A Conditional Mutant Deficient in Hypoxanthine-guanine Phosphoribosyltransferase and Xanthine Phosphoribosyltransferase Validates the Purine Salvage Pathway of *Leishmania donovani*  

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*Leishmania donovani* cannot synthesize purines de novo and express a multiplicity of enzymes that enable them to salvage purines from their hosts. Previous efforts to generate an *L. donovani* strain deficient in both hypoxanthine-guanine phosphoribosyltransferase (HGPRT) and xanthine phosphoribosyltransferase (XPRT) using gene replacement approaches were not successful, lending indirect support to the hypothesis that either HGPRT or XPRT is crucial for purine salvage by the parasite. We now report the genetic confirmation of this hypothesis through the construction of a conditional *Δhgprt/Δxprt* mutant strain that exhibits an absolute requirement for 2′-deoxycoformycin, an inhibitor of the leishmanial adenine aminohydrolase enzyme, and either adenine or adenosine as a source of purine. Unlike wild type parasites, the *Δhgprt/Δxprt* strain cannot proliferate indefinitely without 2′-deoxycoformycin or with hypoxanthine, guanine, xanthine, guanosine, inosine, or xanthosine as the sole purine nutrient. The *Δhgprt/Δxprt* mutant infects murine bone marrow-derived macrophages <5% as effectively as wild type parasites and cannot sustain an infection. These data establish genetically that either HGPRT or XPRT is absolutely essential for purine acquisition, parasite viability, and parasite infectivity of mouse macrophages, that all exogenous purines are funneled to hypoxanthine and/or xanthine by *L. donovani*, and that the purine sources within the macrophage to which the parasites have access are HGPRT or XPRT substrates.

*Leishmania donovani* is a protozoan parasite that is the etiologic agent of visceral leishmaniasis, a devastating and often fatal disease in humans. *Leishmania* spp. are digenetic, existing in both insect vector and mammalian forms. The flagellated, motile, extracellular promastigote proliferates in the midgut of phlebotomine sandfly family members, whereas the nonflagellated, nonmotile, intracellular amastigote resides in phagolysosomes of macrophages and other reticuloendothelial cells within the vertebrate host. Because of the absence of effective vaccines, chemotherapy has offered the only avenue of defense for the treatment and prevention of leishmaniasis and other parasitic diseases. Unfortunately, drug therapy for leishmaniasis is compromised by toxicity, expense, prolonged and invasive routes of administration, and resistance. Thus, the need for new and more efficacious drugs is acute.

The institution of an effective parasite-specific therapeutic regimen for the treatment of leishmaniasis, or for that matter any parasitic disease, depends upon exploiting fundamental biochemical or metabolic differences between parasite and host. Perhaps the most striking metabolic disparity between parasites and their mammalian hosts is the avenue by which they synthesize purine nucleotides. Whereas mammalian cells can generate the purine ring de novo, all of the protozoan parasites studied to date are incapable of synthesizing the purine ring (1). As a consequence, each genus of parasite has evolved a unique complement of purine salvage enzymes that enables it to scavenge host purines (1, 2).

*Leishmania* expresses a number of purine salvage enzymes. These enzymes include hypoxanthine-guanine phosphoribosyltransferase (HGPRT), 3 adenine phosphoribosyltransferase (APRT), xanthine phosphoribosyltransferase (XPRT), and adenosine kinase (AK), and the genes encoding all four enzymes, phosphorylases, and deaminases (1, 11). Thus, the purine salvage pathway of *Leishmania* is divagating and redundant, and this metabolic complexity, as well as the diploid nature of the parasite, has hindered a thorough characterization of the pathway.

The ability of *Leishmania* to carry out efficient homologous gene replacement (12, 13) and take up foreign DNAs (14), however, can overcome these impediments and enables the genetic dissection of complex metabolic pathways such as that for purine acquisition. Implementing targeted gene replacement strategies, *L. donovani* promastigotes deficient in HGPRT, APRT, XPRT, and/or AK were created in almost every conceivable combination (5, 15, 16), although it was not possible to create a *Δhgprt/Δxprt* double mutant in any genetic background. These genetic studies underscored our central hypothesis governing purine metabolism that either HGPRT or XPRT is both necessary and sufficient for all of purine acquisition by *L. donovani*. The inability to create the *Δhgprt/Δxprt* double knock-out, however, was negative evidence that did not confirm the premise.

