Regulation of Differentiation to the Infective Stage of the Protozoan Parasite *Leishmania major* by Tetrahydrobiopterin

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A critical step in the infectious cycle of *Leishmania* is the differentiation of parasites within the sand fly vector to the highly infective metacyclic promastigote stage. Here, we establish tetrahydrobiopterin (H_4B) levels as an important factor controlling the extent of metacyclogenesis. H_4B levels decline substantially during normal development, and genetic or nutritional manipulations showed that low H_4B caused elevated metacyclogenesis. Mutants lacking pteridine reductase 1 (PTR1) had low levels of H_4B, remained infectious to mice, and induced larger cutaneous lesions (hypervirulence). Thus, the control of pteridine metabolism has relevance to the mechanism of *Leishmania* differentiation and the limitation of virulence during evolution.

During its infectious cycle, the human pathogenic protozoan *Leishmania* spp. alternates between flagellated promastigote forms, which grow within the alimentary tract of the sand fly vector, and aflagellate amastigotes, which replicate within acidified phagolysosomes of vertebrate host macrophages. A critical step is metacyclogenesis, the differentiation of the noninfective procyclic promastigote to the highly infective metacyclic form within the sand fly (*1*). Several genes showing metacyclic-specific expression have been identified (*2*); however, the molecular mechanism(s) underlying the initiation and control of this vital step remain undefined. Here, we identify decreases in intracellular H_4B levels as an important factor controlling the extent of metacyclogenesis.

Leishmania spp., in contrast to their mammalian and sand fly hosts, are pteridine auxotrophs (*3*). Consequently, these parasites use a versatile pteridine salvage network comprising transporters with specificity for folate and biopterin [FT1 and BT1, respectively (*4–7*)]. Once internalized, two pteridine reductases, one specific for folate (dihydrofolate reductase–thymidylate synthase or DHFR-TS) and a second with broader specificity (pteridine reductase 1 or PTR1), respectively reduce folate and biopterin into the active forms, tetrahydrofolate and H_4B (*3, 8–10*). Both DHFR-TS and PTR1 are essential for promastigote growth in vitro, and DHFR-TS is essential in vivo (*9, 11–13*).

We investigated whether *L. major* parasites lacking PTR1 [*ptr1*$^-$, obtained by gene-knockout methodology (*9*)] were able to induce infections in mice. After promastigotes were grown to stationary phase (as occurs normally in parasite development in the sand fly and is required for metacyclogenesis), they were inoculated into the footpads of BALB/c mice. H_4B is synthesized de novo in mammals, occurs in serum at levels able to support parasite growth in vitro, and is abundant in macrophages (*9, 14, 15*); for these reasons, we anticipated that the *ptr1*$^-$ parasites might be rescued by salvage of host H_4B. Remarkably, the *ptr1*$^-$ parasite population showed increased virulence, as judged by the rate of lesion formation (Fig. 1A). This arose from loss of PTR1 alone, as reintroduction of *PTR1* restored lesion progression to wild-type levels (Fig. 1A). Increased *ptr1*$^-$ lesion formation reflected increased parasite burden; 13 days after infection *ptr1*$^-$ lesions had more parasites than wild-type lesions by a factor of more than 50 (*16*). The virulence of the *ptr1*$^-$ parasite population suggested that, in agreement with previous data, inhibition of PTR1 alone is not a promising chemotherapeutic strategy (*3, 17*).

Microscopic examination showed that stationary-phase *ptr1*$^-$ cultures contained elevated levels of metacyclic promastigotes, which are morphologically distinct (Fig. 2A) (*1*). Because the onset and rate of lesion formation depend on the number of metacyclic parasites inoculated (*1*), we postulated that the increased virulence of the *ptr1*$^-$ line arose from the elevated levels of infectious metacyclics. Because pteridine transporters are down-regulated during development (*4*), we proposed that this was a manifestation of a cellular process occurring normally during development, and that decreased pteridine levels were critical for metacyclogenesis.

