom of panel B), at least two independent cell extracts were examined, with multiple points falling within the linear range of the assay. The means and standard deviations are shown. (B) Northern blot analysis of RNAs from different stages and/or species of *Leishmania*. Five micrograms of total RNA from each sample was analyzed and hybridized with a *L. major* PTR1 full-length coding region probe; the ethidium bromide stained gel showing the three rRNAs (the large subunit rRNA of *Leishmania* known to be cleaved into two large fragments) is provided as a loading control.

Analysis of cultured *L. mexicana* amastigotes demonstrated for the first time the presence of PTR1 activity in this stage of the parasite, at levels about fourfold lower than in promastigotes. Unlike promastigotes, amastigote PTR1 levels did not decline in stationary phase (Fig. 3A).

3.3. Growth-phase and developmental regulation of DHFR-TS

The abundance of DHFR-TS in crude cell lysates was determined by stoichiometric binding of [^{3}H]-methotrexate [41,44,45]. All species contained similar levels of DHFR-TS in logarithmic phase promastigotes, ranging between 10 and 16 pmol mg^{-1} soluble protein. Levels in stationary phase were only slightly less than found in log phase (Fig. 4A). *L. mexicana* amastigotes expressed tenfold less DHFR-TS than promastigotes, and in both stages there was no significant difference in DHFR-TS levels between cells in the logarithmic stage and after 48 h in stationary phase. Overall these results are consistent with previous studies of DHFR-TS in *Leishmania* [4,20,45,52].

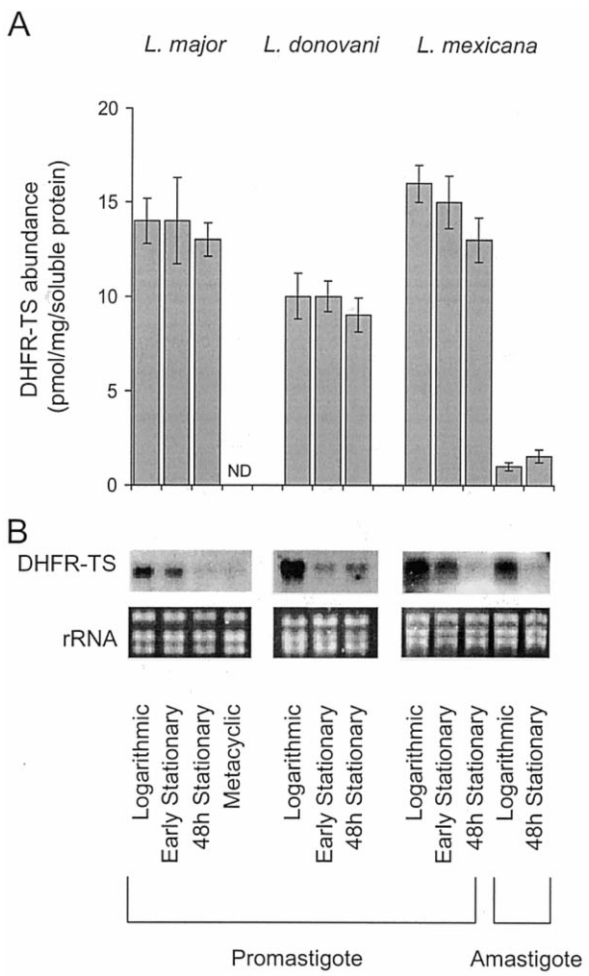

Fig. 4. Expression of DHFR-TS and mRNAs in growth and development. (A) The abundance of DHFR-TS was determined by quantitative binding of [^{3}H]-methotrexate as described in the methods. Values represent the average of two independent extracts each measured in duplicate. (B) Northern blot analysis of total RNA hybridized with the *L. major DHFR-TS* full-length coding region probe, as described in the legend to Fig. 3B. ND, not done.

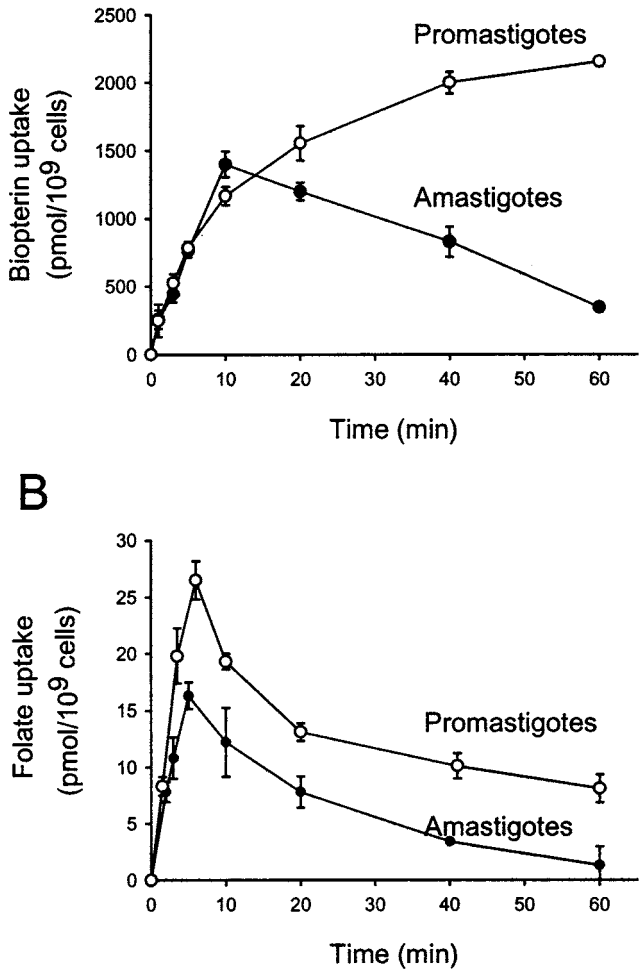

Fig. 5. Kinetics of biopterin and folate accumulation by *L. mexicana*. The net accumulation of (A) biopterin or (B) folate of logarithmic promastigotes (○) or logarithmic amastigotes (●) is shown (means and S.D.s of three samples each). External concentrations of biopterin and folate were 200 and 10 μM, respectively.