We have now isolated and characterized a conditional *Δhgprt/Δxprt* mutant strain that did not confirm the premise.

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3 The abbreviations used are: HGPRT, hypoxanthine-guanine phosphoribosyltransferase; APRT, adenine phosphoribosyltransferase; XPRT, xanthine phosphoribosyltransferase; AK, adenosine kinase; dCF, 2′-deoxycoformycin; AAH, adenine aminohydrolase; UTR, untranslated region; DME-L, Dulbecco’s Modified Eagle-Leishmania; PBS, phosphate-buffered saline.
line of *L. donovani* that was selected by targeted gene replacement in the presence of 2'-deoxycoformycin (dCF), an inhibitor of the leishmanial adenine aminohydrolase (AAH) enzyme (11), and adenine as a source of purine. The creation and characterization of this Δ*hgprt*/Δ*xprt* mutant confirms our main supposition that either HGPRT or XPRP is absolutely essential for purine acquisition and parasite viability.

**EXPERIMENTAL PROCEDURES**

**Materials**—[8-14C]Adenine (50 mCi/mmol), [8-14C]adenosine (53 mCi/mmol), [8-14C]guanine (55 mCi/mmol), [8-14C]guanosine (50 mCi/mmol), [8-14C]hypoxanthine (51 mCi/mmol), [8-14C]inosine (52 mCi/mmol), and [8-14C]xanthine (53 mCi/mmol) were all purchased from Moravek Biochemicals (Brea, CA). dCF was obtained from the National Cancer Institute (Bethesda, MD). All restriction and DNA-modifying enzymes and all other chemicals and reagents were of the highest quality commercially available.

**Axenic Parasite Cell Culture**—The *L. donovani* strain 152D originated with Dr. Dennis Dwyer (NIH) and was adapted for growth as axenic amastigotes as described (17). A clonal derivative of this strain, LdBob (17), was provided by Dr. Stephen Beverley (Washington University, St. Louis, MO). LdBob promastigotes were cultured at 26 °C in purine-replete M199-based medium as detailed (17) or in a modified Dulbecco’s Modified Eagle-Leishmania (DME-L) medium (18) that lacks bovine serum albumin and is supplemented with 10% dialyzed fetal bovine serum, 1 mM glutamine, 1× RPMI 1640 (1640 vitamin mix, 10 μM folate, 100 μM adenine, and 20 μM dCF. Axenic amastigotes were cultured at 37 °C as described (17). The characterization of LdBob strains harboring single Δ*hgprt*, Δ*xprt*, or Δ*xprt* lesions is currently under review.

**Targeting and Episomal Constructs**—The flanking regions from the *HGPRT*, *APRT*, and *XPRP* loci and the oligonucleotides used for their amplification by the PCR have been described (5, 15). The construction and authentication of the pX63-HYG-Δ*hgprt*, pX63-NEO-Δ*xprt*, and pX63-HYG-Δ*xprt* targeting vectors employed in the previous allelic replacements of the *HGPRT* and *XPRP* loci have also been described (5, 15). The pX63-NEO-Δ*hgprt*/Δ*xprt* construct was created by replacing the 5′-untranslated region (UTR) of *XPRT* in the pX63-NEO-Δ*xprt* vector with the 5′-UTR of the *HGPRT*. The pX63-PHLEO-Δ*xprt* replacement plasmid was generated by excising the 5′- and 3′-UTRs of *XPRT* from pX63-HYG-Δ*xprt* and inserting them into the appropriate sites within pX63-PHLEO (19).

