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Spermidine Synthase Is Required for Virulence of *Leishmania donovani*▽

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Genetic lesions in the polyamine biosynthetic pathway of *Leishmania donovani*, the causal agent of visceral leishmaniasis, are conditionally lethal mutations that render the insect vector form of the parasite auxotrophic for polyamines. Recently, we have demonstrated that a Δ*odc L. donovani* null mutant lacking ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine biosynthesis, was profoundly compromised in its ability to infect mice, indicating that ODC is essential for the infectious mammalian stage of the parasite and further validating the enzyme as a possible drug target. To assess whether other components of the polyamine biosynthetic pathway were also essential for parasite virulence, a cell line deficient in spermidine synthase (SPDSYN), the enzyme that converts putrescine to spermidine, was created by double-targeted gene replacement within a virulent *L. donovani* background. This Δ*spdsyn* strain was auxotrophic for polyamines, required spermidine for growth in its insect vector form, and was adversely impacted in its ability to infect mice. These findings establish that SPDSYN, like ODC, is essential for maintaining a robust infection in mammals and indicate that pharmacologic inhibition of SPDSYN, and perhaps all components of the polyamine biosynthetic pathway, is a valid therapeutic strategy for the treatment of visceral and, potentially, other forms of leishmaniasis.

Leishmania donovani, a digenetic protozoan parasite, is the etiological agent of visceral leishmaniasis, a devastating and invariably fatal disease when left untreated. This unicellular eukaryote lives as the extracellular, flagellated promastigote within its insect vector, the phlebotomine sandfly, and resides as the intracellular, aflagellar amastigote within the phagolysosomes of infected macrophages and other reticuloendothelial cells of the mammalian host. There is no reliable vaccine for leishmaniasis, and, consequently, drugs are the only avenue by which the disease can be treated. Regrettably, the existing collection of antileishmanial drugs is far from ideal, primarily due to their lack of selectivity toward the metabolic machinery of the parasite. Drug toxicity, as well as increased drug resistance (24, 40), reinforces the need to develop new therapeutics and to identify and validate new drug targets.

One metabolic pathway that has drawn considerable attention in proposing new antiparasitic therapies is that for the synthesis of polyamines, ubiquitous aliphatic cations (putrescine, spermidine, spermine) that are known to play critical roles in a variety of fundamental cellular processes such as growth, differentiation, and macromolecular synthesis (5, 32, 33, 57). Polyamines also act as precursors for the production of trypanothione, a molecule present in trypanosomatids that is involved in combating oxidative stress (21). The root of this interest in the polyamine biosynthesis pathway originates from the observation that DL-α-difluoromethylornithine (DFMO) has proven effective in curing West African sleeping sickness caused by *Trypanosoma brucei gambiense*, a protozoan parasite phylogenetically similar to *Leishmania* (4, 14, 20, 56, 59). DFMO is a suicide inhibitor of ornithine decarboxylase (ODC), the enzyme that catalyzes the conversion of ornithine to putrescine (39). This drug is also effective at killing other genera of protozoan parasites *in vitro* (1, 9, 27, 49), including *Leishmania* promastigotes (35, 37, 41, 49, 55), and markedly ameliorates but does not eliminate short-term *L. donovani* infections in mice (30, 37, 45) and hamsters (42). In addition, inhibitors of a second enzyme in the polyamine pathway, *S*-adenosylmethionine decarboxylase (ADOMETDC), the enzyme that produces the decarboxylated *S*-adenosylmethionine substrate for the spermidine synthase (SPDSYN) reaction, are also effectual antitrypanosomal agents (2, 3, 6, 8, 9, 16, 18, 58).

The polyamine biosynthetic pathway of *Leishmania* is comprised of four enzymes: arginase (ARG), ODC, ADOMETDC, and SPDSYN. The genes encoding all four enzymes have been cloned, and conditionally lethal gene knockouts have been created in *L. mexicana* and *L. major* (Δ*arg* mutant only) (23, 43, 48, 52) and in *L. donovani* (Δ*odc*, Δ*spdsyn*, and Δ*adometdc* mutants) (12, 34, 50, 51) via double-targeted gene replacement. Growth studies with the mutant promastigotes revealed that each knockout was auxotrophic for polyamines as a consequence of the gene deletion events and that this nutritional deficiency could be circumvented by propagation in medium supplemented with an appropriate source of polyamine or polyamine precursor. Thus, an intact polyamine biosynthetic pathway is essential for the viability and growth of the promastigote stage of the *Leishmania* parasite.

