Disruption of the trypanothione reductase gene of *Leishmania* decreases its ability to survive oxidative stress in macrophages

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Parasitic protozoa belonging to the order Kinetoplastida contain trypanothione as their major thiol. Trypanothione reductase (TR), the enzyme responsible for maintaining trypanothione in its reduced form, is thought to be central to the redox defence systems of trypanosomatids. To investigate further the physiological role of TR in *Leishmania*, we attempted to create TR-knockout mutants by gene disruption in *L. donovani* and *L. major* strains using the selectable markers neomycin and hygromycin phosphotransferases. TR is likely to be an important gene for parasite survival since all our attempts to obtain a TR null mutant in *L. donovani* failed. Instead, we obtained mutants with a partial trisomy for the TR locus where, despite the successful disruption of two TR alleles by gene targeting, a third TR copy was generated as a result of genomic rearrangements involving the translocation of a TR-containing region to a larger chromosome. Mutants of *L. donovani* and *L. major* possessing only one wild-type TR allele express less TR mRNA and have lower TR activity compared with wild-type cells carrying two copies of the TR gene. Significantly, these mutants show attenuated infectivity with a markedly decreased capacity to survive intracellularly within macrophages, provided that the latter are producing reactive oxygen intermediates.

Keywords: gene targeting/intracellular survival/*Leishmania*/oxidative stress/trypanothione reductase

Introduction

Parasitic protozoa of the order Kinetoplastida are the causative agents of several medically important tropical diseases including sleeping sickness (*Trypanosoma brucei rhodesiense* and *T. b. gambiense*), Chagas disease (*T. cruzi*) and visceral (kala-azar) and cutaneous (oriental sore) leishmaniasis (e.g. *Leishmania donovani* and *L. major*, respectively). Among many other metabolic peculiarities, trypanosomatids maintain their intracellular redox balance by a mechanism that is completely different from that of their insect vectors and mammalian hosts. They lack glutathione reductase (GR) which in nearly all other

organisms is responsible for the maintenance of an intracellular reducing environment important for the reduction of disulfides, the detoxification of peroxides and the synthesis of DNA precursors (Fairlamb *et al.*, 1985; Schirmer and Schulz, 1987). Instead, they possess a unique system using *N*¹,*N*⁸-bis(glutathionyl)spermidine (trypanothione) and its metabolic precursor *N*¹-glutathionylspermidine as their main thiols. These glutathione conjugates are kept in the reduced state by trypanothione reductase (TR) (Fairlamb *et al.*, 1985; Shames *et al.*, 1986; Fairlamb and Cerami, 1992). TRs are members of the NADPH-dependent flavoprotein oxidoreductase family and are structurally and mechanistically related to GRs (Shames *et al.*, 1986; Krauth-Siegel *et al.*, 1987). Human GR and all parasite TRs have mutually exclusive substrate specificities (Shames *et al.*, 1986; Henderson *et al.*, 1987; Krauth-Siegel *et al.*, 1987; Cunningham and Fairlamb, 1995; Krauth-Siegel and Schöneck, 1995), providing a route for the design of selective inhibitors against the parasite enzymes.

During its infective cycle in the vertebrate host, *Leishmania* must survive the rigorous oxidizing environment of the macrophage. TR and its subordinate thiols are proposed to play a vital role in maintaining an intracellular reducing environment and in protecting these parasites against oxidative damage, arising both internally as a result of their aerobic metabolism and externally by the immune response of the mammalian host (Fairlamb *et al.*, 1985; Shames *et al.*, 1986). Selective inhibition of TR is therefore potentially an attractive strategy to incapacitate these parasites.

In view of the pivotal role and uniqueness of TR in the management of oxidative stress, we have attempted to generate attenuated *Leishmania* strains with decreased or null TR activity that could eventually be useful for the development of live vaccines. In order to generate attenuated strains and to investigate further the physiological role of TR, we have genetically engineered TR mutants by gene disruption in pathogenic *Leishmania* species and analysed the phenotype of the resulting mutants with respect to intracellular survival of the parasites within macrophages.

