Plasticity in chromosome number and testing of essential genes in Leishmania by targeting
(tetraploid/population biology/aneuploidy/dihydrofolate reductase-thymidylate synthase/protozoan parasite)

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ABSTRACT We attempted to generate homozygous dhfr-ts (dihydrofolate reductase-thymidylate synthase) knockouts in virulent Leishmania major, an asexual diploid protozoan parasite. Transfection of a neo (neomycin phosphotransferase) targeting fragment yielded heterozygous replacement lines with high efficiency. However, second transfections with a hgy (hygromycin B phosphotransferase) targeting fragment in the presence of metabolites shown to rescue homozygous knockouts in attenuated Leishmania did not yield the expected dhfr-ts thymidine auxotrophs obtained previously with attenuated lines. Molecular karyotype, Southern blot, and flow cytometric DNA content analysis of clonal transfectants revealed three classes: (i) genomic tetraploids, containing two wild-type dhfr-ts chromosomes and one neo and one hgy replacement chromosome; (ii) aneuploid trisomic lines with one wild-type dhfr-ts and one neo and one hgy replacement chromosome; (iii) diploids bearing homologous integration of the targeting fragment without replacement. Aneuploid and tetraploid lines predominated. This confirms the common impression that natural populations of Leishmania are often aneuploid. The remarkable ability of these parasites to undergo and tolerate changes in chromosome number suggests a general method for testing whether genes are essential for growth in vitro, as the ability of Leishmania to simultaneously undergo homologous gene replacement while retaining wild-type genes by increasing chromosome number provides a diagnostic and positive experimental result. Our results show that virulent Leishmania require at least one copy of dhfr-ts and argue that DHFR-TS plays an unanticipated role in addition to its role in the de novo synthesis of thymidine. These results also have implications for genetic tests of the organization of Leishmania populations.

It is now possible by DNA transfection to engineer trypanosomatid parasites that overexpress or lack a given gene product. Since trypanosomatids are generally diploid and it is currently either impossible (Leishmania) or difficult (Trypanosoma brucei) to perform sexual crosses, homologous gene knockouts are usually obtained by the process of double gene replacement with independent selectable markers (1). This approach is limited to genes whose function is not essential during in vitro culture; however, many interesting parasite-specific molecules are known or likely to be nonessential for growth in vitro (2, 3).

One approach to the study of essential genes is to use situations in which knockouts are conditionally lethal. We have shown that homologous knockouts of the Leishmania major dhfr-ts (dihydrofolate reductase-thymidylate synthase) locus can be obtained if parasites are provided with thymidine (1, 4), in keeping with the expected role of DHFR-TS in intermediary metabolism. The dhfr-ts knockout will facilitate studies of folate metabolism and antifolate chemotherapy, and conditionally lethal mutants like these thy- (thymidine negative) auxotrophs may have important applications in the development of safe, avirulent lines that could be used as potential vaccination strains, as could parasites lacking molecules essential for infectivity in vivo but nonessential for growth in vitro (1). Since virulent Leishmania may provide superior vaccination potential relative to attenuated lines, we attempted to develop homologous knockouts of dhfr-ts in virulent lines. To our surprise, we were unable to obtain the desired mutants, despite the ease with which such knockouts were obtained with attenuated lines (1). Instead, virulent Leishmania parasites possess a remarkable ability to undergo changes in chromosome number, permitting retention of one or more chromosomes containing dhfr-ts despite the success of the double targeting.

MATERIALS AND METHODS

Virulent L. major were LV39 clone 5 (Rho/SU/39/P) and Friedlin (MHOM/JL/80/Friedlin); attenuated lines were LT252 clone CC-1 (MHOM/IR/83/IR; ref. 5) and CB rev3 (6, 7). Cells were grown in M199 medium and transfected by electroporation and plating as described (1, 4, 5). KS supplements were added in transfections expected to yield dhfr-ts lines (4). Virulent parasites recovered from lesions were cultured in NNN medium (7), transformed into protomastigotes, and grown <2 weeks prior to transfection. Stationary-phase parasites (5 × 106) were injected subcutaneously into the foot of BALB/c mice in triplicate, and development of lesions followed (7). General molecular methods were performed as described (1, 4, 5, 8). Gene-specific hybridization probes were as follows: dhfr-ts, 0.8-kb Xho I fragment (nt 658-1483; ref. 8); neo (neomycin phosphotransferase), 0.9-kb Spe I fragment from pSpeNE0A (5); hgy (hygromycin B phosphotransferase), a PCR-derived cassette (1); probe P, 1.6-kb EcoRI/EcoRV fragment (8). DNA content was measured by flow cytometry using ethanol-fixed cells (9). Heat-inactivated RNase (150 μl; 10 mg/ml) was added to fixed cells in 1 ml of phosphate-buffered saline (PBS) and incubated 30 min at 37°C. Cells were washed, resuspended for 1 h in 0.5 ml of propidium iodide (50 mg/ml in PBS), and washed once before flow cytometry.