To generate episomal constructs of *HGPRT* and *XPRT*, the two genes were amplified by PCR and inserted into the Smal-BamHI restriction sites within the pXG-BSD blasticidin resistance expression plasmid generously provided by Dr. Stephen Beverley. The complementation vectors were designated pXG-BSD-HGPRT and pXG-BSD-XPRT, respectively. The other two episomal constructs, pSNBR-HYG-Δ*hgprt*(A209–211) and pXG-BSD-ΔALK, which lack a peroxisomal targeting signal 1 and therefore produce cytosolic hgprt and xprt, respectively, were generated as described (7, 8). pSNBR-HYG-Δ*hgprt*(A209–211) will now be referred to as pSNBR-HYG-Δ*hgprt*ΔSKV.

**Gene Replacements and Complemented Lines**—All genetic manipulations were conducted on LdBob promastigotes. Single Δ*hgprt*, Δ*xprt*, and Δ*xprt* knock-outs were created in LdBob using the same targeting constructs, protocols, and gene replacement strategies employed previously for the generation of these mutants within an avirulent *L. donovani* background (5, 15). The Δ*hgprt*/Δ*xprt* double knock-out was generated after three sequential rounds of targeted gene replacement. The pX63-NEO-Δ*hgprt*/Δ*xprt*, pX63-HYG-Δ*hgprt*, and pX63-PHLEO-Δ*xprt* plasmids were linearized with HindIII and BglII and transfected into 5 × 10⁷ parasites using reported electroporation conditions (17). Homologous integrations were selected by plating parasites on semisolid medium containing selective concentrations of either Geneticin, hygromycin, or phleomycin (19), as appropriate for the drug resistance marker of the targeting cassette. The HGPRT/Δ*hgprt*/Δ*xprt* and Δ*hgprt*/Δ*xprt*/Δ*xprt* lines were generated and maintained in the M199-based medium, whereas the Δ*hgprt*/Δ*xprt* null mutant was selected and maintained in the modified MDE-L medium described above. The HGPRT/Δ*hgprt*/Δ*xprt*, Δ*xprt*/Δ*xprt*, and Δ*hgprt*/Δ*xprt* lines were all maintained continuously under selective pressure in the drugs for which they contained resistance markers.

The Δ*hgprt*/Δ*xprt* line was also transfected separately with pXG-BSD-HGPRT, pXG-BSD-XPRT, or pXG-BSD-ΔALK, and transfectants selected in 20 μg/ml blasticidin and either 100 μM hypoxanthine or 100 μM xanthine to generate the complemented lines Δ*hgprt*/Δ*xprt*/Δ*xprt* (pXG-BSD-HGPRT), Δ*hgprt*/Δ*xprt* (pXG-BSD-XPRT), and Δ*xprt*/Δ*xprt*/Δ*xprt* (pXG-BSD-ΔALK), respectively. These cell lines are designated Δ*hgprt*/Δ*xprt*/Δ*xprt* (pXG-BSD-HGPRT), Δ*hgprt*/Δ*xprt*/Δ*xprt* (pXG-BSD-XPRT), and Δ*xprt*/Δ*xprt*/Δ*xprt* (pXG-BSD-ΔALK). The Δ*hgprt*/Δ*xprt* cell line that was transfected with pSNBR-HYG-Δ*hgprt*ΔSKV was selected in 50 μg/ml hygromycin and 100 μM hypoxanthine and is designated Δ*hgprt*/Δ*xprt*/Δ*xprt* (pSNBR-HYG-ΔSKV).

**DNA Manipulations and Western Blotting**—Isolation of genomic DNA and Southern blotting were performed by conventional protocols (20). Monospecific antibodies to purified recombinant *L. donovani* HGPRT, APRT, and XPRP proteins have been described (3–5), and Western blotting protocols were carried out as conveyed (20). Mouse monoclonal antibody to the amastigote-specific A2 protein (21) was generously provided by Dr. Greg Matlashewski of McGill University Faculty of Medicine, Montreal, Quebec, Canada.