Despite the extensive genetic and biochemical characterization of the null mutants in their promastigote stage, only recently have polyamine gene functions been assessed in the

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infectious amastigote stage of the parasite. The evaluation of polyamine gene function in amastigotes has also been hampered by the fact that the initially characterized Δ*odc*, Δ*adometdc*, and Δ*spdsyn L. donovani* knockouts were created within an attenuated wild-type strain of *L. donovani* that had lost its capacity to infect macrophages or rodents, therefore precluding functional evaluation of these genes as virulence determinants (34, 50, 51). Thus, an effort to reconstruct the genetic lesions in polyamine biosynthesis genes in a virulent *L. donovani* background was initiated.

Creation of a Δ*odc* lesion in a wild-type clone of this virulent strain dramatically compromised the ability of the parasite to infect mouse livers and spleens (12). Moreover, episomal complementation of the chromosomal lesion in *ODC* restored parasite burdens in infected organs to near wild-type levels, proving that the virulence defect in the Δ*odc* knockout was triggered by the polyamine gene deletion event (12). Recent studies also found that exogenous administration of putrescine to mice infected with Δ*odc L. donovani* partially restored virulence in the mutant parasites (45), further substantiating that the virulence defect in the Δ*odc* knockout is due to a lack of polyamine biosynthetic capacity and not to a secondary unknown function of *ODC*. The fact that a defect in the *ODC* gene so profoundly compromises the virulence of *L. donovani* suggests that other enzymes in the polyamine biosynthesis pathway might be critical virulence determinants as well.

To address the fundamental question of whether other components of the polyamine biosynthesis pathway are essential for *L. donovani* to trigger an infection in mammals, we have recapitulated the Δ*spdsyn* genotype within a virulent strain of *L. donovani* and evaluated the capacity of the Δ*spdsyn* mutant to infect mice. Here we report that parasite burdens in livers and spleens of mice infected with the knockout parasites are significantly lower than those in livers and spleens of mice infected with the wild-type strain. These studies demonstrate that *L. donovani* amastigotes require SPDSYN activity to sustain a robust infection in mice and indicate that SPDSYN and perhaps all of the enzymes in the polyamine pathway are realistic therapeutic targets for the treatment of leishmaniasis.

MATERIALS AND METHODS

Parasite cell culture. Parasites used in this study were derived from the wild-type LdBob strain of *L. donovani* (29) obtained from Stephen M. Beverley (Washington University, St. Louis, MO). LdBob promastigotes were routinely cultivated at 26°C, pH 6.9, in previously described medium (29) with 10% chicken serum or 5% Serum Plus (Gibco Cell Culture, Carlsbad, CA). Wild-type parasites were cultured in the absence of supplementation, while the Δ*spdsyn* mutants were supplemented either with 100 μM spermidine alone or with 100 μM spermidine to which 50 μg/ml hygromycin and 20 μM puromycin were added to maintain appropriate selective pressure. The "add-back" strain, Δ*spdsyn1*(pXG-BSD-*SPDSYN*), was routinely propagated in 20 μg/ml blasticidin to maintain the multicopy episomal plasmid containing the full-length *SPDSYN* gene.

Construction of Δ*spdsyn* knockouts. The cloning and sequencing of *SPDSYN* and its adjacent flanking regions from an *L. donovani* cosmid library have been reported previously (50). The *SPDSYN*/*spdsyn* heterozygotes were constructed using the same drug resistance cassette, pX63-HYG-Δ*spdsyn*, employed in the derivation of the Δ*spdsyn* mutants in the previously described avirulent DI700 strain of *L. donovani* (50). The Δ*spdsyn* null mutants were created using the drug resistance cassette pX63-PAC-Δ*spdsyn*, which was constructed using the same methods used to construct pX63-HYG-Δ*spdsyn* (50). These targeting vectors contain the hygromycin phosphotransferase and puromycin *N*-acetyltransferase markers flanked by sequences derived from the 5′ and 3′ untranslated regions of *SPDSYN*. Linear targeting DNAs were excised from plasmid by restriction digest with HindIII and BglII and gel purified prior to transfection as described previously (34). Wild-type *L. donovani* promastigotes were transfected via electroporation with linearized pX63-HYG-Δ*spdsyn* according to standard protocols using a GenePulser XCell (Bio-Rad) system and previously described parameters (54). Transfected parasites were plated on semisolid agar containing 50 μg/ml hygromycin, colonies were expanded, and homologous recombination was confirmed by Southern blotting. One *SPDSYN*/*spdsyn* heterozygote clone was then subjected to a second round of transfection using the excised targeting construct from pX63-PAC-Δ*spdsyn* and plated on semisolid agar containing 50 μg/ml hygromycin, 20 μM puromycin, and 100 μM spermidine. Colonies were expanded, and Southern blot analysis was employed to confirm the deletion of both wild-type *SPDSYN* alleles. Two independent Δ*spdsyn* clones, Δ*spdsyn1* and Δ*spdsyn2*, were chosen for further analysis.

