Ornithine Decarboxylase Gene Deletion Mutants of Leishmania donovani*

(Received for publication, September 25, 1998, and in revised form, November 13, 1998)

Yuqui Jiang‡‡, Sigrid C. Roberts‡‡, Armando Jardim‡, Nicola S. Carter‡, Sarah Shih‡‡, Mark Ariyanayagam‡, Alan H. Fairlamb‡, and Buddy Ullman‡‡**

From the ‡Department of Biochemistry and Molecular Biology, Oregon Health Sciences University, Portland, Oregon 97201-3098 and *Department of Biochemistry, University of Dundee, Dundee DD1 4HN, Scotland, United Kingdom

A knockout strain of Leishmania donovani lacking both ornithine decarboxylase (ODC) alleles has been created by targeted gene replacement. Growth of Δodc cells in polyamine-deficient medium resulted in a rapid and profound depletion of cellular putrescine pools, although levels of spermidine were relatively unaffected. Concentrations of trypanothione, a spermidine conjugate, were also reduced, whereas glutathione concentrations were augmented. The Δodc L. donovani exhibited an auxotrophy for polyamines that could be circumvented by the addition of the naturally occurring polyamines, putrescine or spermidine, to the culture medium. Whereas putrescine supplementation restored intracellular pools of both putrescine and spermidine, exogenous spermidine was not converted back to putrescine, indicating that spermidine alone is sufficient to meet the polyamine requirement, and that L. donovani does not express the enzymatic machinery for polyamine degradation. The lack of a polyamine catabolic pathway in intact parasites was confirmed radiometrically. In addition, the Δodc strain could grow in medium supplemented with either 1,3-diaminopropane or 1,5-diaminopentane (cadaverine), but polyamine auxotrophy could not be overcome by other aliphatic diamines or spermine. These data establish genetically that ODC is an essential gene in L. donovani, define the polyamine requirements of the parasite, and reveal the absence of a polyamine-degradative pathway.

Polyamines are cationic compounds that play essential roles in cell proliferation, differentiation, and macromolecular synthesis (1–3). Ornithine decarboxylase (ODC)1 catalyzes the conversion of ornithine to putrescine (1,4-diaminobutane) and is the initial and rate-limiting enzyme in polyamine biosynthesis in most organisms (4). The ODC enzyme of protozoan parasites is a polyamine-degradative pathway.

phase of African sleeping sickness caused by Trypanosoma brucei gambiense (3, 6). DFMO is also active against T. b. rhodesiense and T. congolense in murine models and has proven effective against other genera of protozoan parasites in vivo and in vitro, including Plasmodia (7), Giardia (8), and Leishmania (9). DFMO blocks the de novo synthesis of both putrescine and spermidine in T. brucei, and to dissect the polyamine pathway in intact parasites, a null mutant of L. donovani has been created by double-targeted gene replacement in which both wild type ODC alleles have been sequentially eliminated. The phenotypic dissection of the parasites in which the ODC copy number has been genetically altered has established the essential role of ODC in polyamine metabolism, reveals significant discrepancies between the polyamine pathway of the parasite and host cells, has important implications in understanding the therapeutic relevance of the polyamine pathway, and supports a general strategy for the creation of attenuated strains for vaccine development in prophylaxing leishmaniasis.

MATERIALS AND METHODS

Materials, Chemicals, and Reagents—[COOH-14C]Ornithine (50–60 mCi/mmol) was obtained from Moravek Biochemicals (Brea, CA), whereas [14C]spermidine trihydrochloride (113 mCi/mmol) and [14C]spermine tetrahydrochloride (115 mCi/mmol) were procured from Amersham Pharmacia Biotech. Diamines were purchased from Sigma. DFMO was a gift from the Merrell Dow Research Institute (Cincinnati, OH). The pX63-NEO and pX63-HYG plasmids used in the parasite transfections and pSNBR used in the subcloning of a cosmid fragment were generously provided to this laboratory by Dr. Stephen M. Beverley (Washington University, St. Louis, MO). Purified L. donovani ODC was furnished by Dr. Margaret A. Phillips (University of Texas Southwest Medical Center, Dallas, TX). All other materials, chemicals, and reagents used in these experiments have been described previously (14–16) and were of the highest quality commercially available.

Cell Culture and Preexisting Cell Lines—L. donovani promastigotes, the extracellular insect vector form of the parasite, were grown in DME-L, a completely defined culture medium especially designed for the cultivation of Leishmania (17). In specified experiments, cells were propagated in a modified DME-L medium, DME-L-CS, in which the bovine serum albumin component of DME-L was replaced with 10%
chicken serum. The DI700 cell line is a wild type clone of the Sudanese 1S strain of L. donovani that was used for DNA isolation and library construction and as a recipient strain in all initial transfections. For the purposes of the genetic manipulations reported in this study, DI700 is denoted ODC+/n, in which + refers to the wild type allele. Growth rate determinations were performed in the absence of polyamines and diamines were carried out as described previously (9, 14).