RESULTS

Replacement of One Allele of dhfr-ts in Virulent Leishmania. Virulent L. major (LV39 clone 5) were transfected with a linear targeting fragment containing the neo selectable marker joined to DHFR-TS flanking sequences (Fig. 1A), as described for attenuated lines (4). Eight of nine clones

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possessed the planned homologous replacement, while the remaining clone contained unintegrated extrachromosomal circular DNA (Table 1).

The molecular karyotype of the neo/− heterozygous replacement was the same as the parental line (Fig. 2A, lanes 5 and 9 vs. lane 5; others not shown). A neo probe identified a 500-kb chromosome only in the neo/+ lines (Fig. 2C, lanes 6 and 9), and a dfr-ts probe identified a similarly sized chromosome in these and the parental line (Fig. 2B, lanes 6 and 9 vs. lane 5). Southern blots of DNAs digested with Not I and hybridized with a flanking probe showed the presence of the expected 14.5-kb replacement fragment (Fig. 1A) in neo/+ lines (Fig. 1B, lanes 6 and 9), as well as the 21-kb wild-type dfr-ts fragment (Fig. 1B, lanes 6 and 9 vs. lane 5). Southern blots of Not I-digested DNAs similarly revealed a 20-kb replacement fragment in the neo/+ lines with the flanking probe (Fig. 1C, lanes 6 and 9) in addition to the 21-kb wild-type dfr-ts fragment (Fig. 1C, lanes 6 and 9 vs. lane 5). Similar results were obtained with attenuated Leishmania replacement lines (Figs. 1B and C and 2A−C, lanes 1 and 2; ref. 4). These data confirmed the presence of the planned heterozygous replacement in the virulent transfecants.

Virulence of the Heterozygous Lines. The infectivity of the eight heterozygous neo/+ replacement lines was assessed in susceptible mice. Three lines failed to give a lesion after 2.5 months, while the remaining five lines developed lesions that progressed somewhat less rapidly than the parental virulent lines (data not shown). This shows that the loss of one copy of dfr-ts does not drastically reduce infectivity. Leishmania are known to lose virulence during culture in vitro, accounting for its loss in some transfecants (unpublished data).

Second-Round Targeting. Three virulent heterozygous neo/+ replacement clones (E18-6C1, E18-7C5, and E18-7A3) were chosen for a second round of transfection with a hyg targeting fragment (Fig. 1A), with protocols and medium supplements that had been used successfully in dfr-ts knockouts in attenuated lines (1).

From clone E18-7C5 hygromycin-resistant colonies were not obtained, although circular plasmid transfections gave several hundred colonies. From clones E18-6C1 and E18-7A3 a total of 11 hygromycin-resistant lines were obtained. The transfection efficiency (colonies per μg of DNA) was ≈5% that of control circular DNAs, comparable to that obtained previously (1). All 11 lines were prototrophic for thymidine (Table 1). In contrast, analogous transfections of an attenuated neo/+ replacement line yielded many thymidine auxotrophs (12/20; Table 1; ref. 1).

Tetraploids. The molecular karyotypes of the five hygromycin-resistant clonal lines from clone E18-7A3 (termed twice-targeted lines) were the same as the parental lines (Fig. 2A, lanes 7 and 8 vs. lanes 5 and 6). Southern blots showed

Table 1. Targeting of dfr-ts in virulent and attenuated L. major

<table>
<thead>
<tr>
<th>Round</th>
<th>Line</th>
<th>Colonies tested</th>
<th>Replacements</th>
<th>% replacements</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>LV39 (V)</td>
<td>9</td>
<td>8 NA</td>
<td>89</td>
</tr>
<tr>
<td></td>
<td>Friedlin (V)</td>
<td>7</td>
<td>3 NA</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td>CC-1 (AV)</td>
<td>27</td>
<td>21 NA</td>
<td>78</td>
</tr>
<tr>
<td></td>
<td>C, Reb (AV)</td>
<td>4</td>
<td>3 NA</td>
<td>75</td>
</tr>
<tr>
<td>2</td>
<td>LV39 (E18-7A3; V)</td>
<td>5 5*</td>
<td>0 100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>LV39 (E18-6C1; V)</td>
<td>6 4*</td>
<td>0 67</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Friedlin (E18-6C1; V)</td>
<td>4 4</td>
<td>0 60</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CC-1 (AV)</td>
<td>20</td>
<td>12 12</td>
<td>60</td>
</tr>
</tbody>
</table>

ND, not done; NA, not applicable; V, virulent line; AV, attenuated line.