Creation of Δ*spdsyn*(p*SPDSYN*) add-back parasites. The Δ*spdsyn1*-knockout line was functionally complemented by transfecting a chimeric plasmid containing the *L. donovani SPDSYN* open reading frame ligated into the pXG-BSD leishmanial expression plasmid (28). The add-back line was selected in medium containing 20 μg/ml blasticidin and lacking polyamine and was designated Δ*spdsyn1*(pXG-BSD-*SPDSYN*) according to the generally accepted genetic nomenclature for *Leishmania* (17).

Southern and Western blot analyses. For Southern blot analysis, genomic DNA from logarithmic-growth-phase wild-type and Δ*spdsyn* parasites was prepared using a DNeasy kit (Qiagen Inc.) according to the manufacturer's protocol. Genomic DNA was digested with either SalI and probed with a 1.0-kb fragment of the *SPDSYN* coding region or digested with XhoI and probed with a 1.2-kb fragment of the *SPDSYN* 5′ flanking region as described previously (50).

For Western blot analysis, parasite lysates were prepared from logarithmic-phase wild-type, Δ*spdsyn*, and Δ*spdsyn1*(pXG-BSD-*SPDSYN*) cell lines and were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (38) and blotted onto Immun-Blot polyvinylidene difluoride membranes (Bio-Rad). The membranes were probed with monospecific polyclonal antibody raised to the purified *L. donovani* SPDSYN (50, 53) or with commercially available anti-α-tubulin mouse monoclonal antibody (DM1A) (Calbiochem).

Growth phenotypes of Δ*spdsyn* promastigotes. Wild-type, Δ*spdsyn1*, and Δ*spdsyn1*(pXG-BSD-*SPDSYN*) promastigotes were inoculated at a density of 5×10^4 cells/ml into growth medium containing 5% Serum Plus and appropriate drug selections. Cultures were maintained at 26°C with 5% CO_2. Δ*spdsyn1* parasites were starved of exogenous polyamines 2 days prior to growth phenotyping, and upon inoculation, 100 μM spermidine or 100 μM putrescine was added as appropriate. Parasites were enumerated after 4 days by hemacytometer. The inoculation density was subtracted from the final cell density, as cell growth and multiplication were of primary interest.

Infectivity studies with mice. Groups of five 6-week-old female BALB/c mice (Charles River Laboratories) were each inoculated by tail vein injection with 5×10^6 of either wild-type, Δ*spdsyn1*, or Δ*spdsyn1*(pXG-BSD-*SPDSYN*) stationary-phase promastigotes grown in medium containing 10% chicken serum. Prior to the mouse injections, each of the three strains were passaged through mice for 10 days in order to eliminate parasites that had become attenuated in response to prolonged culture. At 4 weeks postinjection, livers and spleens were harvested and passaged through 70-μm-mesh-size cell strainers to create single-cell suspensions. To quantify parasite loads, limiting dilution assays were performed as described previously (13). Serial 4-fold dilutions of liver and spleen homogenates in 96-well microtiter plates were cultivated at 26°C with 5% CO_2 in medium containing 5% Serum Plus with 100 μM spermidine supplementation for the Δ*spdsyn* strain. Growth in individual wells was assessed after 3 weeks by visual inspection, and graphs displaying mean parasite burdens and standard deviations from five animals were constructed. Note that all mouse studies were carried out in compliance with guidelines established by the Institutional Animal Care and Use Committee at Oregon Health and Science University.