3.4. Kinetics of biopterin and folate uptake

The time-dependence of biopterin accumulation was examined in *L. mexicana*, *L. donovani* and *L. major*. Typical profiles for log-phase *L. mexicana* promastigotes and amastigotes are shown in Fig. 5A (similar data obtained with *L. donovani* and *L. major* promastigotes are not shown). Promastigotes rapidly accumulated [^{3}H]-biopterin, with linear kinetics over the first 5 min and reaching a plateau value after 60 min (Fig. 5A, open circles), as seen previously [3,9]. A different profile was observed with log-phase amastigotes, with an initial linear phase for the first 10 min, followed by a steady decrease to about 25% of the peak values (Fig. 5A, closed circles).

The time-dependence of folate accumulation was examined in *L. mexicana*, *L. donovani* and *L. major*. A typical profile for log-phase *L. mexicana* axenic amastigotes and promastigotes is shown in Fig. 5B

(similar data obtained with *L. donovani* and *L. major* promastigotes are not shown). As seen with biopterin, [^{3}H]-folate accumulation was rapid and accumulated with linear kinetics for the first 5 min, until a maximal concentration was attained. Subsequently, intracellular [^{3}H]-folate decreased until after 60 min, only 30 and 8% of the maximal levels remains for promastigotes and amastigotes, respectively.

Thus while promastigotes show simple uptake kinetics for biopterin, amastigotes with biopterin and both stages with folate exhibit more complicated profiles. Similar results were reported previously in murine leukemia cell L1210 [53] and *L. major* [41]. In murine cells this profile was attributed to the presence of radiolabeled breakdown products of folate, however, in *L. major* this was ruled out by carefully purifying all radiolabeled ligands [41]; similar procedures were followed here (Section 2). The source of this phenomenon is not known, and probably reflects some aspect of pteridine metabolism as yet uncharacterized [41]. As in previous studies, we used the initial linear uptake phase as a measure of transporter activity (Figs 6A, 7A). Previous studies of mutant promastigotes showed that all biopterin uptake activity can be attributed to the transporter encoded by *BT1* [8,9] (Cunningham and Beverley, unpublished data), while 99% of folate uptake in *L. donovani* can be attributed to the transporter encoded by *FT1* (Moore and Beverley, and Moore et al., manuscripts in preparation).

3.5. Growth phase and developmental regulation of pteridine accumulation

Log-phase promastigotes of all three species displayed similar rates of biopterin uptake, ranging between 94 and 210 pmol^{-1} min^{-1} 10^9 cells^{-1} (Fig. 6A). These values agree well with previous studies of *L. donovani* and *L. major* [3,8] (Moore et al., manuscript in preparation) and *L. tarentolae* (data not shown). As cells entered stationary phase biopterin uptake declined, to 16–27% of log-phase values after 48 h in stationary phase (Fig. 6A). Cultured *L. mexicana* amastigotes showed 30% higher rates of biopterin uptake than promastigotes. As with promastigotes, biopterin transport activity decreased in stationary phase amastigotes, albeit to a lesser extent (67% maximal; Fig. 6A).

Similar results were obtained with folate uptake in all three species. Log-phase promastigotes showed the highest rate of uptake, ranging from 6.2 to 23 pmol^{-1} min^{-1} 10^9 cells^{-1}. *L. major* and *L. donovani* exhibited rates of folate uptake about threefold higher than *L. mexicana* (Fig. 7A). As parasites entered stationary phase, the rate of folate uptake declined, to 10–22% of log-phase values (Fig. 7A). Log-phase *L. mexicana* amastigotes accumulated folate with rates comparable to that of promastigotes (5.4 pmol^{-1} min^{-1} 10^9 cells^{-1}; Fig. 7A), and as with biopterin, this rate declined by 30% in stationary phase amastigotes.

3.6. Regulation of BT1 RNA expression

The *L. major* Friedlin V1 *BT1* gene was cloned and sequenced. An open reading frame of 1893 bp was identified, predicting a BT1 protein of 631 residues which showed 9.3% and 11.8% amino acid difference with *L. donovani* and *L. mexicana* BT1, respectively [8–10]. Southern blot data showed that this gene was single copy in *L. major* and corresponded to the *BT1*

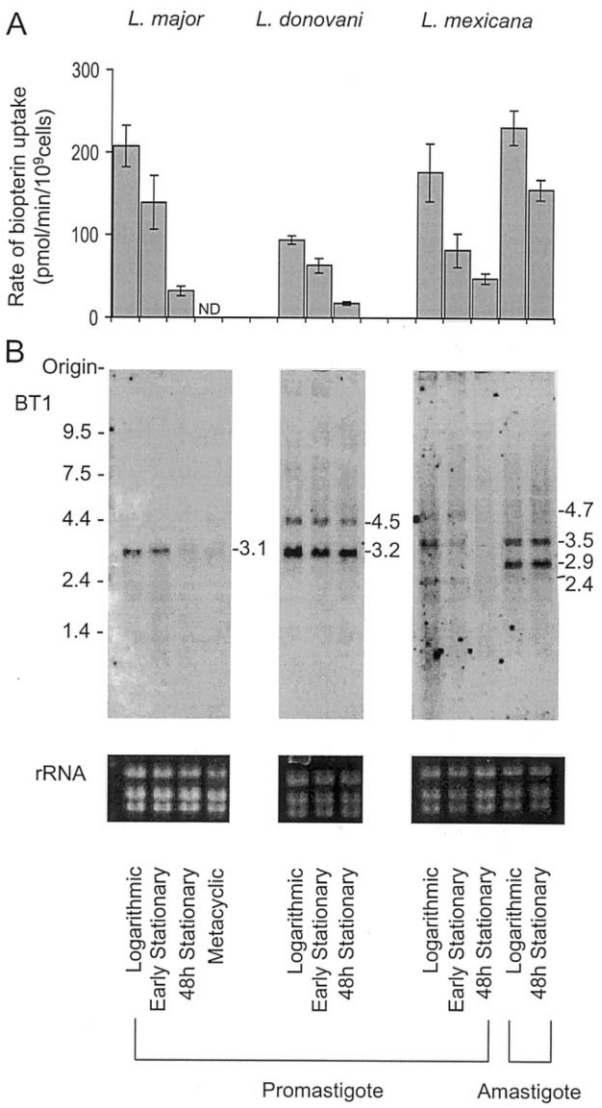

Fig. 6. Biopterin uptake and *BT1* RNA during growth and development. (A) The initial rate of biopterin uptake was measured as described in the methods. Each determination represents the average of triplicate determinations of three different experiments; the means and standard deviations are shown. The external biopterin concentration was 200 μM. ND, not done. (B) Northern blot analysis of total RNA hybridized with the *L. major BT1* full-length coding region probe, as described in the legend to Fig. 3B. RNA standards are shown to the left and *BT1* transcript sizes to the right.