Results

Inactivation of the *L. donovani* TR gene by gene disruption

The TR gene is single copy in the *Leishmania* genome (Taylor *et al.*, 1994) and, since *Leishmania* is generally considered to be diploid and no sexual crosses have been achieved, two successive rounds of gene targeting should be sufficient to create null mutants for TR. In an attempt to inactivate both TR alleles in *L. donovani*, we have created two disruption constructs. These consist of the

Fig. 1. Inactivation of *L. donovani* *TR* alleles. (A) Schematic drawing of the *TR* locus in *L. donovani* and its relevant restriction sites (S, *SacII*; B1, *BalI*). Integration of the *hyg* cassette into the *TR* gene must generate two additional *SacII* fragments of 1.3 and 2.83 kb. Upon integration of the *neo* disruption construct at the homologous locus, the genomic *SacII* fragment would increase from 2.9 to 3.8 kb. The 3.1 kb S-S' fragment corresponds to a new restriction fragment generated following genomic rearrangement leading to a translocation of part of the *TR* locus on a 1200 kb chromosome (see C and text). (B) Southern blot analysis of *L. donovani* wild-type and *TR* single and double targeted mutants. DNAs were digested with *SacII*, electrophoresed through an agarose gel and hybridized independently with *TR*-, *hyg*- and *neo*-specific probes. Lanes 1, *L. donovani* wild-type; 2, *L. donovani* *TR/TR::hyg* mutant; 3, *L. donovani* *TR/TR::neo*; and 4, *L. donovani* *TR/TR::hyg/TR::neo* double targeted transfectant. (C) Molecular karyotype of *L. donovani* *TR* mutants. Chromosomes were separated by pulsed-field gel electrophoresis using a Bio-Rad CHEF-DR III apparatus at 5 V/cm, 120° separation angle and switch times varying from 35 to 120 for 30 h. Blots were hybridized to the same probes as above. Lanes 1 and 2 are as in (B) and lane 3 corresponds to the *L. donovani* *TR/TR::hyg/TR::neo* mutant.

insertion of the neomycin phosphotransferase (*neo*) or the hygromycin phosphotransferase (*hyg*) expression cassettes (Papadopolou et al., 1994a) into the unique *BalI* site of the 1.25 kb PCR fragment of the *TR* coding region subcloned into the pSP72 vector (Promega) in order to form *TR::neo* and *TR::hyg*, respectively (see Figure 1A).

In a first round of targeting, the *TR::hyg* disruption construct was transfected into *L. donovani*. Cells growing in the presence of hygromycin B were cloned on semi-solid agar and the DNA of clones was digested with *SacII* and analysed by Southern blots hybridized to specific *TR* and *hyg* probes. Two *SacII* fragments of 2.6 and 2.9 kb were recognized by the *TR* probe in wild-type *L. donovani* (Figure 1B, lane 1). Integration of the *hyg* cassette into the *TR* gene should replace one of the 2.9 kb fragments with two new restriction fragments of 1.3 and 2.83 kb (see Figure 1A) while leaving the 2.6 kb fragment intact. If only one allele is disrupted, then the 2.6 and 2.9 kb bands corresponding to the second non-targeted *TR* allele should be visible as well. Hybridization to *TR* and *hyg* probes (Figure 1B, lanes 2) confirmed that the expected single disruption with the *hyg* gene had occurred. Likewise, transfection of wild-type cells with the *TR::neo* construct resulted in successful disruption of one *TR* allele, generat-

ing the predicted 3.8 kb *SacII* fragment (Figure 1B, lanes 3).

A clone of the *TR/TR::hyg* transfectant was selected for the second round of targeting using the *TR::neo* disruption construct. Transfectants resistant to G418 and hygromycin B were obtained and clones were analysed by hybridization with probes specific for *TR*, *neo* and *hyg*. The *neo* gene was indeed integrated into the *TR* locus because the predicted 3.8 kb *SacII* fragment was obtained following hybridization to *neo* and *TR* probes (Figure 1B, lanes 4). Although *TR* gene disruption by *neo* and *hyg* took place, as clearly shown from the hybridization studies, one *TR* allele remained intact since the 2.9 kb genomic *SacII* fragment was still present in the double targeted mutant (Figure 1B, lane 4). Moreover, a novel 3.1 kb *SacII* band hybridizing to the *TR* probe was detected in this transfectant (Figure 1B, lane 4), most likely resulting from a genomic rearrangement. Digestions with other restriction enzymes also revealed new fragments hybridizing to the *TR* probe that did not co-migrate with bands present in the wild-type strain (not shown), indicating that a genomic rearrangement must have occurred upstream of the *TR* gene.