**Enzyme Assays**—2 × 10⁹ parasites were washed two times in phosphate-buffered saline (PBS), resuspended in 50 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 1 mM phosphoribosylpyrophosphate, and protease inhibitor mixture, lysed by sonication, and fractionated by centrifugation at 9,740 × g at 4 °C. Radiolabel incorporation assays were performed on cell-free lysates of promastigote extracts as described (22), whereas amastigote enzymatic assays were conducted with lysates that were not fractionated by centrifugation. These assays measure the rates of radiolabeled preformed purine nucleoside and nucleobases into phosphorylated, anionic metabolites, i.e. nucleotides and nucleic acids.

**Purine Metabolism in Live Cells**—The rates of conversion of radiolabeled purine into nucleotides were measured using the earlier described DE-81 filter disk method (22). Cells were washed with PBS and resuspended at a density of 1 × 10⁹ cells/ml in either promastigote or amastigote medium containing 2 μM radiolabeled purine but lacking bovine serum albumin, FBS, and hemin. At each time point 1 × 10⁹ cells were removed, washed once in PBS, lysed in 1% Triton X-100, and spotted onto DE-81 filter disks. Parasite metabolism of various radiolabeled purines was quantified by liquid scintillation.

**Growth Phenotypes**—To assess the abilities of genetically manipulated strains to grow in various different purine sources, all parasites were washed several times with PBS, resuspended in modified MDE-L medium lacking purine, and incubated at 26 °C for 4 h before they were seeded at a density of 5 × 10⁹ cells/ml in 1.0-mL aliquots of modified MDE-L containing 100 μM purine and 5% dialyzed FBS. Amastigotes were seeded at a density of 5 × 10⁹ cells/ml into amastigote medium containing 20% FBS and 100 μM purine, incubated for 7–10 days, and counted by hemacytometer.

**Macrophage Infections**—Stationary phase promastigotes were washed two times in purine-free promastigote medium and resuspended in
Dulbecco’s modified Eagle’s medium supplemented with 4 mM l-glutamine, 1.5 g/liter sodium bicarbonate, 4.5 g/liter glucose, and 10% FBS. 2 × 10⁶ bone marrow-derived mouse macrophages, from Balb/c mice, and 2 × 10⁶ promastigotes were placed in 4-well Lab-TekII CHAMBER SLIDES (Nalge Nunc International Corp., Naperville, IL) containing 1.0 ml of macrophage growth medium and incubated at 37 °C in a humidified 5% CO₂ incubator. After 16 h, adherent macrophages were washed 10 times in PBS to eliminate residual extracellular promastigotes after which fresh growth medium was added and then again 24 h later. After an additional 24-h incubation, the chambers were washed three times with PBS, and macrophages were stained using the Diff-Quik kit (International Medical Equipment Inc., San Marcos, CA). Parasites were visualized on a Zeiss Axiovert 200 M scope (Carl Zeiss Microimaging, Thornwood, NY) using 60× oil immersion light and photographed with an AxioCam MRm camera (Zeiss), and parasites were enumerated. Color photographs of parasitized macrophages were visualized on a Zeiss Axioskop microscope using 40× oil immersion and photographed with a Leica DC 300 camera (Leica Camera AG, Solms, Germany).

RESULTS

The Δhgprt/Δxprt knock-out was created after three rounds of transfection with drug resistance cassettes carrying 5' and 3' UTRs of HGPRT and XPRT (Fig. 1). Because HGPRT and XPRT are colocalized within a 4359-bp EcoRI fragment in the L. donovani genome (5), the first copy of both genes was displaced with X63-NEO-Δhgprt/Δxprt (linearized pX63-NEO-Δhgprt/Δxprt), a construct containing the 5' UTR of HGPRT and the 3' UTR of XPRT, to create the HGPRT/hgprt/XPRT/xprt double heterozygote. The heterozygote was then transfected with X63-HYG-Δhgprt to generate the Δhgprt/XPRT/xprt line, and the latter was transfected with X63-PHEL-Δxprt to create the Δhgprt/Δxprt double knock-out (Fig. 1). The last round of transfection was performed in medium containing 20 μM dCF and 100 μM adenosine, whereas the HGPRT/hgprt/XPRT/xprt and Δhgprt/XPRT/xprt progenitors of the double knock-out were isolated in medium lacking dCF and containing 100 μM adenosine as a purine source. 50–100 drug-resistant colonies were obtained within days after the first two cycles of transfections, but surprisingly only two barely visible colonies were obtained after 4 weeks following the last round of transfection in the adenine-dCF medium. Both colonies were picked and expanded in liquid culture medium containing adenosine and dCF.