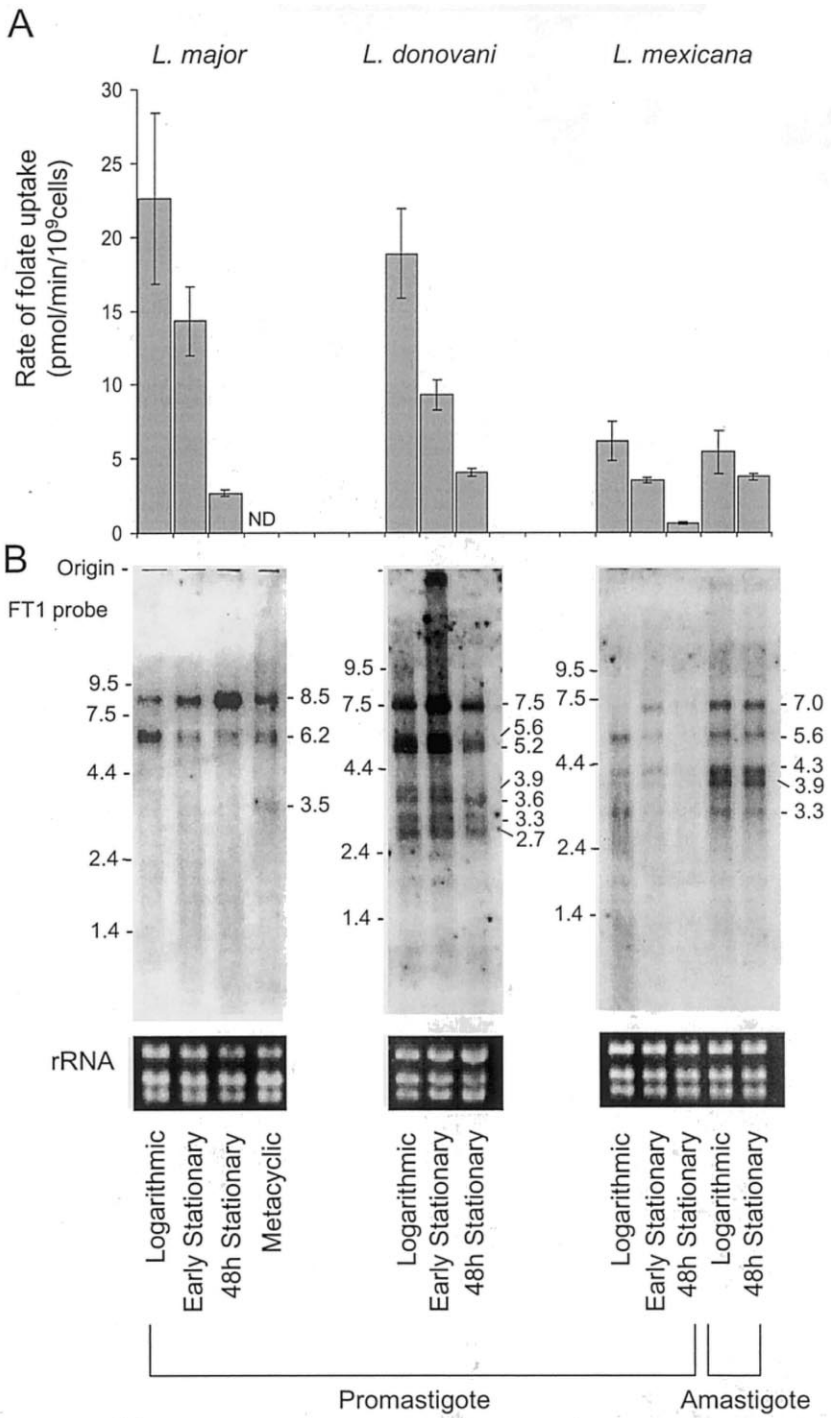

Fig. 7. Folate uptake and expression of FT1-related RNAs during growth and development. (A) The initial rate of folate uptake was measured as described in the methods. Each determination represents the average of triplicate determinations of three different experiments; the means and standard are deviations shown. The external folate concentration was 10 μM. ND, not done. (B) Northern blot analysis of total RNA hybridized with the *L. donovani* DI700 *FT1* full-length coding region probe, as described in the legend to Fig. 3B. RNA standards are shown to the left and transcript sizes to the right.

gene recently found on chromosome 35 (Fig. 8A; [75] in press). Similarly, a single copy of *BT1* was found in the strains of *L. mexicana* and *L. donovani* studied here, as described in other strains of these species (Fig. 8A; Refs. [8,9] (Moore et al., manuscript in preparation).

Northern blot analysis showed that while only a single *BT1* mRNA was evident in *L. major*, there were two in *L. donovani* and four in *L. mexicana* (Fig. 6B). Given that *BT1* is a single copy gene in all species, the multiple transcripts must be precursors or arise from

alternative RNA processing. Notably, in *L. mexicana* the 3.5 kb RNA was expressed in both stages, the 4.7 and 2.4 kb RNAs were predominately expressed in promastigotes, and the 2.9 kb RNA was restricted to amastigotes (Fig. 6B).

In *L. major* and *L. mexicana* promastigotes, the overall levels of BT1 RNA(s) followed biopterin (BT1) uptake activity, with log phase showing higher levels than stationary phase promastigotes (Fig. 6A, B). In contrast, *L. donovani* BT1 RNA showed little stage variation (Fig. 6B).