To determine how these rearranged fragments might

Fig. 2. Translocation of part of the *TR* locus in *L. donovani* *TR/TR::hyg/TR::neo* mutant leading to partial trisomy for *TR*. (A) A restriction map of a genomic region of a 45 kb part of a 520 kb chromosome containing the *TR* locus. Restriction sites are indicated as C for *Cla*I, RI for *Eco*RI, S for *Sac*II and X for *Xho*I. The two boxes located upstream and downstream of the *TR* gene represent the putative sequences used during translocation leading to the generation of a third *TR* allele. The upstream box is part of the 2.6 kb *Sac*II restriction fragment (see Figure 1B) although its exact position is unknown. (B) Schematic drawing of a region of at least 35 kb within the *TR* locus translocated from the 520 kb (thin line) into a 1200 kb chromosome (thick line). S', X' and RI' represent novel restriction sites generated following the upstream genomic rearrangement. (C) Presence of homologous sequences in the 520 kb *TR* locus-containing chromosome and in the 1200 kb chromosome where translocation occurred. Chromosomes were separated by CHEF electrophoresis as described in Materials and methods and hybridized to probe 1 (see A). Lanes 1, *L. donovani* wild-type; 2, *L. donovani* *TR/TR::hyg*; and 3, *L. donovani* *TR/TR::hyg/TR::neo*. (D) Southern blot of total genomic DNA digested with *Xho*I and hybridized to probe 1. Lanes are as in (C). (E) A CHEF blot hybridizing to probe 2 consisting of a 23 kb *Eco*RI–*Eco*RI fragment located downstream of the *TR* gene to look for the second rearrangement site. Lanes are as in (C).

have arisen, chromosomes of the *TR/TR::hyg/TR::neo* transfectant were resolved by CHEF electrophoresis and the blot was hybridized to the appropriate probes. In wild-type *L. donovani*, *TR* is located on a 520 kb chromosome (Figure 1C), which is in contrast to the 1.1 Mb chromosome reported for another isolate of *L. donovani* (Taylor *et al.*, 1994). In addition to a 520 kb chromosome on which the *TR* gene is normally located, a new chromosome of ~1200 kb hybridized to a *TR*-specific probe (Figure 1C, lane 3). The newly generated *TR* allele was the one targeted by the *neo* gene (see Figure 1C, lane 3), thus leaving an intact *TR* allele at the original chromosomal location (520 kb). In additional independent transfection experiments, we were again unable to generate a null mutant, as the *TR* locus became trisomic (not shown). Thus, our attempts to generate a *TR* null mutant in *L. donovani* have, therefore, failed. These results suggest that the *TR* gene is possibly essential for survival of *L. donovani* promastigotes.

In order to explain the events leading to genomic translocation and aneuploidy for *TR* and to determine the size of the translocated region, we have hybridized a CHEF blot of wild-type *L. donovani* and *TR* disruption mutants to several probes covering a region of ~35 kb containing the *TR* locus. As a first probe, we used a 4.1 kb

*Cla*I–*Eco*RI fragment (probe 1, Figure 2A) containing a large part of the *TR* gene and the putative sequences used for the rearrangement at the 5' end as indicated from the *Sac*II digestion (see Figures 1B and 2A). Even at relatively high stringency, a positive hybridization signal was obtained at the level of a 1200 kb chromosome in the *L. donovani* wild-type and *TR/TR::hyg* strains (Figure 2C, lanes 1 and 2), indicating that sequences homologous to those present at the *TR* locus on the 520 kb chromosome were also found on a 1200 kb chromosome.