Southern blot analysis of the HGPRT/hgprt/XPRT/xprt, Δhgprt/ XPRT/xprt, and Δhgprt/Δxprt strains divulged the new alleles that had been created by the homologous gene replacement events (Fig. 2). The digestion of genomic DNA with EcoRI and hybridization to the HGPRT and XPRT open reading frames revealed the presence of the common 4359-bp restriction fragment in the wild type and HGPRT/hgprt/XPRT/xprt lines and its absence in the Δhgprt/Δxprt null mutant. The Δhgprt/ XPRT/xprt strain, as expected, lacks a band for HGPRT but exhibits a hybridization signal at 5939 bp when probed with the XPRT open reading frame that reflects the appropriate integration of the hygromycin resistance marker within the X63-HYG-Δhgprt cassette into the HGPRT locus. For comparison, a Southern blot analysis of previously isolated single knock-outs of HGPRT, APRT, and XPRT are also depicted, and the signals are appropriate for the expected homologous integrations (Fig. 2).

Western blot analysis of wild type, HGPRT/hgprt/XPRT/xprt, Δhgprt/XPRT/xprt, and Δhgprt/Δxprt extracts confirmed the absence of HGPRT and XPRT protein in strains in which the corresponding gene had been eliminated (Fig. 3). The single Δhgprt, Δaprt, and Δxprt null mutants for which Southern blot data are shown in Fig. 2 also lacked the proteins corresponding to the deleted genes.

The abilities of wild type and Δhgprt/Δxprt promastigote lysates to incorporate individual radiolabeled purines into nucleotides were
assessed over a 15-min time interval (Fig. 4). Whereas wild type promastigotes were capable of incorporating all purine bases and nucleosides tested, the Δhgprt/Δxprt could not convert guanine, guanosine, hypoxanthine, or inosine to nucleotides. The double knock-out could, however, incorporate radiolabeled adenine and adenosine into phosphorylated products during the 15 min assay interval, whether or not dCF was added to the extracts (Fig. 4). [14C]Xanthine conversion to nucleotides could not be measured in promastigote extracts for technical reasons.

The ability of live promastigotes to metabolize various [14C] radiolabeled purine bases and nucleosides was also measured. These results corroborated the results from the enzymatic assays described above. Additionally, the metabolism of [14C]xanthine could be measured in intact wild type cells but not in the double knock-out (Fig. 5). [14C]Adenine and [14C]adenosine metabolism could be measured in both wild type and mutant cells during the 2-h time course in the presence or absence of dCF.

The ability of Δhgprt/Δxprt promastigotes to proliferate in various purine sources was assessed (Fig. 6). Whereas wild type parasites could grow robustly in either hypoxanthine or xanthine, although a limited amount of growth was observed when inosine was added as the purine in the presence of dCF. The Δhgprt/Δxprt parasites incubated in inosine and dCF were also not capable of long-term survival after several additional weeks of incubation. The limited proliferation in inosine was further investigated with immucillin H, an iminoribitol analog of inosine that is a potent inhibitor of the leishmanial inosine-uridine nucleoside hydrolase activity (23). Although immucillin H at 20 μM allowed the Δhgprt/Δxprt double knock-out to replicate for several cell cycles in 100 μM inosine, indefinite growth in the inosine-immucillin H combination could also not be sustained (data not shown).