3.7. Regulation of FT1 family RNA expression

In *L. donovani*, there are at least six genes related to *FT1* present in a single genomic locus, although only

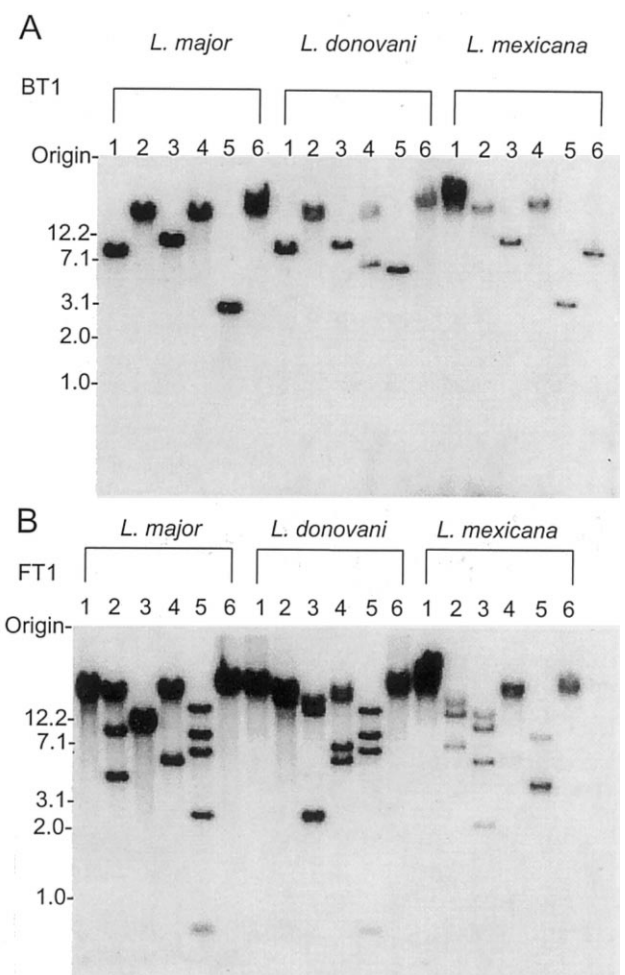

Fig. 8. Southern blot analysis of *L. major*, *L. donovani* and *L. mexicana* genomic DNA. Genomic DNA from the species indicated was digested with restriction enzymes and subjected to Southern blot hybridization. The enzymes used were: lane 1, *Eco*RI; lane 2, *Hin*dIII; lane 3, *Nhe*I; lane 4, *Not*I; lane 5, *Sac*I; and lane 6, *Spe*I. (A) *L. major* V1 *BT1* full-length coding region probe. (B) *L. donovani* DI700 *FT1* full-length coding region probe.

FT1 has been associated with folate transporter activity (Moore and Beverley, manuscript in preparation). While the structure of the *FT1* locus was not determined for *L. major* and *L. mexicana*, Southern blot analysis with six different enzymes suggested that multiple genes are present in these species as well (Fig. 8B). Since gene-specific probes were not available, we used an *L. donovani FT1* probe to assess the expression of transcripts arising from the *FT1* family.

The expression profiles of *FT1*-related RNAs was complex, with three to seven prominent transcripts evident in each species (Fig. 7B). These RNAs may be precursors or arise from alternative RNA processing, however we favor the possibility that they arise from different *FT* gene family members. Despite this complexity, several patterns of RNA expression were evident. Within promastigotes, most *FT1*-related RNAs decreased in abundance after 48 h in stationary phase relative to logarithmic phase (Fig. 7B). However, there were exceptions: in *L. major* an 8.5 kb RNA increased in stationary phase promastigotes while a 3.5 kb RNA was uniquely detected in metacyclic promastigotes (Fig. 7B). Several RNAs showed little or no changes in abundance between log and stationary phase *L. donovani* (2.7, 3.3 and 3.6 kb; Fig. 7B).

Comparisons of *L. mexicana* promastigotes and amastigotes showed that most of the *FT1*-related transcripts detected in promastigotes were shared by amastigotes as well. However, three transcripts (7.0, 4.3 and 3.9 kb) were up-regulated, in some cases nearly tenfold more abundant than in log-phase promastigotes. There was little or no change in *FT1*-related RNA abundance between logarithmic and stationary phase amastigotes (Fig. 7B). Thus, *FT1*-related RNA expression is complex, and likely to involve differential expression and/or processing of individual gene family members.

3.8. Regulation of PTR1 and DHFR-TS mRNA expression

PTR1 is a single copy gene and a single mRNA of 1.5–1.6 kb was detected for all species examined (Fig. 3B) [24,25]. In contrast to PTR1 activity, the abundance of *PTR1* mRNA decreased eight to 13-fold after 48 h in stationary phase in all three species (Fig. 3B), although purified metacyclic stage *L. major* showed *PTR1* mRNA levels closer to log-phase promastigotes (Fig. 3B). Lastly, *PTR1* mRNAs in logarithmic and stationary phase *L. mexicana* amastigotes were equally abundant, and similar to the levels in logarithmic promastigotes (Fig. 3B).

DHFR-TS is a single copy gene, and a single 3.1–3.3 kb mRNA was identified in Northern blot analysis of all species (Fig. 4B) [14]. Maximal mRNA levels were detected in logarithmic promastigotes, which decreased

between six and 20-fold after 48 h in stationary phase. Expression of *DHFR-TS* mRNA in amastigotes resembled promastigotes, with maximal levels being present in logarithmic growing cells and decreasing sixfold after 48 h in stationary phase. Thus, as with PTR1, the decrease in mRNA during promastigotes growth is not reflected by equally large decreases in protein abundance. Furthermore, whilst the mRNA levels in logarithmic amastigotes is approximately 60% of that seen in logarithmic promastigotes, the abundance of protein is tenfold lower.

4. Discussion

Nutritional, biochemical and genetic studies show that both conjugated (folates) and unconjugated (biopterin) pteridines are essential for *Leishmania* growth [3–7,17,20,21,54]. Fortunately for the parasite, both its insect and mammalian hosts have well-developed pathways for the synthesis of unconjugated pterins de novo [27] and/or for the recovery and utilization of folates from the diet [11,12]. Thus, throughout the infectious cycle *Leishmania* has only to solve the task of exploiting the pteridine resources available within its hosts. Correspondingly, our data show that *Leishmania* expresses all known activities required for pteridine salvage and utilization throughout its infectious cycle.