DNA Manipulations and Library Construction—Genomic DNA was isolated from L. donovani promastigotes by standard protocols (14, 15). Southern blot analysis was performed as described previously (14, 15). A cosmids library was prepared from DI700 genomic DNA that was partially digested with Sau3AI, and 30–45-kb fragments were ligated into the BamHI site of the Supercos 1 cosmid vector using protocols described in the brochure from Stratagene (La Jolla, CA).

Isolation of a Cosmid Containing ODC—A cosmid encompassing ODC designated ODC-5L1 was isolated and purified using the L. donovani ODC as a probe and stringent hybridization and wash conditions as described previously (14, 15). The L. donovani ODC was originally isolated from a bacteriophage clone as described previously by this laboratory (14). Restriction mapping of the cosmid was described in the relevant brochure from Stratagene.

Oligonucleotide Primers—Primers used in the amplification of the 5'- and 3'-flanking regions of ODC by the polymerase chain reaction (PCR) are as follows, with their restriction enzyme sites underlined: 5’F5', 5’-CCATGGCATCCGTCGAC-3’; 5’F3', 5’-GAGGACGCCGCG-3’; 3’F3’, 5’-TCCCCGGGGGATGCACCGCCAG-3’; and 3’F3’, 5’-GAAGATCGAGGACCCTTACT-3’.

Molecular Constructs for the Replacement of ODC Alleles—To construct appropriate vectors for the replacement of each wild type ODC allele, a 0.8-kb fragment (5’F) consisting of 410 bp of 5’- untranslated DNA and 390 bp of the ODC open reading frame was ligated into the HindIII-SalI site, and a 2.0-kb fragment of 5’-translated DNA (3’F) was inserted into the Smal-3’SalII site of both the PX63-NEO (18) and PX63-HYG (19) vectors (Fig. 1). Both 5’F and 3’F were amplified by PCR using standard reaction conditions (15 cycles; 94°C for 60 s, 50°C for 30 s, 72°C for 30 s) for the amplification of DNAs from plasmid or cosmid DNA templates (18). 5’F was generated from a 15-kb HindIII-EcoRI fragment of ODC-5L1 encompassing ODC that had been subcloned into pSNBR (20), whereas 3’F was amplified from the 9.8-kb SalI fragment described by Hanson et al. (14). Sense and antisense primers used in the PCR were 5’F5’ and 5’F3’ for 5’F and 3’F5’ and 3’F3’ for 3’, respectively. These PCR products were digested with the appropriate restriction enzymes; 5’F, which has an internal SalI site, was cleaved with HindIII and SalI, whereas 3’F was digested with Smal and HindIII, and ligated sequentially into PX63-NEO and PX63-HYG. The presence of the flanking regions and their orientation in the knockout vectors were confirmed by nucleotide sequencing (21) and restriction mapping. The recombinant PX63-NEO and PX63-HYG vectors containing the ODC flanking regions are designated PX63-NEO-dodc and PX63-HYG-dodc, respectively (Fig. 1, B and C).

Transfections—Parasites were transfected by electroporation using conditions similar to those described previously (18). PX63-NEO-dodc and PX63-HYG-dodc were linearized with HindIII and BglII and gel purified before electroporation. In the construction of the dodc strain, the first wild type ODC allele was replaced with px63-NEO-dodc to create the ODC/dodc heterozygote (designated ODC+/n), whereas the second wild type allele was deleted from the heterozygote with PX63-HYG-dodc to create the homozygous dodc knockout null strain (designated ODC-). Electroporated parasites were maintained in liquid medium for 24 h before plating on a drug-containing semisolid medium. Drug-resistant clones transfected with PX63-NEO-dodc were isolated from plates containing 20 μg/ml Genetin (G418), whereas parasites transfected with PX63-HYG-dodc were selected in 20 μg/ml G418, 50 μg/ml hygromycin, and 100 μg/pseudamine. Colonies isolated after transfer with either PX63-NEO-dodc or PX63-HYG-dodc were picked into 1.0 ml of liquid DME-L containing the relevant selectable and indispensable agents, expanded, and analyzed for the appropriate allelic replacements by Southern blotting. The ODC+/dodc and ODC−dodc transformants were maintained continually in the appropriate selective media unless otherwise indicated.

Western Blotting—Polyclonal antibody to purified L. donovani ODC was generated in rabbits by conventional methods (22). Promastigotes were lysed by sonication, and cell supernatants were prepared by centrifugation at 20,000 g x 10 min. 10 μg of protein from each cell line were fractionated by SDS-polyacrylamide gel electrophoresis (23), blotted onto nitrocellulose membranes using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad), and subjected to Western blot analysis by standard protocols (22).