Hence, we measured metacyclic formation in the *ptr1*$^-$ parasite inoculum (*9*) and in *bt1*$^-$ parasites lacking the biopterin transporter BT1 (*18*). These two mutant parasites are specifically altered in biopterin but not folate metabolism because they retain DHFR-TS and the folate transporter FT1. Because metacyclogenesis occurs when cells progress into stationary phase and exit the cell cycle (*1*), it was necessary to exclude effects arising from changes in growth rate or cell cycle arrest caused by pteridine deficiency. We used growth media containing sufficient reduced biopterin to enable the *ptr1*$^-$ and *bt1*$^-$ parasites (and their complemented derivatives) to grow at the same rate as wild-type parasites (Fig. 1B). Metacyclic formation was determined by agglutination with peanut agglutinin (PNA): Procyclic promastigotes strongly agglutinate, whereas metacyclic parasites do not react (PNA$^-$) (*1*). By this test, both the *ptr1*$^-$ and *bt1*$^-$ lines exhibited numbers of metacyclic parasites that surpassed the wild type by a factor of 4 to 5 (Fig. 1C). The effects were specific to the loss of PTR1 or BT1 because the respective rescued lines behaved like wild-type controls (Fig. 1C).

Metacyclics formed by the *ptr1*$^-$ and *bt1*$^-$ parasites were authentic by several criteria. The reactivity of both procyclic and metacyclic parasites with PNA was identical, as judged by titrations. All PNA$^-$ parasites exhibited typical metacyclic morphology, with thin cell bodies, elongated flagella, and high motility (Fig. 2A). Expression of metacyclic stage–specific genes such as *SHERP* (*19*) was normal in *ptr1*$^-$ and *bt1*$^-$ metacyclics (Fig. 2B). Last, during metacyclogenesis the *Leishmania* surface glycocalyx coat (composed primarily of the glycolipid lipophosphoglycan or LPG) undergoes modifications that lead to shifts from complement sensitivity to complement resistance (*20*). Both log-phase and metacyclic *ptr1*$^-$ and *bt1*$^-$ parasites showed complement-resistant profiles identical to that of the wild type, with metacyclics being more resistant (Fig. 2C). Analysis of purified LPGs from log-phase and stationary-phase parasites showed that LPG underwent stage-specific changes, which again were identical in all lines (*21*).

We measured biopterin levels directly in

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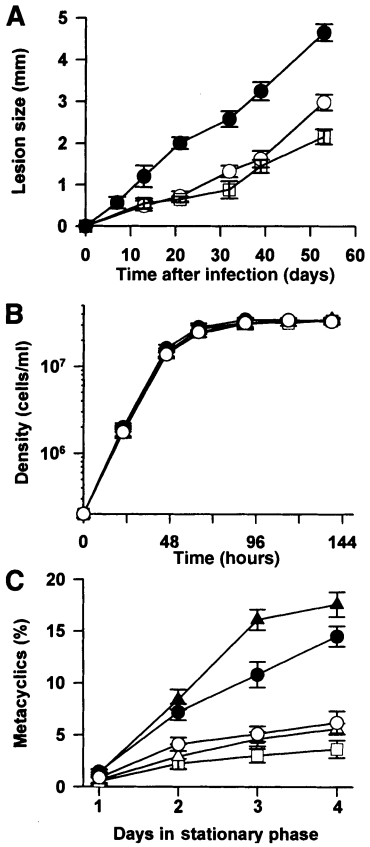