4.1. Pteridine uptake is regulated by growth phase but not development

The first step in pteridine salvage is uptake (Fig. 1). Our data show that both biopterin and folate uptake occurs at high levels in the amastigote stage of *L. mexicana*, comparable to that observed in the promastigote stage (Figs. 6 and 7) [3,8,9,41,55] (Moore et al., and Moore and Beverley, manuscripts in preparation). Since amastigotes normally reside within a parasitophorous vacuole within vertebrate macrophages, we infer that both transporters must act within this cellular compartment during a natural infection. BT1 is the only biopterin transporter known in *Leishmania* (or any other organism currently), and is likely to be responsible for biopterin uptake in vivo. In contrast, *Leishmania* possess a family of transporters related to FT1 (Moore and Beverley, manuscript in preparation), and activity and Northern blot data suggest that one or more members of this family are acting in the amastigote and metacyclic stages (Fig. 7B). In other *Leishmania* gene families, diversity in the structure, activity and regulation of individual members has been observed (such as the glucose transporters and the surface protease gp63 [56,57]). Resolution of this question for the *FT1* gene family awaits the development and application of gene-specific probes, and/or the genetic disruption of the other *FT1*-related genes and tests within the amastigote stage.

Folate and biopterin transport activities are similar in promastigotes and amastigotes (Figs 6A, 7A). In contrast, these activities decline substantially as the dividing parasites enter stationary phase (Figs 6A, 7A) [41]. This effect is strongest in the promastigote stage, where on average an 80% and 85% decrease in BT1 and FT1 activity was observed, respectively. Thus it is evident that *Leishmania* is able to differentially regulate pteridine uptake in response to growth phase and developmental signals. Notably, cessation of growth in *Leishmania* acts as a trigger for differentiation into the infective metacyclic promastigote phase of the parasite [58]. Although the role of biopterin in parasite growth is unknown, down-regulation of folate transporter activity may be associated with the formation of folate polyglutamates which are efficiently retained within the cell [59], and/or the cessation of growth and DNA synthesis.

4.2. Alterations in pteridine uptake associated with adaptation to in vitro culture

While the rate of folate accumulation determined here with *L. donovani* agrees well with previous studies [3], the rate of folate uptake observed in the highly virulent *L. major* strain Friedlin V1 was more than tenfold less than observed previously with another strain, CC1 (23 versus 270 $pmol^{-1}$ min^{-1} 10^9 $cells^{-1}$ [41]). We repeated the studies of *L. major* CC-1 and confirmed the original finding (data not shown). Previous studies have noted differences in antifolate sensitivity and folate pathways between the fully virulent strains of *L. major* (Friedlin V1 and LV39) and attenuated derivatives thereof [60,61], and *L. major* strain CC-1 is itself somewhat less virulent in mouse infections [18]. Similarly, changes in biopterin uptake and *BT1* expression have been associated with adaptation to in vitro culture, due to amplification and/or rearrangements of the *BT1* gene in *L. donovani* [8,10] and *L. major* (Moore et al., manuscript in preparation), a process distinct from alterations seen in methotrexate-selected *L. tarentolae* [9]. These findings underscore the importance that the *Leishmania* parasite places on the maintenance of sufficient intracellular pteridine levels. Moreover, it establishes the need to focus on virulent strains when investigating the role of pteridine metabolism in the natural infectious cycle.

4.3. PTR1 and DHFR-TS are expressed throughout the infectious life cycle

Following entry into the cell, biopterin and folate must be reduced to the active H_4-forms. Biopterin

reduction is carried out exclusively by PTR1, while folate reduction in promastigotes is mediated predominantly by DHFR-TS and to a lesser extent by PTR1 (Fig. 1) [20,22,62]. We found it necessary to develop a more sensitive assay for PTR1 activity that would be free from interference by contaminating activities present in parasite crude extracts. An HPLC-based method was developed, validated, and shown to be superior to the spectrophotometric method used previously by several criteria, including sensitivity and freedom from interference in crude lysates.

During development, DHFR-TS levels in *L. mexicana* amastigotes declined considerably relative to promastigotes, decreasing tenfold (Fig. 4A), comparable to the sixfold decline reported previously in lesion *L. major* amastigotes [45]. In contrast, PTR1 levels declined only threefold in *L. mexicana* amastigotes relative to logarithmic promastigotes (Fig. 3A). The potential implications for chemotherapy are discussed later. In contrast to *L. mexicana*, amastigotes of the related trypanosomatid parasite *Trypanosoma cruzi* do not express PTR1 [63]. This may be related to fact that *T. cruzi* amastigotes reside within the cytoplasm and thus have free access to reduced biopterin in the cytoplasm, unlike *Leishmania* amastigotes which reside within a membrane-enclosed vacuole.

Neither PTR1 nor DHFR-TS showed substantial reductions in expression related to growth phase, in promastigote or amastigotes (Figs 3A, 4A). Overall, our findings indicate that pteridine uptake activities appear to be regulated more strongly by growth phase than development, while pteridine reductase levels are more strongly affected by development than growth phase.

4.4. Post-transcriptional mechanisms play a major role in regulation of pteridine salvage activities

As the first step towards understanding the genetic regulatory mechanisms controlling pteridine salvage, we examined RNA levels during the growth cycle and development of *Leishmania*. In some cases, a reasonable correlation between changes in activity and RNA level were observed (*BT1* in *L. major* and *L. mexicana*; possibly *FT1* in *L. mexicana*). More commonly and somewhat unexpectedly, RNA and activity levels were not strongly correlated. In some cases, activity remained high while RNA levels declined (stationary phase promastigote PTR1 and DHFR-TS levels in all species), while in others activity declined while RNA levels remained high (FT in *L. major* and *L. donovani*). Remarkably, the pattern of RNA/activity regulation showed variation even between species, often while the pattern of activity was conserved (*BT1* in *L. donovani*, relative to *L. major* and *L. mexicana*).

Leishmania, like other trypanosomatid parasites, employ a polycistronic transcriptional mechanism in which large precursors are processed by coupled 5′ trans-splicing and 3′ polyadenylation to form mature monocistronic RNAs [64–66]. Consequently, post-transcriptional mechanisms of gene regulation must play a larger role in general [66], and perhaps this is responsible for the diversity of regulatory patterns seen in the pteridine salvage pathway of *Leishmania*. Several studies have stressed the importance of both translational regulation and protein turn-over (for example, Refs. [67,68]), and these could respectively account for the 'high RNA/low protein' and 'high protein/low RNA' patterns seen in pteridine salvage regulatory patterns noted above. While post-translational modification of proteins may also play a role, preliminary data with anti-PTR1 antisera suggested that protein and activity levels were in good agreement. Interestingly, the monofunctional DHFR and TS from mammals have been shown to bind their respective mRNAs and inhibit translation, suggesting another potential mode of regulation [69,70].