Polyamine Pool Determinations—5.0 × 106 parasites were harvested and extracted for polyamine pool determinations with 20% trichloroacetic acid as described previously (24). An internal 1,7-diaminohexane standard (40 μg/ml) was added to the trichloroacetic acid supernatants. The trichloroacetic acid was extracted with ethyl acetate as reported previously (24), and the samples were dried on a Speed Vac concentrator. Samples were pre-column derivatized with dansyl chloride using previously reported protocols (25), except that the sample volumes were 100 μl. 100 μl of 25% proline were added to scavenges the excess dansyl chloride. The derivatized polyamines were recovered with two ethyl acetate extractions, and the organic layers were pooled and dried. Samples were resuspended in 200 μl of 95% methanol/5% acetic acid, and polyamines were analyzed by high performance liquid chromatography on a Beckman system equipped with a C8 reversed phase column (Bio-Rad) as described previously (25). Relative fluorescence was measured on a Shimadzu RF-535 fluorescence high performance liquid chromatography monitor at excitation and emission wavelengths of 365 and 485 nm, respectively. Peak areas were calculated using a Hewlett Packard HP3396 series II integrator and compared with those of known polyamine standards.

Thiol Pool Measurements—1.0 × 108 cells were prepared for thiol pool determinations and derivatized with monobromobimane as described previously (24). Derivatized thiols were fractionated by high performance liquid chromatography over a Ymae C8 reversed phase column as reported previously (26). Relative fluorescence was measured at excitation and emission wavelengths of 395 and 480 nm, respectively. Peak areas were calculated as described for the polyamine pool determinations.

Radilabeled Polyamine Incorporation Experiments—5.0 × 106 wild type promastigotes were incubated with 2 μCi of [14C]putrescine (113 mCi/mmol) or [14C]spermine (115 mCi/mmol) in 5 ml of DME-L-CS under normal growth conditions for 48 h. Polyamine pools were processed and chromatographed as described above. 1-ml fractions were collected, and radioactivity was quantified by liquid scintillation spectrometry. The positions of the radiolabeled polyamines were determined by co-injection with polyamine standards.

RESULTS

Replacement of the ODC Alleles—To disrupt the ODC locus, a single copy gene in L. donovani (14), each allele was sequentially replaced with a drug resistance cassette. The first ODC allele was replaced with PX63-NEO-dodc to create the ODC+/dodc heterozygote, and the second was replaced with PX63-HYG-dodc to create the null ODC−dodc mutant. In each round of transfection, 4 × 107 promastigotes were transfected with the appropriate linearized DNA fragments, and ~100 drug-resistant colonies were obtained from each plating. The homoyogotes were selected in medium supplemented with 100 μg/pseudamine, because it has been established previously that pharmacologic simulation of a genetic deficiency of ODC in L. donovani by the incubation of intact parasites with DFMO could be circumvented by the addition of the diamine to the culture medium (9). G418 was also added to the hygromycin resistance selections to ensure that the second round of gene targeting yielded cell lines in which PX63-HYG-dodc had replaced the wild type allele of the ODC+/dodc heterozygote.

Southern blot analysis of the ODC+/dodc, ODC−/dodc, and ODC−dodc strains revealed the new alleles that had been constitutive by homologous gene replacement events (Fig. 2). These altered alleles could be effectively discriminated from the wild type allele by the positions of distinct SacI sites located within the endogenous ODC locus, PX63-NEO-dodc, and PX63-HYG-dodc (Fig. 5). These allelic differences are demonstrated in Fig. 2. Digestion of genomic DNA prepared from ODC+/dodc, ODC−/dodc, and ODC−dodc cells with SacI and hybridization with the 0.8-kb 5'-flanking probe 5’F (Fig. 1, Probe A) revealed only the expected wild type hybridization signals at 1.6 and 1.3 kb (Fig. 2A). A novel 3.2-kb band was observed in SacI-digested genomic DNA from ODC−/dodc with a concomitant diminution of the hybridization intensity of the 1.6-kb signal from the 5'-
flanking region of the wild type allele. A similar digestion of ODC<sup>wt</sup> DNA showed the loss of the 1.6-kb fragment from the wild type allele and an increase in the hybridization signal at 3.2 kb. As expected from the restriction maps (Fig. 1), no changes in the 1.3-kb signal were observed (Fig. 2A). A parallel digestion of genomic DNA with SacI-SalI and probing with the 2.0-kb 3<sup>9</sup>F-flanking probe 3<sup>9</sup>F (Fig. 1, Probe B) also confirmed the presence of the new alleles in ODC<sup>+/n</sup> and ODC<sup>n/h</sup> cells and the disappearance of the wild type counterparts. The SacI-SalI band that hybridizes to 3<sup>9</sup>F is 2.3 kb (see Fig. 1), whereas the fragments from the alleles disrupted by pX63-NEO-<i>odc</i> and pX63-HYG-<i>odc</i> are 2.8 kb (Fig. 2B). Finally, to establish that the drug-resistant clones were truly deficient in ODC coding region sequences, genomic DNA from the three genotypes was digested with SalI and probed with a 1.3-kb BamHI-StuI fragment located within the protein coding portion of ODC (Fig. 1, Probe C). As anticipated, a 3.9-kb hybridization signal corresponding to the wild type allele was observed in the ODC<sup>+/+</sup> line. The same fragment, exhibiting approximately half the signal intensity of the wild type 3.9-kb band, was observed in the ODC<sup>+/n</sup> heterozygote, whereas the band was absent in the ODC<sup>n/h</sup> knockout. It is worth noting that after both rounds of transfection, clones displaying genetic events other than simple gene replacements were detected by Southern blotting at a frequency of 20%, but these more complex genetic alterations were not analyzed further.