The Δhgprt/Δxprt transfectants Δhgprt/Δxprt[pHGPRT] and Δhgprt/Δxprt[pXPRT] exhibited the same growth phenotypes as the Δxprt and Δhgprt single mutants, respectively; Δhgprt/Δxprt[pXPRT] parasites could grow on all purines, whereas Δhgprt/Δxprt[pHGPRT] could grow on all purines except guanine, xanthine, and xanthosine (data not shown). dCF was not necessary for growth with adenine or adenosine in the complemented lines. Complementation of the Δhgprt/Δxprt line could also be achieved with episomes expressing cytosolic versions of hgprt and xprt that lacked their glycosomal targeting signals (7, 8). The Δhgprt/Δxprt[pHGPRT] and Δhgprt/Δxprt[pXPRT] cell lines grew robustly in either hypoxanthine or xanthine thus confirming that proper localization of either enzyme is not necessary for enzyme function in the promastigote stage of the parasite (7, 8).

Both wild type and Δhgprt/Δxprt parasites were capable of transformation to axenic amastigotes, as assessed by their expression of A2 proteins, a family of amastigote-specific markers (24). No A2 was observed in the promastigotes. The growth phenotypes of both wild type and knock-out axenic amastigotes were identical to their promastigote counterparts (data not shown). The metabolic capacities of the axenic amastigotes toward sundry purines were also indistinguishable from the promastigote equivalent.

Wild type L. donovani promastigotes were capable of sustaining a robust infection of bone marrow-derived murine macrophages, whereas the Δhgprt/Δxprt knock-out could not (Fig. 7). Parasitemia of the wild type strain was ~21 parasites/macrophage, whereas the double knock-out infectivity was ~1 parasite/macrophage, a 20-fold difference. The inability of Δhgprt/Δxprt cells to proliferate inside macrophages could not be imputed to a failure to infect the mammalian cells, because similar numbers of intracellular parasites were observed for both wild type and Δhgprt/Δxprt parasites in bone marrow-derived macrophages.
Genetic Validation of the Purine Salvage Pathway of L. donovani

A

B

C

D

FIGURE 7. Infection of bone marrow-derived murine macrophages with wild type or Δhgprt/Δxprt parasites. Infections of bone marrow-derived macrophages were accomplished as described under "Experimental Procedures." Uninfected macrophages (A) and macrophages infected with either wild type (B) or Δhgprt/Δxprt (C) parasites are depicted. The large, dark staining area in each cell is the macrophage nucleus (A–C) and the smaller, round bodies within the macrophages are L. donovani amastigotes (B, C). The scale bar represents 50 μm. D, the average number of amastigotes/macrophage is depicted for wild type cells, the Δhgprt/Δxprt mutant, and for the Δhgprt/Δxprt mutant when supplemented with adenine or adenine plus dCF during the course of infection.

and knock-out parasites 4 h postinfection (data not shown). Supplementation of the macrophage medium with either 100 μM adenine or a combination of 100 μM adenine plus 20 μM dCF allowed the Δhgprt/Δxprt mutant to reach a parasite load of 7.5 parasites/macrophage and 12 parasites/macrophage, respectively, indicating that it is possible for the Δhgprt/Δxprt mutant to proliferate within macrophages if vital nutrients are provided to its host cells (Fig. 7D). Both complemented lines, Δhgprt/Δxprt[pHGPRT] and Δhgprt/Δxprt[pXPRT], also sustained robust infections in the macrophages, although the infectivity was lower than that of wild type parasites. Parasite loads of ~8 parasites/macrophage and ~13 parasites/macrophage were observed for Δhgprt/

Δxprt[pHGPRT] and Δhgprt/Δxprt[pXPRT], respectively (data not shown).