Fig. 1. Increased lesion formation and metacyclogenesis in *Leishmania* pteridine mutants. (**A**) Lesion formation. Promastigote cultures were initiated at 2×10^5 log-phase promastigotes per milliliter and grown at 26°C in BH_2-M199 medium, which is M199 medium (*4*) containing 2 μg/ml each of biopterin and H_2B. Stationary-phase cells (taken 48 hours after entry into stationary phase) were isolated by centrifugation, washed with Dulbecco's modified Eagle's medium (DMEM), resuspended at a density of 2×10^7/ml, and kept on ice. Groups of six BALB/c mice (Charles River) were infected subcutaneously with 10^6 stationary-phase promastigotes in their hind footpads, and lesion formation was monitored; means and standard deviations are shown. Similar results were obtained in two additional independent experiments. (**B**) Growth rate. Cells were inoculated at 2×10^5 log-phase promastigotes per milliliter into BH_2-M199 medium; growth was monitored by counting with a hemocytometer. Log-phase doubling times were ~9 hours. (**C**) Metacyclic formation. Stationary-phase promastigotes were prepared as described in (A), resuspended at 10^8 per milliliter in DMEM supplemented with the lectin PNA at 35 μg/ml, and incubated at 26°C for 30 min with gentle agitation. Agglutinated cells were pelleted by centrifugation (5 min, 100*g*), and nonagglutinated metacyclic parasites (PNA^-) were recovered by centrifugation (10 min, 2100*g*) and counted with a hemocytometer. Means and standard deviations of triplicate determinations are shown. In a total of four independent experiments, average percentages of metacyclics were as follows: wild type, 3.4%; *ptr1*$^-$, 14.9%; *bt1*$^-$, 15.3%; *ptr1*$^-$/pXG-PTR1, 3.2%; *bt1*$^-$/pXG-BT1, 5.5%. Symbols: □, wild type; ●, *ptr1*$^-$; ○, *ptr1*$^-$/pXG-PTR1; ▲, *bt1*$^-$; △, *bt1*$^-$/pXG-BT1. All parasites derive from the virulent CC-1 line of *L. major* (*4, 9*).

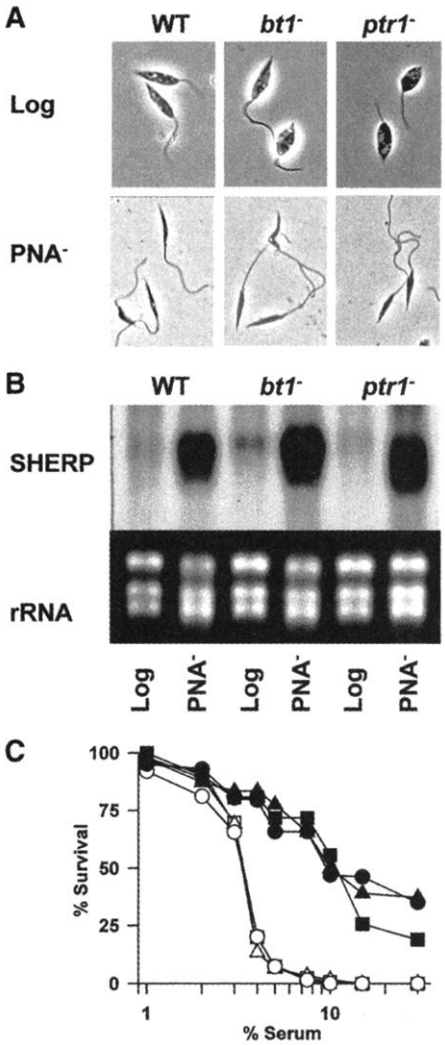

Fig. 2. *bt1*$^-$ and *ptr1*$^-$ metacyclics are comparable to wild-type metacyclics. Parasites were cultured and log-phase (Log) and metacyclic cells (PNA^-) were prepared as described in Fig. 1. (**A**) Morphology of log-phase parasites and PNA^- metacyclics. (**B**) Expression of a metacyclic stage–specific gene. Northern blot hybridization was performed as described (*4*) with the metacyclic stage–specific gene *SHERP* (*19*). Before transfer, the gel was stained with ethidium bromide to control for RNA loading, and this is shown beneath the autoradiogram. (**C**) Sensitivity to human serum–dependent lysis. Parasites were isolated by centrifugation (10 min, 2100*g*), washed free of media, resuspended in phosphate-buffered saline at a final concentration of 4×10^6/ml with varying amounts of human serum, and incubated for 45 min at 26°C. Samples were then placed on ice, and intact motile cells were enumerated and expressed relative to initial density. Averages of duplicate measurements are shown; a second experiment gave similar results. Symbols: wild-type log-phase (□) and PNA^- (■); *bt1*$^-$ log-phase (△) and PNA^- (▲); *ptr1*$^-$ log-phase (○) and PNA^- (●).