RESULTS

Molecular characterization of Δ*spdsyn* lines in LdBob *L. donovani*. Although Δ*spdsyn L. donovani* promastigotes have been described previously, the genetic lesion was created within an avirulent strain that precluded ascertaining whether *SPDSYN* was an indispensable virulence determinant (50). Thus, the Δ*spdsyn* lesion was reconstructed in LdBob, a virulent *L. donovani* strain that is capable of establishing robust visceral infections in mice (11, 12, 19, 29). Two independent

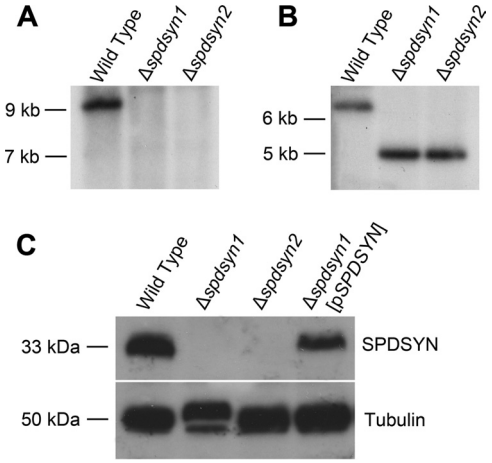

FIG. 1. (A and B) Molecular characterization of the *SPDSYN* locus and SPDSYN expression in the Δ*spdsyn* knockouts. Two micrograms of genomic DNA from wild-type, Δ*spdsyn1*, or Δ*spdsyn2* parasites was digested with SalI and hybridized to a 1.0-kb fragment of the *SPDSYN* coding region (A) or XhoI and hybridized to a 1.2-kb fragment of the 5′ flanking region (B). Molecular size markers are indicated, and equal loading of DNA was verified via ethidium bromide staining. (C) For protein expression analysis, protein lysates from 1.0×10^6 wild-type, Δ*spdsyn1*, Δ*spdsyn2*, and Δ*spdsyn1*(pXG-BSD-*SPDSYN*) [Δ*spdsyn1*(p*SPDSYN*)] promastigotes were fractionated by SDS-PAGE, blotted, and probed with polyclonal antibodies against *L. donovani* SPDSYN. Tubulin expression was analyzed as a loading control.

Δ*spdsyn* clones were isolated after double-targeted gene replacement and designated Δ*spdsyn1* and Δ*spdsyn2*. In order to authenticate the deletion of both *SPDSYN* alleles, Southern blot analysis was performed on genomic DNA prepared from wild-type, Δ*spdsyn1*, and Δ*spdsyn2* parasites using probes to the *SPDSYN* gene coding region (Fig. 1A and B). Ethidium bromide staining confirmed equal loading of chromosomal DNA, and the Southern analysis demonstrated the expected genetic lesion. To further substantiate the gene knockout, expression of the SPDSYN protein was examined by Western blotting in the same three cell lines as well as an add-back line, Δ*spdsyn1*(pXG-BSD-*SPDSYN*) [Δ*spdsyn1*(p*SPDSYN*)] consisting of the Δ*spdsyn1* mutant with a multicopy episome containing the *SPDSYN* open reading frame (Fig. 1C). Polyclonal antisera specific for SPDSYN recognized the expected ~33-kDa protein corresponding to the predicted *SPDSYN* translation product (50) in both wild-type and Δ*spdsyn1*(p*SPDSYN*) lysates, while this band was not detected in extracts prepared from Δ*spdsyn1* and Δ*spdsyn2* lysates (Fig. 1C). The blot was also probed with antitubulin antibody as a loading control.

Δ*spdsyn* parasites are polyamine auxotrophs. A growth assay was performed to determine the reliance of the Δ*spdsyn* knockout cell lines on polyamines, and as anticipated, Δ*spdsyn1* promastigotes were auxotrophic for polyamines. While wild-type parasites had undergone approximately 10 to 11 cell doublings during the course of the growth experiment, the mutant cell line was unable to proliferate in polyamine-deficient medium (Fig. 2). Visual inspection of the Δ*spdsyn1* promastigotes incubated under these nonpermissive conditions at the end of the experiment revealed a few surviving parasites that were misshapen and lethargic. These parasites did not recover when maintained for longer periods of time under nonpermissive conditions. The polyamine auxotrophy of the Δ*spdsyn1* null mutant was circumvented genetically in the Δ*spdsyn1*(p*SPDSYN*) promastigotes by complementation with the covering *SPDSYN* plasmid or nutritionally by supplementation of the culture medium with spermidine (Fig. 2). The polyamine auxotrophy could not, however, be bypassed by the addition of putrescine, the product of ODC and a substrate of SPDSYN (Fig. 2). The Δ*spdsyn2* clone displayed a growth phenotype identical to that of Δ*spdsyn1* but was not examined in as great of detail (data not shown).