In total, these data provide evidence for an unexpected complexity in the mechanisms employed to regulate pteridine salvage at both the RNA and protein levels, amongst genes and amongst different pathogenic *Leishmania*. These pathways will provide a rich grounds for future studies of regulatory mechanisms and diversity amongst parasite species that seemingly live within similar evolutionary niches. Lastly, it suggests that the understanding of the role of these pathways in parasite metabolism will depend heavily upon direct measurement of relevant enzymatic/transporter activities, rather than RNA levels.

4.5. Implications for chemotherapy

In previous studies of the promastigote stage of *L. major*, we hypothesized that PTR1 expression could provide a potential 'metabolic by-pass' of DHFR-TS inhibition, allowing a partial or complete reversal of anti-pteridine inhibition depending upon the relative level of PTR1 expression [20,21]. This is due primarily to the insensitivity of PTR1 to anti-pteridines relative to DHFR, permitting folates to be reduced even under conditions of DHFR inhibition [20,21,71]. Our data extend the PTR1 by-pass model to other pathogenic strains and also to the chemotherapeutically relevant amastigote stage. Firstly, the PTR1 activity in *L. major* promastigotes is 10 to 17-fold lower than in *L. donovani* and *L. mexicana*, and *L. major* is considerably more sensitive to methotrexate. This suggests that the role of PTR1 as a metabolic-bypass is likely to be even greater in *L. donovani* and *L. mexicana* than *L.major* [6,72]. Secondly, the data presented here also show that cultured *L. mexicana* amastigotes express high levels of

PTR1, much higher than observed previously in *L. major* promastigotes (Fig. 3A), again providing ample evidence for the presence of the PTR1 'by-pass' pathway in amastigotes. Moreover, DHFR-TS levels are lower in *L. mexicana* amastigotes relative to promastigotes (Fig. 4A), suggesting that if anything the PTR1-dependent effects will be exacerbated relative to that seen in promastigotes. No PTR1-like activity has been detected in the other protozoa for which anti-folate based chemotherapy has proven successful.

Thus, in order to be effective, anti-folate chemotherapy targeting the macrophage-resident amastigote stage of *Leishmania* will need to take into account the ability of PTR1 to by-pass inhibitors targeted against DHFR-TS. One possibility is the identification of compounds able to inhibit both PTR1 and DHFR-TS simultaneously, or separately. Preliminary studies have identified several lead compounds which inhibit both PTR1 and DHFR enzymes in vitro, and inhibit promastigote growth at sub-micromolar concentrations [71], and one compound inhibited the growth of *L. major* amastigotes within cultured macrophages (Nare B, Beverley SM, unpublished observation). The three-dimensional structures of both *L. major* DHFR-TS and PTR1 have now been solved, and in combination with the structure of the human DHFR, will facilitate the design of new inhibitors selective for parasite but not host enzymes [73,74]. Another possibility involves blocking folate and/or biopterin uptake, through inhibition of the BT1 and/or FT1 transporters, perhaps in conjunction with anti-pteridine reductase inhibitors.

In summary, our studies present a comprehensive picture of the pteridine salvage pathway, across the growth and developmental stages of *Leishmania*. The developmental changes observed pose intriguing questions about the roles of pteridines in the complex life cycle of this parasite and the regulatory mechanisms underlying their control. Furthermore, the demonstration that amastigotes possess an extensive pteridine salvage pathway comparable to that of promastigotes has profound implications for anti-folate based chemotherapy, and may explain the failure to successfully exploit this essential pathway.

Acknowledgements

We thank D. Dobson, T. Ellenberger, F. Gueiros-Filho, L.-F. Lye, J. Moore, B. Nare, K. Zhang, and D. Zilberstein for advice, discussions, permission to mention unpublished data, and comments on this manuscript. Supported by NIH grants AI21903 and AI29646.

References

[1] WHO Expert Committee. The Leishmaniasis. Geneva: WHO, 1984.
[2] Jackson JE, Tally JD, Ellis WY, et al. Quantitative in vitro drug potency and drug susceptibility evaluation of *Leishmania* spp. from patients unresponsive to pentavalent antimony therapy. Am J Trop Med Hyg 1990;43:464–80.
[3] Beck JT, Ullman B. Nutritional requirements of wild-type and folate transport-deficient *Leishmania donovani* for pterins and folates. Mol Biochem Parasitol 1990;43:221–30.
[4] Scott DA, Coombs GH, Sanderson BE. Folate utilisation by *Leishmania* species and the identification of intracellular derivatives and folate-metabolising enzymes. Mol Biochem Parasitol 1987;23:139–49.
[5] Peixoto MP, Beverley SM. In vitro activity of sulfonamides and sulfones against *Leishmania major* promastigotes. Antimicrob Agents Chemother 1987;31:1575–8.
[6] Kaur K, Coons T, Emmett K, Ullman B. Methotrexate-resistant *Leishmania donovani* genetically deficient in the folate-methotrexate transporter. J Biol Chem 1988;263:7020–8.
[7] Trager W. Pteridine requirement of the hemoflagellate *Leishmania tarentolae*. J Protozool 1969;16:372–5.
[8] Lemley C, Yan S, Dole VS, et al. The *Leishmania donovani* LD1 locus gene ORFG encodes a biopterin transporter (BT1). Mol Biochem Parasitol 1999;104:93–105.
[9] Kundig C, Haimeur A, Legare D, Papadopoulou B, Ouellette M. Increased transport of pteridines compensates for mutations in the high affinity folate transporter and contributes to methotrexate resistance in the protozoan parasite *Leishmania tarentolae*. EMBO J 1999;18:2342–51.
[10] Myler PJ, Lodes MJ, Merlin G, de Vos T, Stuart KD. An amplified DNA element in *Leishmania* encodes potential integral membrane and nucleotide-binding proteins. Mol Biochem Parasitol 1994;66:11–20.
[11] Antony AC. The biological chemistry of folate receptors. Blood 1992;79:2807–20.
[12] Sirotnak FM, Tolner B. Carrier-mediated membrane transport of folates in mammalian cells [In Process Citation]. Annu Rev Nutr 1999;19:91–122.
[13] Blakley RL, Benkovic SJ. Chemistry and biochemistry of folates. In: Folates and Pterins, vol. 1. New York: John Wiley, 1984.
[14] Beverley SM, Ellenberger TE, Cordingley JS. Primary structure of the gene encoding the bifunctional dihydrofolate reductase–thymidylate synthase of *Leishmania major*. Proc Natl Acad Sci USA 1986;83:2584–8.
[15] Ferone R, Roland S. Dihydrofolate reductase: thymidylate synthase, a bifunctional polypeptide from *Crithidia fasciculata*. Proc Natl Acad Sci USA 1980;77:5802–6.
[16] Ivanetich KM, Santi DV. Dihydrofolate reductase–thymidylate synthase in protozoa. Exp Parasitol 1990;70:367–71.
[17] Cruz A, Beverley SM. Gene replacement in parasitic protozoa. Nature 1990;348:171–3.
[18] Titus RG, Gueiros-Filho FJ, de Freitas LA, Beverley SM. Development of a safe live *Leishmania* vaccine line by gene replacement. Proc Natl Acad Sci USA 1995;92:10267–71.
[19] Thony B, Auerbach G, Blau N. Tetrahydrobiopterin biosynthesis, regeneration and functions. Biochem J 2000;347:1–16.
[20] Nare B, Hardy LW, Beverley SM. The roles of pteridine reductase 1 and dihydrofolate reductase–thymidylate synthase in pteridine metabolism in the protozoan parasite *Leishmania major*. J Biol Chem 1997;272:13883–91.
[21] Bello AR, Nare B, Freedman D, Hardy L, Beverley SM. PTR1: a reductase mediating salvage of oxidized pteridines and methotrexate resistance in the protozoan parasite *Leishmania major*. Proc Natl Acad Sci USA 1994;91:11442–6.