wild-type and mutant *Leishmania* using a high-performance liquid chromatography (HPLC)–based method (*4*). In log phase, wild-type parasites contained high levels of H_4B, which declined in stationary phase by a factor of 13 (Fig. 3A). This agreed with previous studies showing that BT1-mediated biopterin influx declines in stationary phase by a factor of 6 (*4*). Both the *ptr1*$^-$ and *bt1*$^-$ parasites had significantly lower intracellular levels of H_4B throughout development in vitro: lower than log-phase wild-type parasites by a factor of 20, and comparable with levels seen in stationary-phase wild-type parasites (Fig. 3A). Measurements of total biopterin showed that the effect was specific for H_4B, as biopterin levels in *ptr1*$^-$ parasites declined by less than half in log phase (Fig. 3B). Thus, H_4B levels declined during normal development, whereas *ptr1*$^-$ and *bt1*$^-$ parasites showed constitutively low H_4B levels. This suggests a model in which a signal associated with decreased H_4B levels is executed in stationary phase, when metacyclogenesis occurs.

Leishmania major require very low levels of biopterin for normal growth (*9, 22*), allowing tests of metacyclogenesis across a wide range of biopterin concentrations (8.1 nM to 16.7 μM; Fig. 3C). At external biopterin concentrations above 0.5 μM, metacyclogenesis was maintained at the basal level seen previously (~3%; Fig. 3C). Below this external threshold, the degree of metacyclogenesis increased, reaching more than 13% (Fig. 3C). Conversely, above the external threshold point, internal H_4B levels were relatively high, but below this point they declined steeply (Fig. 3C). Notably, at subthreshold external biopterin levels, internal H_4B declined to values comparable to those seen in stationary-phase wild-type parasites and in the *ptr1*$^-$ and *bt1*$^-$ mutants throughout growth (Fig. 3A). The correspondence of the threshold points for internal H_4B levels and metacyclogenesis provides evidence that decreased H_4B levels are responsible for elevated metacyclogenesis. The threshold effect also explains why overexpression of PTR1 or BT1 [which occurs in the rescued mutants (*5, 9, 18, 23*)] does not suppress metacyclogenesis (Fig. 1C).

The assay shown in Fig. 3C can be conveniently applied to other *Leishmania* strains and growth conditions. Like the CC-1 line, the highly virulent Friedlin strain (clone V1) showed increased metacyclogenesis at subthreshold biopterin levels (10.8% metacyclics when tested at 8.1 nM biopterin, versus 3.1% metacyclics when tested at 16.7 μM biopterin). As anticipated from genetic studies and the enzymatic properties of PTR1 and BT1, no folate-dependent alterations in metacyclogenesis were observed (*24*).

These studies establish that high intracellular H_4B levels act to down-regulate metacyclo-