**ODC Expression in ODC<sup>+/+</sup>, ODC<sup>−/−</sup>, and ODC<sup>+/−</sup> L. donovani**—To evaluate the phenotypic consequences of ODC replacement, ODC activity and protein were measured in wild type and genetically manipulated strains. As shown in Fig. 3,
levels of ODC activity in ODC<sup>+/+</sup>, ODC<sup>−/−</sup>, and ODC<sup>+/−</sup> cells were directly proportional to the ODC copy number. ODC activity in the heterozygous deletion mutant was ~50% that of wild type cells, and no ODC activity could be detected in the ODC<sup>+/−</sup> double knockout. Western blot analysis of ODC<sup>+/+</sup>, ODC<sup>−/−</sup>, and ODC<sup>+/−</sup> cell extracts demonstrated that the observed reduction in ODC activity in the single and double knockouts was consistent with diminished cellular expression of ODC protein (Fig. 4).

**Nutritional Requirements of ODC<sup>+/−</sup> Parasites**—The nutritional requirements of the Δodc parasites were also evaluated. As demonstrated in Fig. 5, the ODC<sup>+/−</sup> double knockout could not grow in DME-L medium in the absence of putrescine, whereas ODC<sup>−/−</sup> grew as proficiently as the ODC<sup>+/+</sup> strain (data not shown). The addition of 200 μM putrescine to the culture medium averted the lethal consequences of ODC deficiency, and the growth rate of ODC<sup>+/−</sup> cells in the presence of putrescine was indistinguishable from that of the ODC<sup>+/+</sup> and ODC<sup>−/−</sup> cell lines (Fig. 5). Putrescine did not affect the growth rate of either the ODC<sup>+/+</sup> or ODC<sup>−/−</sup> cell lines. Parallel results were obtained with all three strains cultivated in DME-L-CS (data not shown). No morphological distinctions were noted among the three strains by light microscopy as long as ODC<sup>+/−</sup> cells were maintained in putrescine-supplemented medium.

The cellular polyamine requirement in ODC<sup>+/−</sup> cells could also be satisfied by the provision of 200 μM spermidine, although supplementation of the medium with equimolar spermidine, a concentration that did not affect the growth of ODC<sup>+/+</sup> parasites, did not support the growth of the knockout (Fig. 6). The experiments establishing whether spermidine and spermine could overcome cellular ODC deficiency were conducted in DME-L-CS medium to avoid the polyamine toxicity attributable to the presence of minute amounts of polyamine oxidase activity in DME-L medium. Interestingly, the polyamine requirement of ODC<sup>+/−</sup> cells could also be fulfilled by supplementing DME-L-CS with either 1,3-diaminopropane or 1,5-diaminopentane (cadaverine) (Fig. 6). However, the growth rate of Δodc cells in DME-L-CS supplemented with either 1,3-diaminopropane or cadaverine was somewhat less than that in medium supplemented with putrescine, although the final cell densities were similar (data not shown). The ODC<sup>+/−</sup> cells could be propagated continuously in DME-L-CS supplemented with 200 μM concentrations of either putrescine, spermidine, 1,3-diaminopropane, or cadaverine for >6 months. The Δodc parasites could not grow in DME-L-CS supplemented with other diamines, including 1,2-diaminoethane, 1,6-diaminohexane, 1,7-diaminohexane, 1,8-diaminoctane, 1,10-diaminodecane, and 1,12-diaminododecane, at concentrations that were nontoxic to wild type parasites.

**Polyamine and Thiol Pool Measurements**—The metabolic consequences of ODC deficiency on polyamine and thiol pools were also evaluated in Δodc cells. Thiol pools were measured, because *Leishmania* and other trypanosomatid protozoa contain millimolar concentrations of the spermidine-containing trypanothione molecule (27), a thiol that likely serves as a general reductant in these parasites (28). As shown in Table I, exponentially growing wild type and ODC<sup>+/−</sup> cells contained commensurate concentrations of putrescine, spermidine, trypanothione, glutathionylspermidine, and glutathione. The
in intracellular putrescine and spermidine pools of the Δodc cells expanded in putrescine-supplemented medium were also comparable to those of wild type and ODC<sup>+/−</sup> cells, although the thiol concentrations were significantly higher. No spermine was detected in any of these cell lines, which is consistent with previous observations that <i>L. donovani</i> lack spermine (9).