*All are tetraploids.
†All are aneuploids.
‡Only 6 lines were analyzed molecularly; 5 were the expected homozygous replacements (1). The other 14 lines were analyzed only for thymidine auxotrophy; 7 were thy− and presumed to be replacements.
that the twice-targeted lines had identically sized chromosome(s) that hybridized with dhfr-ts, neo, and hyg-specific probes (Fig. 2 B-D, lanes 7 and 8), which were the same as the wild-type dhfr-ts chromosome (lane 5). A twice-targeted attenuated thy− auxotroph showed no hybridization with the dhfr-ts probe (Fig. 2B, lane 4; ref. 1).

Southern blots showed that the twice-targeted E18-7A3 lines contained both expected hyg and neo replacements plus wild-type dhfr-ts. Southern blots of Not I/Spe I-digested DNAs with flanking probe P (Fig. 1A) yielded the 21-kb wild-type dhfr-ts fragment (Fig. 1B, lanes 7 and 8 vs. lanes 1 and 5), the 14.5-kb neo replacement fragment (Fig. 1B, lanes 7 and 8 vs. lanes 2 and 6), and the 15.6-kb hyg replacement fragment (Fig. 1B, lanes 7 and 8 vs. lane 3). Similar results were obtained with other digests and probes. The intensity of the 21-kb dhfr-ts band was greater than that of the neo or hyg replacement bands, suggesting that there may be more than one copy of this chromosome. Southern blot analysis of Not I-digested DNAs with flanking probe P ruled out the possibility that these fragments arose by homologous integration of the targeting fragment into the dhfr-ts locus, an event that would give rise to a fragment larger than the normal 21-kb Not I fragment, which was not observed (Fig. 1A; Fig. 1C, lanes 7 and 8 vs. lanes 2, 3, and 6).

This showed that the twice-targeted derivatives of clone E18-7A3 contained one neo replacement, one hyg replacement, and one or more wild-type dhfr-ts chromosomes. One possibility was that these cells had undergone alterations in either chromosome number or ploidy, and the total cellular DNA content was measured by flow cytometry of fixed cells stained with propidium diode. Logarithmic-phase attenuated CC-1 or virulent LV39 clone 5 cells showed the expected pattern of G1, S, and G2 phase cells (Fig. 3 A and B), with G1 and G2 DNA contents of about 130 and 260 fluorescence units (FU), respectively. Stationary-phase cells showed the expected G1 staining pattern (data not shown). All heterozygous neo/+ replacement lines showed similar profiles, both before and after passage in the mouse (Fig. 3 C and D). However, all of the twice-targeted E18-7A3 lines showed a doubling of total DNA content, with a G1 peak of about 270 FU and a G2 peak of 520 FU (Fig. 3E, solid line; other data not shown). Since we previously showed that the attenuated CC-1 line of L. major is diploid for both the dhfr-ts and minixeom chromosomes (1, 4, 10), the DNA content and blotting data confirm that the twice-targeted derivatives of clone E18-7A3 are genomic tetraploids.

Only one of the five tetraploid lines (E23-7A3L1') was infective to mice. Parasites recovered from this lesion retained the tetraploid DNA profile (Fig. 3F), showing that tetraploidy is stable during the amastigote stage. The tetraploid DNA content was also maintained during 3 months of culture in vitro as promastigotes (data not shown).