[22] Wang J, Leblanc E, Chang CF, et al. Pterin and folate reduction by the *Leishmania tarentolae* H locus short-chain dehydrogenase/reductase PTR1. Arch Biochem Biophys 1997;342:197–202.
[23] Luba J, Nare B, Liang PH, Anderson KS, Beverley SM, Hardy LW. *Leishmania major* pteridine reductase 1 belongs to the short chain dehydrogenase family: stereochemical and kinetic evidence. Biochemistry 1998;37:4093–104.
[24] Callahan HL, Beverley SM. A member of the aldoketo reductase family confers methotrexate resistance in *Leishmania*. J Biol Chem 1992;267:24165–8.
[25] Papadopoulou B, Roy G, Ouellette M. A novel antifolate resistance gene on the amplified H circle of *Leishmania*. EMBO J 1992;11:3601–8.
[26] Whiteley JM, Xuong NH, Varughese KI. Is dihydropteridine reductase an anomalous dihydrofolate reductase, a flavin-like enzyme, or a short-chain dehydrogenase? Adv Exp Med Biol 1993;338:115–21.
[27] Blakley R, Benkovic SJ. Chemistry and biochemistry of pterins. In: Folates and Pterins, vol. 2. New York: John Wiley, 1985.
[28] Tietz A, Lindberg M, Kennedy EP. A new pteridine-requiring enzyme system for the oxidation of glyceryl ethers. J Biol Chem 1964;239:4081–90.
[29] Tayeh MA, Marletta MA. Macrophage oxidation of L-arginine to nitric oxide, nitrite and nitrate. Tetrahydrobiopterin is required as a cofactor. J Biol Chem 1989;264:19654–8.
[30] Kwon N, Nathan CF, Stuehr D. Reduced biopterin as a cofactor in the generation of nitrogen oxides by murine macrophages. J Biol Chem 1989;264:20496–501.
[31] Peters W, Richards WHG. Antimalarial Drugs. Berlin, New York: Springer-Verlag, 1984.
[32] Long PL. The Biology of the Coccidia. Baltimore: University Park Press, 1982.
[33] Grossman PL, Remington JS. The effect of trimethoprim and sulfamethoxazole on *Toxoplasma gondii* in vitro and in vivo. Am J Trop Med Hyg 1979;28:445–55.
[34] Neal RA, Croft SL. An in-vitro system for determining the activity of compounds against the intracellular amastigote form of *Leishmania donovani*. J Antimicrob Chemother 1984;14:463–75.
[35] Mattock NM, Peters W. The experimental chemotherapy of leishmaniasis. II. The activity in tissue culture of some antiparasitic and antimicrobial compounds in clinical use. Ann Trop Med Parasitol 1975;69:359–71.
[36] Abdi YA. Handbook of Drugs for Tropical Parasitic Infections, second ed. London; Bristol, PA: Taylor & Francis, 1995.
[37] Nare B, Luba J, Hardy LW, Beverley S. New approaches to *Leishmania* chemotherapy: pteridine reductase 1 (PTR1) as a target and modulator of antifolate sensitivity. Parasitology 1997;114:S101–10.
[38] Kapler GM, Coburn CM, Beverley SM. Stable transfection of the human parasite *Leishmania major* delineates a 30-kilobase region sufficient for extrachromosomal replication and expression. Mol Cell Biol 1990;10:1084–94.
[39] Pan AA. *Leishmania mexicana*: serial cultivation of intracellular stages in a cell-free medium. Exp Parasitol 1984;58:72–80.
[40] Sacks DL, da Silva RP. The generation of infective stage *Leishmania major* promastigotes is associated with the cell-surface expression and release of a developmentally regulated glycolipid. J Immunol 1987;139:3099–106.
[41] Ellenberger TE, Beverley SM. Biochemistry and regulation of folate and methotrexate transport in *Leishmania major*. J Biol Chem 1987;262:10053–8.
[42] Meek TD, Garvey EP, Santi DV. Purification and characterization of the bifunctional thymidylate synthetase–dihydrofolate reductase from methotrexate-resistant *Leishmania tropica*. Biochemistry 1985;24:678–86.
[43] Aronow B, Kaur K, McCartan K, Ullman B. Two high affinity nucleoside transporters in *Leishmania donovani*. Mol Biochem Parasitol 1987;22:29–37.
[44] Haber DA, Beverley SM, Kiely ML, Schimke RT. Properties of an altered dihydrofolate reductase encoded by amplified genes in cultured mouse fibroblasts. J Biol Chem 1981;256:9501–10.
[45] Kapler GM, Beverley SM. Transcriptional mapping of the amplified region encoding the dihydrofolate reductase–thymidylate synthase of *Leishmania major* reveals a high density of transcripts, including overlapping and antisense RNAs. Mol Cell Biol 1989;9:3959–72.
[46] Fukushima T, Nixon JC. Analysis of reduced forms of biopterin in biological tissues and fluids. Anal Biochem 1980;102:176–88.
[47] Medina-Acosta E, Cross GA. Rapid isolation of DNA from trypanosomatid protozoa using a simple 'mini-prep' procedure. Mol Biochem Parasitol 1993;59:327–9.
[48] Sambrook J, Fritsch EF, Maniatis T. MolecularCloning: A Laboratory Manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press, 1989:1–1000.
[49] Church GM, Gilbert W. Genomic sequencing. Proc Natl Acad Sci USA 1984;81:1991–5.
[50] Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal Biochem 1983;132:6–13.
[51] Ryan KA, Dasgupta S, Beverley SM. Shuttle cosmid vectors for the trypanosomatid parasite *Leishmania*. Gene 1993;131:145–50.
[52] Kundig C, Leblanc E, Papadopoulou B, Ouellette M. Role of the locus and of the resistance gene on gene amplification frequency in methotrexate resistant *Leishmania tarentolae*. Nucl Acids Res 1999;27:3653–9.
[53] Henderson GB, Suresh MR, Vitols KS, Huennekens FM. Transport of folate compounds in L1210 cells: kinetic evidence that folate influx proceeds via the high-affinity transport system for 5-methyltetrahydrofolate and methotrexate. Cancer Res 1986;46:1639–43.
[54] Cruz A, Coburn CM, Beverley SM. Double targeted gene replacement for creating null mutants. Proc Natl Acad Sci USA 1991;88:7170–4.
[55] Beck J, Ullman B. Characterization of a mutant clone of *Leishmania donovani* deficient in folate transport. Adv Exp Med Biol 1989:533–538.
[56] McCoy JJ, Beetham JK, Ochs DE, Donelson JE, Wilson ME. Regulatory sequences and a novel gene in the msp (GP63) gene cluster of *Leishmania chagasi*. Mol Biochem Parasitol 1998;95:251–65.
[57] Burchmore RJ, Landfear SM. Differential regulation of multiple glucose transporter genes in *Leishmania mexicana*. J Biol Chem 1998;273:29118–26.
[58] Sacks DL, Perkins PV. Identification of an infective stage of *Leishmania* promastigotes. Science 1984;223:1417–9.
[59] Santi DV, Nolan P, Shane B. Folylpolyglutamates in *Leishmania major*. Biochem Biophys Res Commun 1987;146:1089–92.
[60] Cotrim PC, Garrity LK, Beverley SM. Isolation of genes mediating resistance to inhibitors of nucleoside and ergosterol metabolism in *Leishmania* by overexpression/selection. J Biol Chem 1999;274:37723–30.
[61] Cruz AK, Titus R, Beverley SM. Plasticity in chromosome number and testing of essential genes in *Leishmania* by targeting. Proc Natl Acad Sci USA 1993;90:1599–603.
[62] Papadopoulou B, Roy G, Mourad W, Leblanc E, Ouellette M. Changes in folate and pterin metabolism after disruption of the *Leishmania* H locus short chain dehydrogenase gene. J Biol Chem 1994;269:7310–5.
[63] Robello C, Navarro P, Castanys S, Gamarro F. A pteridine reductase gene ptr1 contiguous to a P-glycoprotein confers resistance to antifolates in *Trypanosoma cruzi*. Mol Biochem Parasitol 1997;90:525–35.