The removal of Δodc cells from putrescine-supplemented DME-L-CS precipitated a rapid depletion of cellular putrescine pools, although the levels of spermidine remained relatively constant after 12 days of maintenance in medium lacking putrescine (Fig. 7A). Reduction in ODC<sup>+/−</sup> cell numbers was not observed until day 7 after removal of the exogenous putrescine, and some augmentation in the cell density was observed through the first 4 days of incubation (Fig. 7). This marginal cell proliferation could be attributed to the fact that <i>Leishmania</i> accommodate sufficient polyamine pools to maintain viability and enable minimal growth in the absence of both polyamine biosynthesis and polyamine salvage. However, throughout the incubation period in unsupplemented DME-L-CS, levels of trypanothione in Δodc cells were much lower than those observed in ODC<sup>+/+</sup>, ODC<sup>+/−</sup>, or ODC<sup>+/+</sup> parasites grown in putrescine-containing medium, although trypanothione levels remained relatively constant, albeit low, after 24 h in the absence of putrescine (Fig. 7B). Glutathionylspermidine levels fluctuated slightly in the Δodc cell line maintained in medium lacking polyamine, whereas cellular glutathione concentrations increased fairly substantially, i.e. ~2-fold.

Polymamines and their conjugates were also measured in ODC<sup>+/−</sup> cells that had been propagated for >3 weeks in putrescine-deficient DME-L-CS supplemented with spermidine. Under these conditions, putrescine concentrations in the Δodc strain were 4% of those of wild type parasites, whereas spermidine and trypanothione pools were comparable. Polymamine and thiol pools were also measured in Δodc parasites grown in either 1,3-diaminopropane or cadaverine (Table I). Both diamines were accumulated by the ODC<sup>+/−</sup> cells, and a concomitant depletion of naturally occurring polymamines was observed. Cellular putrescine pools were negligible, and spermidine pools were markedly depleted in Δodc cells maintained in either 1,3-diaminopropane or cadaverine compared with the knockout parasites grown in putrescine or wild type parasites. The null mutant grown in either spermidine, spermine, or cadaverine also appeared to accumulate small amounts of 1,3-diaminopropane. The origin of this anomaly could be imputed to contaminants in the spermidine, spermine, and cadaverine additives. Trypanothione pools in ODC<sup>+/−</sup> cells grown in DME-L-CS supplemented with either 1,3-diaminopropane or cadaverine were also very low (2.0 and 10.5% of the levels found in putrescine-supplemented Δodc parasites; Table I).

**Polyamine Catabolism in <i>L. donovani</i>—The failure of spermidine supplementation to augment cellular putrescine pools of Δodc parasites suggested that <i>L. donovani</i> lack a polyamine-degradative pathway. To verify this conjecture, wild type parasites were incubated with either <sup>14</sup>C)<sub>S</sub>spermidine or <sup>14</sup>C)<sub>S</sub>spermine to determine whether the polymamines could be catabolized. As demonstrated in Fig. 8, <i>L. donovani</i> do not convert extracellular spermidine or spermine to putrescine, although each radiolabel is accumulated by the parasites in unaltered form. Intracellular <sup>14</sup>C)<sub>S</sub>spermidine is observed in promastigotes that had been incubated with <sup>14</sup>C)<sub>S</sub>spermine, but this could be ascribed to a trace <sup>14</sup>C)<sub>S</sub>spermidine contaminant present in the radiolabeled spermine (data not shown).

**DISCUSSION**

The creation of a Δodc strain of <i>Leishmania</i> by double-targeted gene replacement establishes that ODC plays an essential housekeeping function in this genus of protozoan parasite. Using independent drug resistance cassettes, ODC coding sequences were expunged from wild type <i>L. donovani</i> in two discrete steps, and the presumptive heterozygote generated after the first round of transfection and selection appeared to contain only one intact ODC allele as measured by loss of the hybridization signal and a ~50% reduction of ODC activity and protein as compared with the ODC<sup>+/+</sup> parent. These data confirmed previous results obtained by Southern blotting that <i>L. donovani</i> is diploid at the ODC locus, and that ODC is a single copy gene (14). The observation that Δodc cells cannot survive without putrescine supplementation of the culture medium demonstrates genetically that ODC and polymamines are indispensable to <i>L. donovani</i> and underscores the potential of the