Aneuploidy. The molecular karyotypes of the twice-targeted lines derived from the heterozygous neo/+ replacement line E18-6C1 showed no obvious differences (Fig. 2A, lanes 10–12), and Southern blots showed chromosomes hybridizing with the dhfr-ts, neo, and hyg probes whose sizes were similar to each other and the dhfr-ts chromosomes present in the parental lines (Fig. 2, lanes 10–12 vs. lanes 5 and 9). Southern blots of digested DNAs revealed two different phenotypes: group I (lines E33-5A1, -SB2, -5C1, and -6B4) exhibited Not I/Spe I fragments diagnostic for wild-type dhfr-ts and both the planned neo and hyg replacements (Fig. 1A; Fig. 1B, lanes 10 and 11; data not shown). Not I digestion showed that insertion (without replacement) of the hyg targeting fragment had not occurred (Fig. 1C, lanes 10 and 11). In contrast, in the two group II lines (E23-6C1L1, E33-7C5) the 3.3-kb hyg targeting fragment had inserted into homologous sequences 5' of the first-round neo replacement. This yielded a larger 23-kb Not I fragment and retention of the 21-kb dhfr-ts fragment (Fig. 1C, lane 12).
Flow cytometry of lines from both groups showed that all had the standard amount of DNA (Fig. 3E, dashed line; other data not shown). Since the 500-kb dhfr-ts chromosome constitutes only 1% of the entire parasite genome, an extra chromosome would not be detectable. This indicated that the group I derivatives were aneuploid, containing one or more normal dhfr-ts chromosomes and two modified chromosomes bearing neo and hgy replacements. We expected that aneuploidy of the dhfr-ts chromosome would cause increased staining in the gel shown in Fig. 2A. However, chromosome size polymorphisms between the LV39 and CC-1 lines have resulted in comigration of one or more CC-1 chromosomes with the dhfr-ts chromosome of the LV39 line (Fig. 2A, lanes 1-4 vs. lane 5). The copy number of the LV39 dhfr-ts chromosome is thereby obscured. All three of the aneuploid clones tested showed virulence comparable to the parental E18-6C1 line (data not shown).

Double Gene Targeting in a Second Virulent Line. 

The studies described above failed to yield the desired homozygous knockout of dhfr-ts. This was not unique to the LV39 line, as transfections of a second virulent line of L. major (Friedlin) yielded similar results. After the first round of transfection with the neo targeting fragment (Fig. 1A), three of seven lines contained the planned heterozygous replacement (Table 1). One of these clones (E43-6B4) was passed through mice and transfected with the linear hgy targeting fragment (Fig. 1A), yielding four hygromycin-resistant lines. None was auxotrophic for thymidine (Table 1).

D I S C U S S I O N

We were unable to obtain complete inactivation of the dhfr-ts locus in virulent L. major by methods that were successful in attenuated lines (1). This could happen if either dhfr-ts was essential in virulent Leishmania, or if methodological problems associated with gene targeting prevented success. Our data argue against procedural difficulties: first, both the neo and hgy targeting fragments functioned well in the first round in the virulent lines (Table 1). This rules out effects arising from polymorphisms between the targeting fragment and the chromosomal target. Second, the exclusive recovery of dhfr-ts lines in the second-round transfections of virulent Leishmania was statistically significant (0 thy"/15 twice-targeted virulent lines vs. 12 thy"/20 twice-targeted attenuated lines; P < 0.005; Table 1). Third, the twice-targeted L. major resorted to extreme genetic measures to avoid complete loss of dhfr-ts. The majority of the lines analyzed (9/11; Table 1) underwent alterations in chromosome number, either tetraploidy or tetraploidy. One would more commonly expect integration without replacement or formation of extrachromosomal circular DNA's by self-ligation (4). Fourth, transfections expected to yield homozygous dhfr-ts knockouts were performed in the presence of metabolites known to rescue DHFR- and/or TS-deficient mutants of Escherichia coli cultured mammalian cells, and attenuated Leishmania (1, 4, 11, 12). Last, the inability to obtain homozygous dhfr-ts replacement was not restricted to a single virulent line.

Alterations in Chromosome Number. The frequent recovery of tetraploid and aneuploid lines in the twice-targeted virulent Leishmania was unanticipated. Several workers have suspected that aneuploidy occurs in natural populations of Leishmania (reviewed in ref. 13), although these inferences were made from ethidium bromide-stained chromosomes where the contribution of chromosomal size polymorphisms was unknown. Nonetheless, 10% of random lines show evidence suggestive of aneuploidy for one or more chromosomes (ref. 13; unpublished data). Our work using genetic manipulations to mark sister chromosomes proves this for the dhfr-ts chromosome, and, in combination with previous studies, confirms the prevailing impression of widespread aneuploidy in Leishmania. In contrast, tetraploid Leishmania have not been reported previously but could occur in nature. Hybrid parasites with increased DNA content have been reported in sexual crosses of T. brucei in tsetse flies, including triplexes (reviewed in refs. 14 and 15). Spontaneous fusion of Leishmania cells has been observed (16); however, these cells have not been characterized. Experimental sexual crosses have not been successful in Leishmania (17), although natural hybrids are occasionally reported (18, 19); the ploidy of these has not been determined. Natural tetraploids could arise by fusion or by rare and as yet unidentified sexual processes.