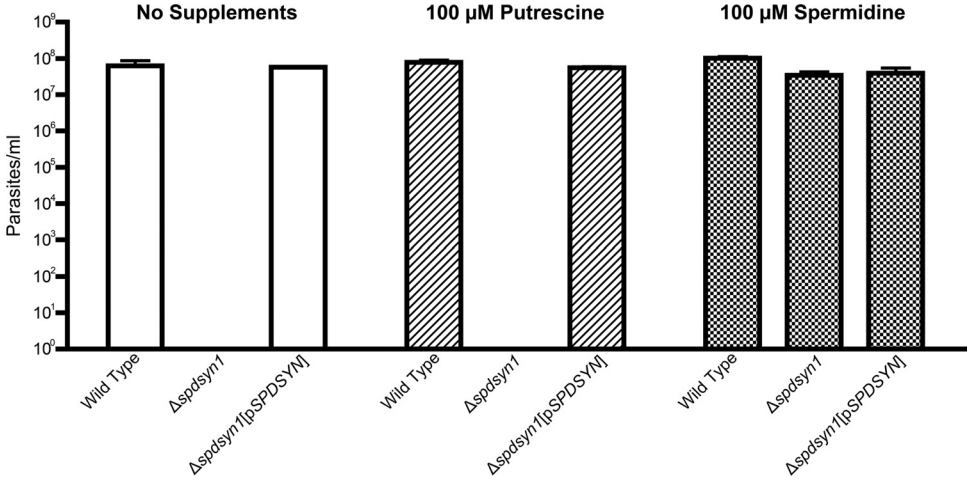

FIG. 2. Growth phenotype of Δ*spdsyn* promastigotes. Wild-type, Δ*spdsyn1*, and Δ*spdsyn1*(pXG-BSD-*SPDSYN*) [Δ*spdsyn1*(p*SPDSYN*)] promastigotes were incubated in their respective growth media in the absence or presence of 100 μM spermidine or 100 μM putrescine. Δ*spdsyn1* parasites were starved of exogenous polyamines 2 days prior to the start of the growth assay. Parasites were inoculated at 5×10^4 cells/ml, maintained at 26°C with 5% CO_2, and enumerated after 4 days. Reported cell densities represent final cell densities minus inoculation densities. Final cell densities of Δ*spdsyn1* parasites grown under nonpermissive conditions were significantly lower than those of the initial inocula, and thus, growth was zero.

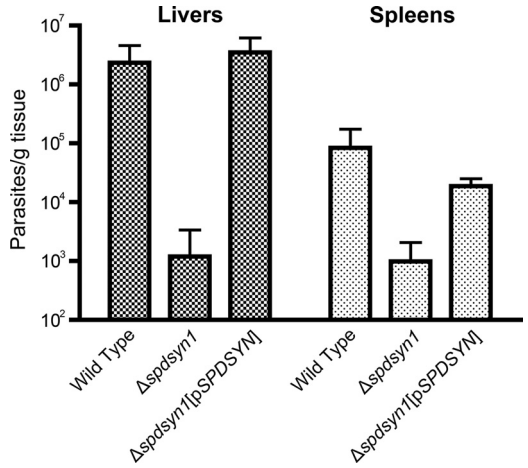

FIG. 3. Parasite burdens in mice. Three separate cohorts of BALB/c mice were infected with either wild-type, Δ*spdsyn1*, or Δ*spdsyn1*(pXG-BSD-*SPDSYN*) [Δ*spdsyn1*(p*SPDSYN*)] stationary-phase promastigotes as described in Materials and Methods. Mice were killed after 4 weeks, and parasite loads in liver and spleen preparations were determined by limiting dilution. The experiment was repeated three times with similar conclusions.