**Table I**

**Polyamine and thiol levels in wild type and mutant <i>L. donovani</i>**

| Cell line | Additions | GSH | GS-SPD | T/SH2 | PUT | SPD | CAD | 1,3 DP | SPM |
|-----------|-----------|-----|--------|-------|-----|-----|-----|--------|-----|
| ODC<sup>+/+</sup> | None | 2.15 ± 0.37 | 0.33 ± 0.07 | 8.23 ± 1.08 | 5.00 ± 0.06 | 2.60 ± 0.06 | ND | ND | ND |
| ODC<sup>+/−</sup> | None | 3.31 ± 0.32 | 0.42 ± 0.07 | 5.31 ± 0.43 | 4.92 ± 0.44 | 2.80 ± 0.20 | ND | ND | ND |
| ODC<sup>+/−</sup> | 1,3-Diaminopropane | 4.41 ± 1.00 | 0.01 ± 0.01 | 0.26 ± 0.06 | 0.06 ± 0.04 | 0.30 ± 0.04 | ND | 19.80 ± 0.24 | ND |
| ODC<sup>+/−</sup> | Putrescine | 9.81 ± 1.80 | 1.23 ± 0.41 | 12.88 ± 2.75 | 3.07 ± 0.37 | 3.14 ± 0.34 | ND | ND | ND |
| ODC<sup>+/−</sup> | Cadaverine | 2.87 ± 0.22 | 0.05 ± 0.03 | 1.36 ± 0.03 | 0.04 ± 0.02 | 0.40 ± 0.06 | 4.86 ± 0.48 | 0.11 ± 0.06 | ND |
| ODC<sup>+/−</sup> | Spermine | 6.91 ± 1.23 | 0.54 ± 0.10 | 7.04 ± 0.79 | 0.19 ± 0.08 | 3.02 ± 0.32 | ND | ND | 0.46 ± 0.03 |
| ODC<sup>+/−</sup> | Spermidine | 7.50 ± 3.10 | 0.33 ± 0.21 | 3.09 ± 0.96 | 0.76 ± 0.02 | 2.42 ± 0.16 | 1.60 ± 0.10 | 0.36 ± 0.02 | ND |

**Fig. 6** Growth of Δodc mutants in DME-L-CS medium supplemented with various polyamines and diamines. The ability of ODC<sup>+/−</sup> cells to grow in DME-L-CS in the presence of 200 μM concentrations of putrescine ( ), spermidine ( ), spermine ( ), 1,3-diaminopropane ( ), or cadaverine ( ) is compared.
polyamine pathway as a therapeutic target. The requirement for ODC is consistent with previous observations that DFMO toxicity in *L. donovani* can be bypassed by the addition of putrescine to the culture medium (9). Furthermore, the growth phenotype of the \( D_{odc} \) strain authenticates that ODC is the sole enzyme that initiates polyamine biosynthesis in *L. donovani*. This conclusion is an important distinction, because *Escherichia coli* and plants can synthesize putrescine from arginine via an arginine decarboxylase-agmatine ureohydrolase pathway (2). Moreover, there have been reports (29, 30), although unconfirmed (31, 32), that *T. cruzi*, a protozoan parasite related to *Leishmania*, expresses an arginine decarboxylase activity that can be targeted by specific inhibitors.

Genetic studies have also demonstrated that ODC is indispensable for long-term proliferation and viability of *T. brucei* (33). After replacement of both ODC alleles with drug resistance cassettes, ODC-deficient *T. brucei* incubated without polyamines growth arrested at the G1-S-phase transition of the cell cycle. Interestingly, a small percentage of the arrested \( D_{odc} \) population remained viable 7–8 weeks after putrescine withdrawal (33). ODC-deficient mutants of mammalian cells (34) and *Neurospora crassa* (35) also could not thrive in the absence of polyamine supplement, confirming that ODC activity is mandatory for the growth of these cells. However, ODC is not essential for *E. coli* (36), which has an alternative pathway for polyamine synthesis, or for some mutant strains of *Saccharomyces cerevisiae* that are both ODC and polyamine deficient (37). Moreover, recent studies have strongly implied that *T. cruzi* lack a polyamine biosynthetic pathway altogether and are therefore obligate scavengers of polyamines (31, 32).

Although the present results establish a strict requirement for polyamines in *L. donovani*, the precise mechanism by which ODC deficiency triggers lethality has not been definitively established. Removal of \( D_{odc} \) cells from putrescine-supplemented medium triggered a rapid and virtually complete depletion of cellular putrescine pools without a concomitant diminution in spermidine levels. A similar obliteration of putrescine pools is also observed when wild type *L. donovani* are treated with DFMO (9). Incubation of ODC\(^{+/+}\) parasites in the absence of exogenous polyamines also prompted a rapid decrease in the
cellular levels of trypanothione, which were subsequently maintained at a low level, whereas concentrations of glutathione increased steadily throughout the prolonged incubation. The depletion of trypanothione could be ascribed either to the reduced flux through the polyamine biosynthetic pathway or to trypanothione turnover to replenish spermidine pools. It is possible that this augmentation of glutathione is a cellular compensation mechanism to preserve the reductive potential of the intracellular environment when trypanothione pools are reduced. The trypanothione depletion and glutathione elevation in putrescine-depleted ODCh L. donovani parallels results obtained with T. brucei in which glutathionylspermidine and trypanothione levels were both substantially depleted and glutathione levels were augmented after DFMO treatment (26). Thus, cessation of parasite growth in polyamine-deficient medium correlates with depletion of both the putrescine and trypanothione pools.