Several questions remain unanswered. For example, why were the clones so different in the second round of targeting? Potential explanations are adventitious genetic differences among the +/neo clones or environmental factors such as small differences in culture conditions or growth phase. Given the frequency of tetraploids arising from clone E177A3, one expects that preexisting aneuploids and tetraploids should be common. However, only ~1% of the virulent wild-type or first-round Leishmania have tetraploid DNA contents (Fig. 3 B-D). Both tetraploids and aneuploids exhibited =2:1 ratios of the +, neo, and hgy alleles (Fig. 1B); yet, since one copy of dhfr-ts is sufficient for viability and normal growth, 1:2:1 or 1:1:2 parasites should also have been recovered. This asymmetry may reflect a bias for replacement of a neo allele by the hgy targeting fragment, which could occur after tetraploidization via endoreduplication, or in a +/+neo heterozygote followed immediately by fusion with another +/+neo cell (this latter model could account for the lack of correlation between the number of preexisting tetraploids and targeting frequencies). It seems unlikely that biased replacement could preclude the recovery.
of double knockouts, as the frequency of replacement was similar in both rounds of gene targeting.

Role of DHFR-TS in Virulent *Leishmania*. As the lack of virulent *dhfr-ts* knockouts cannot be attributed to procedural or chromosomal sources, we conclude that virulent *Leishmania* cannot tolerate the loss of DHFR-TS. One may imagine that this could arise from trivial sources, such as reduced transport of the key metabolite thymidine. However, virulent parasites transport and incorporate thymidine into DNA (20). This argues that DHFR-TS plays an additional unanticipated role in virulent lines directly or in combination with other enzymes.

Currently, we can only speculate about new roles for DHFR-TS in virulent parasites. Previous biochemical characterizations of attenuated and virulent *Leishmania* have shown many differences, including reduced synthesis and/or alterations in the structure of the major surface carbohydrate lipopolysaccharide (LPS), which plays an important role in parasite infectivity (reviewed in ref. 21). LPG and other *Leishmania* surface glycolipids are anchored to the surface by ether-linked lipids (21, 22), which are known to turn over in *Leishmania* (23, 24). In some organisms, cleavage of this family of lipids requires a reduced pterin cofactor (25, 26), and one may speculate that this step could link the DHFR-TS pathway with LPG and virulence. Accumulation of ether-linked lysophospholipids is known to be toxic to *Leishmania* (23, 24), and perhaps failure to cleave these lipids is toxic in virulent but not attenuated parasites. Other possibilities may arise from unique aspects of folate and pterin metabolism in *Leishmania*, which differ significantly from other organisms (27–29).

**Chromosome Number Plasticity May Permit Tests of Essential Genes.** The general ability of *Leishmania* to tolerate changes in chromosome number suggests a unique approach for testing whether genes are essential, which would be useful since *Leishmania* lacks an experimentally manipulable sexual cycle. First, heterozygous replacement lines are developed (the order in which the neo and hyg markers are used is unimportant (1)). If a gene is nonessential, or conditionally so, a second round of targeting will yield the desired homozygous knockout (1). If the gene is essential, homozygous knockouts will be lethal; however, the remarkable ability of *Leishmania* to undergo changes in chromosome number will permit the recovery of cells simultaneously bearing wild-type and both neo and hyg replacement chromosomes.

We propose that this is a diagnostic criterion for essentiality of gene function, as one is scoring a positive result and not just failure to obtain knockouts. Since *Leishmania* appear to be generally tolerant of chromosome number changes (28), this approach should be applicable at loci located on other chromosomes. Other manipulations that exploit chromosome number plasticity, such as parasexual fusions or crosses may eventually be feasible.

**Chromosome Number Variability and the Genetic Structure of *Leishmania* Populations.** One common finding in genetic surveys of natural populations of *Leishmania* is that the frequency of heterozygotes is much less than expected for randomly mating populations (30, 31). These and other data have led to a clonal, asexual model for the genetic structure of *Leishmania* populations. However, the ability of virulent *Leishmania* to undergo changes in chromosome number suggests another mechanism for generating homoyzogosity. Consider a chromosomal heterozygote A/B in which aneuploidy occurs, yielding A/B/B progeny. If aneuploidy persists only transiently, the A/B/B progeny will eventually revert to diploidy and generate either A/B or B/B offspring. The B/B offspring will now be homozygous throughout this chromosome regardless of the occurrence of sexual crossing. Over evolutionary time and different chromosomes, this process would affect most of the genome. Since most population genetic tests assume constant diploidy, their application to "facultative" or "transient" aneuploids such as *Leishmania* must be viewed with caution. In this regard, linkage disequilibrium criteria may be less sensitive to uncertainty arising from chromosome number variability (31).

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