We tested the possibility of whether homologous genomic repeated sequences could also be used for the downstream rearrangement site. No homology between the upstream and the putative downstream point of rearrangement has been detected. Indeed, the only rearranged fragment hybridizing to probe 1 (even at longer exposure) was the 3.3 kb *Xho*I–*Xho*I' representing the upstream rearrangement in the double *TR*-targeted mutant (Figure 2B and D, lane 3). To try to pinpoint the putative downstream rearrangement site, we used a large probe of 23 kb located 9 kb downstream of the *TR* gene (probe 2, Figure 2A). No rearranged fragments were observed using probe 2 in the double targeted *TR* mutant compared with wild-type (data not shown), suggesting that the second rearrangement occurred further downstream. The intense

hybridization signal (Figure 2E, lane 3) observed at the level of the 1200 kb chromosome for the *TR/TR::hyg/TR::neo* mutant indicated that the region covered by probe 2 was translocated along with *TR*.

Decreased intracellular survival of the *L. donovani* *TR/TR::hyg* and *TR/TR::hyg/TR::neo* mutants

Not only the single but also the double targeted *L. donovani* *TR* mutants that we have generated by gene disruption contain one wild-type *TR* allele. To look at the effect of the loss of one *TR* copy in our mutants, we first examined *TR* mRNA levels in both promastigote and amastigote stages of the parasite. *L. donovani* stationary phase promastigotes were differentiated *in vitro* into amastigotes as described in Materials and methods. Differentiation into amastigotes was monitored by hybridizing RNAs (not shown) to an amastigote A2-specific probe (Charest and Matlashewski, 1994). Northern blot analysis of total RNAs isolated from *L. donovani* wild-type and *TR* mutants hybridized to a *TR*-specific probe showed that parasites either with the *TR/TR::hyg* or the *TR/TR::hyg/TR::neo* background produce lower amounts of *TR* mRNA than

wild-type cells (Figure 3A). Densitometric scanning of the RNA bands and normalization with the tubulin RNA levels used as control indicated that there is 3- to 5-fold less RNA in the *TR* mutants than in wild-type *Leishmania*. Furthermore, no stage-specific expression of the *TR* gene was observed in wild-type *Leishmania* (see Figure 3A).

To ascertain whether decreased levels of *TR* mRNA in the disruption mutants correlated with decreased *TR* activity, we have measured enzymatic activities in control and *TR* mutant strains. A 56% decrease of *TR* enzymatic activity was detected in the single and the double targeted *L. donovani* *TR* mutants compared with the control cells (see Table I). As *TR* is the enzyme that maintains trypanothione in its reduced form (Fairlamb *et al.*, 1985), we examined whether a decrease in *TR* activity correlates with an alteration in thiol levels. No statistically significant differences in levels of trypanothione, glutathionylspermidine or glutathione were observed between controls and *TR*-targeted mutants (Table I).

Given the putative importance of *TR* in the detoxification of lethal oxygen metabolites that are produced by the macrophage following infection, we investigated whether our *TR* disruption mutants with less *TR* activity than wild-type cells have the same ability to survive intracellularly. To test this, we used an *in vitro* system to infect either murine or human macrophages with *Leishmania* promastigotes. Both the *L. donovani* *TR/TR::hyg* and *TR/TR::hyg/TR::neo* mutants were tested. In the case of human monocytes differentiated into macrophages, a significant decrease in the percentage of parasitized cells was observed with the single and double targeted *TR* mutants as early as 24 h following infection (Figure 4A). Moreover, although the infection rate was maintained throughout the experiment for the control *L. donovani-neo* transfectant, this was not the case for the *TR/TR::hyg* and *TR/TR::hyg/TR::neo* mutants where infectivity levels decreased steadily to reach only 12% after 72 h (Figure 4A). Similarly, the number of amastigotes per macrophage decreased drastically from nine at 6 h infection to ~0.5 amastigote/cell after 72 h for the *TR/TR::hyg* and *TR/TR::hyg/TR::neo* mutants (see Figure 4B). Indeed, the loss of one *TR* allele has dramatic consequences on the intracellular viability of the parasite. To prove that the observed phenotype was due solely to the disruption of *TR*, we have transfected into the *L. donovani* *TR/TR::hyg* a *neo* expression vector carrying a 2.2 kb insert containing the *TR* gene of *L. donovani infantum* and 650 bp of upstream sequence (see Materials and methods). This recombinant strain regained its ability to survive inside