Circumvention of the conditionally lethal odc mutation can be achieved by the addition of either putrescine or spermidine to the culture medium. Given that the null mutant propagated in spermidine-supplemented medium exhibited a profound reduction of the putrescine pool, the most obvious inference is that L. donovani does not require intracellular putrescine to survive, and that spermidine is both sufficient and necessary to satisfy the polyamine requirement of the parasite. Spermine, a major polyamine of mammalian cells, is not detected in L. donovani (9), supporting the lack of a spermine synthase activity. Moreover, exogenous spermine does not satisfy the polyamine requirement of the odc promastigotes, a distinction from what is observed in mammalian cells in which exogenous spermine can circumvent a genetic deficiency in ODC activity (34). In mammalian cells, spermine is converted to spermidine and spermidine is converted to putrescine by an identical two-enzyme pathway (38) consisting of spermidine/spermine N2-acetyltransferase and polyamine oxidase. The inability of odc cells to use spermine as a polyamine source or to replenish their putrescine pools from extracellular spermidine implied that L. donovani lacks the spermidine/spermine N2-acetyltransferase/polyamine oxidase pathway altogether. This was then confirmed radioimetrically using both [14C]spermidine and [14C]spermine. Considering that parasitic nematodes (39) and E. coli (40) can acetylate naturally occurring polyamines, the lack of a counterpart pathway in L. donovani is unusual. Thus, it seems that the equilibrium between the putrescine and spermidine pools in L. donovani is governed exclusively by spermidine synthase activity, unlike mammalian cells, in which intracellular polyamines are regulated by a delicate balance among the anabolic enzymes, spermidine and spermine synthase, and the catabolic enzymes, spermidine/spermine N2-acetyltransferase and polyamine oxidase. Although the polyamine aminopropyltransferases have not been generally embraced as therapeutic targets, the limited complement of polyamine enzymes in L. donovani provides a rational basis for the utilization of spermidine synthase inhibitors (41) in conjunction with exogenous spermidine/spermine in therapies.

The polyamine requirement of odc cells can also be fulfilled by either 1,3-diaminopropane or cadaverine, because the polyamine auxotrophs can grow continuously in DME-L-CS supplemented with either diamine, and each is taken up and accumulated by the parasites. Essentially no putrescine was detected in the knockout cells incubated with either diamine, which supports the contention that putrescine is not an essential metabolite for L. donovani. Small quantities of spermidine, however, were observed in the 1,3-diaminopropane- and cadaverine-propagated cultures. The source of this spermidine is unclear, although it may be present in the supplemented culture medium in insufficient amounts to support the growth of the odc cells. Glutathionylspermidine and trypanothione levels were also markedly reduced compared with either wild type parasites or odc cells propagated in putrescine-supplemented medium. Although previous investigators detected significant amounts of homotrypanothione, the cadaverine-containing analog of trypanothione, in T. cruzi (31), we were unable to distinguish homotrypanothione and trypanothione in our high performance liquid chromatography system. These data imply that these thiols are not essential for the continual propagation of L. donovani promastigotes, at least in the absence of environmental insult.

The ability to create polyamine auxotrophs of Leishmania by deletion of the ODC locus suggests that ODC could be targeted by live parasite vaccination strategies against leishmaniasis. A similar vaccine-based approach has been inaugurated using thymidine auxotrophs of L. major in which the dihydrofolate reductase-thymidylate synthase locus has been replaced. These dihydrofolate reductase-thymidylate synthase null mutants induce protective immunity in mice and do not precipitate disease (42). We are currently evaluating the ability of our odc to infect and proliferate within human macrophages, the cell type in which the human stage of the parasite resides.