Δ*spdsyn L. donovani* has reduced virulence *in vivo*. Virulence studies in BALB/c mice were carried out to determine the effects of a genetic deficiency of *SPDSYN* and to further characterize the SPDSYN enzyme as a potential therapeutic target. Groups of five BALB/c mice were inoculated with either wild-type, Δ*spdsyn1*, or Δ*spdsyn1*(p*SPDSYN*) promastigotes, and after 4 weeks parasite loads were quantified in livers and spleens, the two organs most affected by a visceralizing *Leishmania* infection. The ability of the Δ*spdsyn1*-knockout parasites to infect mice was severely compromised (Fig. 3). The average parasite burdens in livers and spleens of mice infected with wild-type parasites were 3 and 2 orders of magnitude higher, respectively, than those in livers and spleens of mice infected with Δ*spdsyn* parasites. The virulence defect of the knockout lines was rescued by episomal complementation with parasitemias in livers and spleens of Δ*spdsyn1*(p*SPDSYN*)-infected mice equivalent to those obtained in mice inoculated with wild-type parasites (Fig. 3). It should be noted that the discrepancy in the absolute numbers of wild-type parasites between livers and spleens on a per-gram basis is typical for *L. donovani* infections (10, 22, 36, 44).

DISCUSSION

Previous studies with *Leishmania* null mutants have established that each component of the polyamine biosynthetic pathway, ARG, ODC, ADOMETDC, and SPDSYN, is indispensable for the viability and growth of the promastigote stage of the parasite (34, 48, 50–52). Whether all of these enzymes are also essential for the amastigote to maintain an infection is less clear, because virulence data with knockout strains from several species have not offered a consistent conclusion. Parasite burdens and lesion sizes of mice infected with Δ*arg L. mexicana* (23) or Δ*arg L. major* (43, 48) were only somewhat lower than those of mice infected with the corresponding wild-type line from which these mutants were derived. In contrast, parasite loads in mice infected with Δ*odc L. donovani* (12) were dramatically reduced by many orders of magnitude compared to those in mice inoculated with the wild-type progenitor. Whether the differences in the impact of the genetic lesions can be ascribed to the nature of the genetic lesion, to species differences, or to differences in the cutaneous and visceral environments in which the species reside is unknown. Resolution of this fundamental question requires the evaluation of mutations of the same polyamine pathway gene in the different *Leishmania* species, i.e., a comparison of a Δ*odc* mutation in *L. donovani*, *L. mexicana*, and *L. major*.

To establish whether null mutations in the *L. donovani* polyamine biosynthetic pathway other than *ODC* could also affect the virulence of the organism, a null mutant at the *SPDSYN* locus was created in a virulent wild-type background. The Δ*spdsyn* mutation conferred polyamine auxotrophy (Fig. 2) and caused a substantial reduction in parasite burdens in mice compared to the wild type (~3 orders of magnitude reduction in livers and 2 orders of magnitude reduction in spleens) (Fig. 3), although the extent of the virulence defect in mice was less than that previously observed for Δ*odc L. donovani* (12).

It should be noted that livers and spleens from mice inoculated with Δ*spdsyn* parasites retained a small population of persistent parasites, ~10^3 per liver and spleen, after the 4-week infectivity experiment (Fig. 3). The mechanism of this persistence is unclear and is difficult to analyze at the molecular level since the persistent parasites can be resurrected only under permissive growth conditions *in vitro* (Fig. 2). One could speculate that this persistence is analogous to the situation of dying but enduring Δ*spdsyn* parasites observed under nonpermissive growth conditions *in vitro* (Fig. 2) and infer that it takes >4 weeks to kill amastigotes by polyamine starvation *in situ*. However, whether these persistent Δ*spdsyn* amastigotes would eventually expire is difficult to assess because BALB/c mice eventually clear *L. donovani* parasites, with parasitemias beginning to decrease at 4 to 8 weeks postinfection (60). A more suitable model for assessing long-term virulence of this parasite is the Syrian golden hamster, a rodent capable of mimicking the human form of visceral leishmaniasis (22). Testing Δ*spdsyn* virulence in hamsters is a logical next step in SPDSYN target validation.