Fig. 3. *TR* mRNA expression in the wild-type and single and double *TR* targeted mutants of *L. donovani*. (A) Northern blot of total RNAs isolated from the promastigote and amastigote stages of *L. donovani* wild-type and transfectants and hybridized to a *TR*-specific probe. Each track contains ~10 µg of total RNA. Lanes 1, *L. donovani* wild-type; 2, *TR/TR::hyg* single knockout mutant; and 3, *TR/TR::hyg/TR::neo* double targeted transfectant. (B) The same blot was stripped off and re-hybridized with the *T. brucei* α -tubulin probe to monitor the amount of RNA layered in each lane.

Table I. Trypanothione reductase (*TR*) activity and thiol content in *Leishmania* control and *TR* mutants

Cells	Genotype	Enzyme activity (U/mg)		Ratio <i>TR</i> /ALAT	Thiol content (nmol/10 ⁸ cells)		
		<i>TR</i>	ALAT ^a		GSH ^b	GspdSH ^c	T[SH] ₂ ^d
<i>L. donovani</i>	<i>TR/TR</i>	0.212 ± 0.023	0.117 ± 0.007	1.81	3.10 ± 0.33	1.67 ± 0.12	5.78 ± 1.33
<i>L. donovani</i>	<i>TR/TR::hyg</i>	0.090 ± 0.005	0.112 ± 0.023	0.80	3.55 ± 1.65	1.91 ± 0.26	4.91 ± 1.40
<i>L. donovani</i>	<i>TR/TR::hyg/TR::neo</i>	0.077 ± 0.016	0.097 ± 0.003	0.79	2.90 ± 0.24	1.30 ± 0.17	5.30 ± 0.17
<i>L. major</i>	<i>TR/TR</i>	0.437 ± 0.041	0.337 ± 0.022	1.30	ND ^e	ND	ND
<i>L. major</i>	<i>TR/TR::hyg</i>	0.096 ± 0.005	0.114 ± 0.002	0.84	ND	ND	ND

^aALAT, alanine aminotransferase used as an internal control; ^bGSH, glutathione; ^cGspdSH, glutathionylspermidine; ^dT[SH]₂, trypanothione; ^eND, not determined.

Fig. 4. Infectivity of the *L. donovani* TR disruption mutants toward human monocytes differentiated into macrophages. Human macrophages were incubated with stationary phase *Leishmania* parasites (20:1, parasite to cell ratio) for 6 h as described in Materials and methods. After this initial incubation, free parasites were washed and incubation was followed for 24, 48 and 72 h. At these fixed time points, cell cultures were dried and stained with Diff Quick in order to determine the level of infection. ■ Infection with *L. donovani*-neo, a transfectant containing a neo vector used as a control, ● *L. donovani* TR/TR::hyg single disruption mutant, ◇ *L. donovani* TR/TR::hyg/TR::neo double targeted mutant, △ *L. donovani* TR/TR::hyg transfected with a neo expression vector carrying a 2.2 kb *SphI*-*SphI* genomic fragment containing only the TR gene (see Materials and methods) to revert the mutant phenotype. (A) The percentage of infected macrophages and (B) the total number of amastigotes within 100 macrophage cells with time. The average and standard deviation of four independent experiments are shown.

Table II. Production of superoxide anion (nmol O₂⁻/h/10⁶ cells) by murine (J774) and human macrophages following infection with *L. donovani*

	1 h		2 h		4 h		6 h	
	Human	J774	Human	J774	Human	J774	Human	J774
PMA	11.2 ^a	<0.001	4.09	<0.001	1.28	<0.001	0.32	<0.001
<i>L. donovani</i>	13.1	<0.001	5.03	<0.001	0.82	<0.001	0.17	<0.001
<i>L. donovani</i> TR/TR::hyg/TR::neo	7.4	<0.001	4.89	<0.001	1.37	<0.001	0.11	<0.001

^aAverage of three independent experiments.

macrophages to approximately wild-type levels (Figure 4), strongly suggesting that the loss of one TR copy was responsible for the decreased intracellular survival observed in the mutants.