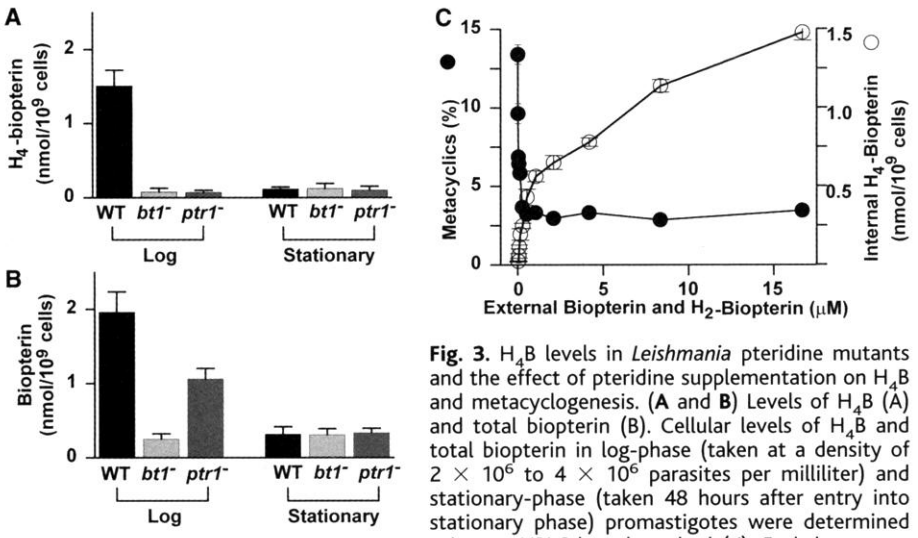

Fig. 3. H_4B levels in *Leishmania* pteridine mutants and the effect of pteridine supplementation on H_4B and metacyclogenesis. (**A** and **B**) Levels of H_4B (A) and total biopterin (B). Cellular levels of H_4B and total biopterin in log-phase (taken at a density of 2×10^6 to 4×10^6 parasites per milliliter) and stationary-phase (taken 48 hours after entry into stationary phase) promastigotes were determined using an HPLC-based method (*4*). Each bar represents the mean and standard deviation of three independent experiments, each comprising 6 to 12 measurements. Residual formation of H_4B in the *ptr1*$^-$ line arises from a third reductase activity that is able to reduce H_2-biopterin at a low rate (*3, 9, 23*). Residual biopterin levels in the *bt1*$^-$ parasites may arise from passive diffusion or entry through the folate transport system (*4–7*). The limit of detection was 0.04 nmol per 10^9 cells (*4*). (**C**) Effect of external biopterin on metacyclogenesis. To equilibrate pteridine pools, we serially passaged *L. major* wild-type promastigotes twice in M199 medium containing varying levels of 1:1 biopterin and H_2-biopterin (the *x* axis shows total biopterin concentration). During the third passage, internal H_4B levels (○) in log-phase cells were measured (*4*), and percentages of metacyclics (●) present on the third day of stationary phase were determined by PNA agglutination. Cells grew equally well under all conditions shown, with log-phase doubling times of ~9 hours. Means and standard deviations for triplicate determinations in a single experiment are shown. From four independent experiments, 13.1 ± 0.6% metacyclics were obtained when grown in 8.1 nM biopterins, versus 3.8 ± 0.6% metacyclics in 16.7 μM biopterins.

genesis in *Leishmania* to a constitutive, basal level. Dipteran insects synthesize high levels of pteridines, including H_4B (*15, 25*), and probably provide a pteridine-rich environment similar to that used in our studies. Thus, it is likely that down-regulation of the BT1 transporter, which occurs during stationary phase, controls internal biopterin levels during metacyclogenesis in the sand fly (*4*). Biopterin is essential for *L. major* growth (*9, 22*), and in these studies the nutritional requirement for biopterin was fully satisfied (Figs. 1B and 3C). Hence, we have identified a new and second role for biopterin in *Leishmania*, that of regulating parasite differentiation to the infective metacyclic stage. Potentially, H_4B could act directly as a signaling molecule, because H_4B and other pteridines have been implicated in cellular differentiation and signal transduction in other organisms (*26, 27*). Alternatively, H_4B may serve as a cofactor for other enzymes (as yet unidentified in *Leishmania*) (*14, 15*). It is also clear that biopterin levels must act in concert with other factors (including growth phase) that determine the basal level of metacyclogenesis evident in Fig. 3.

Our studies have implications for the criteria used to study parasite virulence in animal models. Typically, the search for genes implicated in virulence has focused on those whose loss is associated with decreased virulence, pathology, or infectivity. In contrast, the *ptr1*$^-$ *Leishmania* population exhibits "hypervirulence" after inoculation into BALB/c mice, manifesting as increased lesion formation and parasite numbers (Fig. 1A). Relatively few loci whose loss confers increased virulence have been described in pathogens, and their study may prove illuminating about both the process of virulence gene identification and the manner by which they serve to down-regulate virulence in standard experimental tests. Limiting pathology and pathogen replication may be important in evolutionary terms for transmission and persistence (*28*). Thus, *PTR1*, by acting to limit pathogenesis in the mammalian host, may act to increase host survival and hence parasite transmission, and as such would represent a new class of genes that limit virulence. However, this hypothesis must await further proof and careful testing to confirm that loss of *PTR1* is not deleterious within other stages of the infectious cycle.

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