The reason for the discrepancy in the virulence deficit triggered by the Δ*odc* and Δ*spdsyn* lesions in an otherwise isogenic background is not known but could be ascribed to differences in the putrescine and spermidine contents of the phagolysosome in which visceral macrophages reside. It is likely that *L. donovani* amastigotes require both putrescine and spermidine for optimal proliferation, as has been shown for promastigotes (34). If, for example, the phagolysosomal environment contains pools of spermidine but not putrescine, then a Δ*odc* lesion would be expected to have far more deleterious consequences to the parasite than a Δ*spdsyn* defect, because *L. donovani* lacks a back-conversion pathway from spermidine to putrescine but can readily synthesize spermidine from putrescine through ODC. The conjecture that the putrescine and spermidine pools of the phagolysosome are different can be further explored by determining the virulence properties of Δ*adometdc L. donovani* parasites, which, like Δ*spdsyn* parasites, require spermidine but not putrescine for survival (51).

It should be noted that Δ*odc L. donovani* promastigotes are

much more susceptible to putrescine withdrawal than Δ*spdsyn L. donovani* promastigotes are to spermidine withdrawal (personal observations). This intriguing observation is mirrored in *Trypanosoma brucei*, where genetic investigations showed that depletion of putrescine results in a more rapid cell death than spermidine depletion (62). Although the cellular functions of polyamines are not completely understood, it appears to be evident that putrescine is more than just a precursor for the production of spermidine, which could account for the discrepancy in the magnitude of the virulence deficit caused by the Δ*odc* and Δ*spdsyn* lesions.

Another explanation for the Δ*odc* and Δ*spdsyn* virulence discrepancy is the possibility that the amastigote form of the parasite exhibits different capabilities in transport of putrescine versus spermidine. *Leishmania* promastigotes express robust polyamine transport activities (7), which can account for the ability of putrescine to rescue Δ*odc* parasites (12) and spermidine to enable Δ*spdsyn* parasite growth (Fig. 2), but less is known about transport in the amastigote stage. It should be noted that the only leishmanial polyamine transporter that has been identified at the molecular level is enormously downregulated in the amastigote stage of the parasite (31). However, axenic amastigotes of Δ*odc L. donovani* were able to proliferate in 200 μM putrescine (12), and Δ*spdsyn L. donovani* axenic amastigotes survive in 200 μM spermidine (personal observation), suggesting that the polyamine transport machinery remains somewhat functional in the infectious stage of the parasite, but the extent is unknown. The crippling effects of Δ*odc* or Δ*spdsyn* mutations on the establishment of virulence by *L. donovani*, together with the at least minimally functional polyamine transport activities in the amastigote stage, suggest that the phagolysosomal compartment in which the parasite resides does not provide sufficient exogenous polyamine to bypass a genetic defect in polyamine biosynthesis.

A pairwise alignment of the *L. donovani* and human SPDSYN primary structures revealed an ~56% identity (50), and the amino acids in the human crystal structure that make contact with the substrate (61) are conserved in the parasite enzyme. However, similarities in the active site or ligand-binding pockets of the *L. donovani* and human SPDSYNs do not preclude this enzyme from potential drug targeting. For example, DFMO, the specific ODC inhibitor used in West African sleeping sickness chemotherapy, displays selectivity for the metabolic machinery of *T. brucei* due to discrepant stabilities of the parasite and human ODC enzymes (25, 26, 47) and not to disparities in the affinity of DFMO for the two proteins (46). Furthermore, differential susceptibility to potential inhibitors could be caused by the presence of a parasite-specific allosteric site on the leishmanial SPDSYN or to other discrepancies in the parasite polyamine pathway, such as the lack of the mammalian back-conversion pathway that transforms spermine to spermidine (15).

The finding that an additional lesion in the polyamine biosynthetic pathway dramatically moderates *L. donovani* virulence implies that the virulence deficit of Δ*odc L. donovani* is not specific to the lesion but rather is specific to a more generalized polyamine biosynthetic defect. Moreover, the fact that two independent genetic lesions in the polyamine biosynthesis pathway both cause a striking decrease in the capacity of *L. donovani* to infect mouse livers and spleens provides strong support that the cause of the avirulence is due to a lack of polyamine biosynthetic capacity and not to a secondary unknown function of the *ODC* or *SPDSYN* gene in the amastigote. This study demonstrates that Δ*spdsyn* parasites are greatly reduced in their ability to infect a mammalian host and further validates additional enzymatic components of the polyamine biosynthetic pathway as potential targets for the treatment of visceral and perhaps other forms of leishmaniasis.

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