REFERENCES

1. Pegg, A. E., and Williams-Ashman, H. G. (1981) in Polyamines in Biology and Medicine (Morris, D. R., and Martin, L. J., eds), pp. 3–32, Marcel Dekker, Inc., New York.
2. Tahor, C. W., and Tahor, H. (1984) Annu. Rev. Biochem. 53, 749–790.
3. Zunin, V., and Pegg, A. E. (1988) Progress in Polyamine Research. Nobel Biochemical Pharmacological and Clinical Aspects. Advances in Experimental Medicine and Biology, Vol. 250, Plenum Press, New York.
4. Pegg, A. E. (1986) Biochem. J. 234, 249–262.
5. Metcalf, B. W., Bey, P., Danzijn, C., Casera, P., and Yevert, J. P. (1978) J. Am. Chem. Soc. 200, 2531–2533.
6. Bacchi, C. J., and McCann, P. P. (1987) Inhibition of Polyamine Metabolism (McCann, P. P., Pegg, A. E., and Sjoerdsma, A., eds), pp. 317–344, Academic Press, New York.
7. Bitonti, A. J., McCann, P. P., and Sjoerdsma, A. (1987) Exp. Parasitol. 64, 237–243.
8. Gillin, F. D., Reiner, D., and McCann, P. P. (1988) J. Protozool. 36, 161–163.
9. Kaur, K., Emmett, K., McCann, P. P., Sjoerdsma, A., and Ullman, B. (1986) J. Protozool. 33, 518–521.
10. Giffin, B. F., McCann, P. P., Bitonti, A. J., and Bacchi, C. J. (1986) J. Protozool. 33, 238–243.
11. Cruz, A., and Beverley, S. M. (1990) Nature 348, 171–173.
12. Tobin, J. F., Laban, A., and Wirth, D. F. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 864–868.
13. Phillips, M. A., Coffino, P., and Wang, C. C. (1987) J. Biol. Chem. 262, 8721–8727.
14. Hanson, S., Adelman, J., and Ullman, B. (1992) J. Biol. Chem. 267, 2350–2359.
15. Wilson, K., Collart, F. R., Huberman, E., Stringer, J. R., and Ullman, B. (1991) J. Biol. Chem. 266, 1667–1671.
16. Allen, T., and Ullman, B. (1993) Nucleic Acids Res. 21, 5431–5438.
17. Iovannisci, D. M., and Ullman, B. (1988) J. Parasitol. 69, 633–636.
18. LeBowitz, J. H., Coburn, C. M., McMahon-Pratt, D., and Beverley, S. M. (1990) Proc. Natl. Acad. Sci. U.S.A. 87, 9736–9740.
19. Cruz, A., Coburn, C. M., and Beverley, S. M. (1991) Proc. Natl. Acad. Sci. U.S.A. 88, 7170–7174.
20. Callahan, H. L., and Beverley, S. M. (1992) J. Biol. Chem. 267, 24165–24168.
21. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74, 5463–5467.
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
23. Laemmli, U. K. (1970) Nature 227, 680–685.
24. Shim, H., and Fairlamb, A. H. (1988) J. Gen. Microbiol. 134, 807–817.
25. Rojas-Chaves, M., Hellmud, C., and Walters, R. D. (1996) Mol. Biochem. Parasitol. 75, 261–264.
26. Fairlamb, A. H., Henderson, G. B., Bacchi, C. J., and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 185–191.
27. Fairlamb, A. H., Henderson, G. B., and Cerami, A. (1986) Mol. Biochem. Parasitol. 21, 247–257.
28. Henderson, G. B., Fairlamb, A. H., and Cerami, A. (1987) Mol. Biochem. Parasitol. 24, 39–45.
29. Kierszenbaum, F., Wirth, J. J., McCann, P. P., and Sjoerdsma, A. (1987) Proc. Natl. Acad. Sci. U.S.A. 84, 4578–4582.
30. Majumder, S., Wirth, J. J., Bitonti, A. J., McCann, P. P., and Kierszenbaum, F. (1992) J. Parasitol. 78, 371–374.
31. Hunter, K. J., Le Queenne, S. A., and Fairlamb, A. H. (1994) Eur. J. Biochem. 228, 159–165.
32. Ariyanayagam, M. R., and Fairlamb, A. H. (1997) Mol. Biochem. Parasitol. 84, 111–121.
33. Li, P., Hua, S. B., Wang, C. C., and Gottesdiener, K. M. (1996) Mol. Biochem. Parasitol. 84, 257–243.
34. Steglich, C., and Scheffler, I. E. (1982) *J. Biol. Chem.* **257**, 4603–4609
35. Paulus, T. J., Kiyona, P., and Davis, R. H. (1982) *J. Bacteriol.* **152**, 291–297
36. Hafner, E. W., Tabor, C. W., and Tabor, H. (1979) *J. Biol. Chem.* **254**, 12419–12426
37. Cohn, M. S., Tabor, C. W., and Tabor, H. (1980) *J. Bacteriol.* **145**, 791–799
38. Casero, R. A., Jr., and Pegg, A. E. (1993) *FASEB J.* **7**, 653–661
39. Wittich, R. M., and Walter, R. D. (1990) *Mol. Biochem. Parasitol.* **38**, 13–18
40. Tabor, C. W. (1968) *Biochem. Biophys. Res. Commun.* **30**, 339–342
41. Coward, J. K., and Pegg, A. E. (1987) *Adv. Enzyme Regul.* **26**, 107–113
42. Titus, R. G., Gueiros-Filho, F. J., De Freitas, L. A. R., and Beverley, S. M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 10267–10271