DISCUSSION

The purine salvage pathway of Leishmania is myriad and complex, although determining which of the many routes of purine salvage are functional has eluded genetic dissection (1, 25). Mutational and gene replacement schemes in L. donovani have demonstrated that none of the four known enzymes capable of converting host purine nucleobases or nucleosides to the nucleotide level, HGPRT, APRT, XPR, or AK, is essential by itself (5, 15, 16, 26). Furthermore, the ability to generate viable Δhgprt/Δxprt/ak− (16) and Δxprt/Δhgprt/ak− L. donovani promastigotes reveals that the parasite can rely on either a functional XPR or HGPRT activity for all of its purine nutritional requirements. Indeed, it has been possible to generate mutant parasites by targeted gene replacement in every conceivable combination except mutants accommodating a combined Δhgprt and Δxprt genotype. These results were the basis for our fundamental hypothesis that either HGPRT or XPRT is necessary and sufficient for Leishmania parasites to salvage purines, maintain viability, and sustain proliferation. The hypothesis was sustained by the inability to generate a Δhgprt/Δxprt double knock-out even within a genetic background complemented with an episomal copy of either HGPRT or XPR.4

We now report genetic proof of our principal hypothesis by the creation and characterization of a conditional Δhgprt/Δxprt double knock-out. Taking advantage of the colocalization of the HGPRT and XPR genes in the leishmanial genome (5), a Δhgprt/Δxprt mutant was selected after three rounds of targeted gene replacement, with the final transfection being achieved in the presence of 100 μM adenine and 20 μM dCF, an inhibitor of the L. donovani AAH (11). The Δhgprt/Δxprt mutant is absolutely reliant on the presence of dCF and either adenine or adenosine as a purine source for lasting survival and growth. No other naturally occurring purine tested could enable indefinite, long term proliferation of either stage of the parasite. However, several rounds of replication by the double knock-out could be sustained with adenine or adenosine in the absence of dCF, but this proliferation could not be perpetuated interminably (Fig. 6). The ability of the Δhgprt/Δxprt to undergo several cell divisions in adenine or adenosine can be ascribed to the time interval required for AAH to convert the 6-aminopurine source to hypoxanthine. This contention is buttressed by short term radiolabel incorporation experiments demonstrating that Δhgprt/Δxprt lysates (Fig. 4), as well as intact parasites (Fig. 5), were perfectly capable of converting adenine or adenosine to the nucleotide level via APRT.

Eventually, however, the actions of AAH convert adenine or adenosine, which is cleaved to adenosine by L. donovani promastigotes (11, 22, 26), to hypoxanthine, a dead end nutrient for L. donovani expressing an activity capable of salvaging the nucleoside. Although adenosine kinase activity has not been detected in Leishmania (1), a nucleoside phosphotransferase activity capable of phosphorylating the pyrazolopyrimidine nucleoside analogs allopurinol riboside and formycin B, has been observed in L. donovani promastigotes (27, 28). Whether this phosphotransferase is also capable of recognizing inosine is not known.

To date, there are no effective vaccines to protect against visceral leishmaniasis (29). Generating a strain with intrinsic attenuating mutation(s) could theoretically be exploited as a live attenuated vaccine for immunizing against the disease is a valid alternative strategy to control

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leishmaniasis rather than the conventional paradigm of treatment with toxic drugs. Previous studies using noninfectious Leishmania tarentolae or attenuated strains of Leishmania major have demonstrated that these lines are capable of triggering a protective immune response against further challenge by virulent Leishmania in susceptible rodents (29, 30). The inability of the Δhxprt/Δxprt knock-out to sustain an infection of murine macrophages bolsters this mutant strain as a candidate for such a live vaccine strategy against visceral leishmaniasis. The Δhxprt/Δxprt mutant infects macrophages at a level <5% of that of wild type parasites. Preliminary results indicate that the overall infection rate of the Δhxprt/Δxprt mutant in macrophages can be increased by the addition of either adenine alone or, a combination of adenine and dCF, to the growth medium. Thus, it should be theoretically feasible to sustain an infection of the Δhxprt/Δxprt strain in susceptible mammals by dietary supplementation with adenine and/or dCF until a protective immune response has been established. The strain could then be eliminated by withdrawal of the dietary additions. It is important, of course, to determine the stability of the mutant genotype and phenotype. Genetic studies are currently underway to determine whether the Δhxprt/Δxprt double mutant is stable and does not revert by down-regulating its A2H activity thereby allowing the parasite to presumably grow on adenine or adenosine alone.

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