[64] Ullu E, Tschudi C, Gunzl A. *Trans*-splicing in trypanosomatid protozoa. In: Smith DF, Parsons M, editors. Molecular Biology of Parasitic Protozoa. Oxford: IRL Press, 1996:115–33.
[65] LeBowitz JH, Smith HQ, Rusche L, Beverley SM. Coupling of poly(A) site selection and trans-splicing in *Leishmania*. Genes Develop 1993;7:996–1007.
[66] Stiles JK, Hicock PI, Shah PH, Meade JC. Genomic organization, transcription, splicing and gene regulation in *Leishmania*. Ann Trop Med Parasitol 1999;93:781–807.
[67] Gonzalez J, Ramalho-Pinto FJ, Frevert U, et al. Proteasome activity is required for the stage-specific transformation of a protozoan parasite. J Exp Med 1996;184:1909–18.
[68] Fong D, Chang KP. Tubulin biosynthesis in the developmental cycle of a parasitic protozoan, *Leishmania mexicana*: changes during differentiation of motile and nonmotile stages. Proc Natl Acad Sci USA 1981;78:7624–8.
[69] Chu E, Koeller DM, Casey JL, et al. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. Proc Natl Acad Sci USA 1991;88:8977–81.
[70] Ercikan-Abali EA, Banerjee D, Waltham MC, Skacel N, Scotto KW, Bertino JR. Dihydrofolate reductase protein inhibits its own translation by binding to dihydrofolate reductase mRNA sequences within the coding region. Biochemistry 1997;36:12317–22.
[71] Hardy LW, Matthews W, Nare B, Beverley SM. Biochemical and genetic tests for inhibitors of *Leishmania* pteridine pathways. Exp Parasitol 1997;87:157–69.
[72] Scott DA, Coombs GH, Sanderson BE. Effects of methotrexate and other antifolates on the growth and dihydrofolate reductase activity of *Leishmania* promastigotes. Biochem Pharmacol 1987;36:2043–5.
[73] Gourley DG, Luba J, Hardy LW, Beverley SM, Hunter WN. Crystallization of recombinant *Leishmania major* pteridine reductase 1 (PTR1) [In Process Citation]. Acta Crystallogr D Biol Crystallogr 1999;55:1608–10.
[74] Knighton DR, Kan CC, Howland E, et al. Structure of and kinetic channelling in bifunctional dihydrofolate reductase–thymidylate synthase. Nat Struct Biol 1994;1:186–94.
[75] Sunkin SM, McDonagh P, Cunningham MH, Beverley SM, Stuart K, Myler PJ. Conservation of the LD1 region in Leishmania includes DNA implicated in LD1 amplification. Mol Biochem Parasitol 2001;113:315–21.