The intracellular survival of *L. donovani* TR/TR::hyg and TR/TR::hyg/TR::neo mutants was also studied in the murine macrophage-like J774 cell line. However, in this cell line, the mutant's intracellular survival was not affected and parasites grew similarly to the control (not shown). To try to explain this intriguing difference, we looked at macrophage function related to intracellular parasite killing that could influence the phenotype of the TR mutants, such as the production of superoxide anion. The oxidation of cytochrome *c* was measured in the supernatants of cells stimulated with *Leishmania*, or phorbol myristate acetate as a positive control, as described in Materials and methods. Our results indicate that, following stimulation with *Leishmania* cells, the murine macrophage-like J774 cell line shows no detectable levels of superoxide anion, in contrast to the human macrophages that produce high amounts of this reactive oxygen metabolite (see Table II). Thus, the intracellular survival of *Leishmania* with diminished TR activity seems to decrease only in macrophages capable of producing superoxide anions.

Phenotype of *L. major* TR/TR::hyg mutant

To test whether the results observed in *L. donovani* would be similar in other pathogenic species, we have inactivated one allele of the *L. major* TR gene by targeted gene disruption. Since isogenic DNA appears to be necessary for efficient gene targeting in *Leishmania* (Papadopolou and Dumas, in preparation), we amplified the TR gene of *L. major* by PCR and introduced the hyg expression cassette into the *BalI* unique site of the gene. The resulting construct was then transfected into *L. major*. As shown by Southern blot hybridization of total *XhoI* DNA digests of *L. major* wild-type and TR/TR::hyg transfectant, the integration of the hyg gene into one TR allele leads to an increase in size of one genomic fragment from 8 to 9.2 kb (Figure 5B, lane 2). Less TR activity was found in this mutant as observed with *L. donovani* disruption mutants (Table I). The *L. major* TR/TR::hyg disruption mutant was tested further for its capacity to infect human macrophages. As observed for *L. donovani*, the *L. major* transfectant missing one TR allele showed an important decrease in parasite infectivity. Indeed, 1.1 amastigotes/cell were detected at 72 h post-infection in *L. major* TR/TR::hyg mutant compared with five amastigotes/cell at the beginning of infection (Figure 5C).

Fig. 5. Phenotype of *L. major* TR/TR::hyg mutant. (A) Schematic drawing of the *L. major* TR locus with its XhoI restriction sites. (B) Generation of a *L. major* TR disruption mutant by gene targeting. Southern blot analysis of total DNAs from *L. major* wild-type (lane 1) and *L. major* TR/TR::hyg (lane 2) digested with XhoI and hybridized to TR- and hyg-specific probes. (C) Infection of human macrophages with *L. major* in vitro. This was done as described in Figure 4 and in Materials and methods. The left panel corresponds to the total number of amastigotes in 100 macrophage cells and the right panel to the percentage of infected macrophages with time. The average and standard deviation of four independent experiments are shown.

Discussion

Trypanothione and its related enzymes are thought to be involved in many cellular functions similar to those of glutathione in mammalian cells, such as maintenance of an intracellular reducing environment and defence against damage by oxidants, certain heavy metals (Mukhopadhyay *et al.*, 1996) and possibly xenobiotics (for a review, see Fairlamb and Cerami, 1992). The role of TR in *L. donovani* and *T. cruzi* has been investigated previously by over-expressing TR using transfections (Kelly *et al.*, 1993). Such transfectants were equally susceptible to inhibition by hydrogen peroxide (H_2O_2) as controls, and metabolized H_2O_2 at comparable levels, suggesting that the ability to regenerate trypanothione from trypanothione disulfide is not the rate-limiting step in the metabolism of H_2O_2 (Kelly *et al.*, 1993). To investigate further the physiological role of TR, we attempted to generate TR-knockout mutants by gene disruption. Recently, the function of other *Leishmania* genes has been established using gene targeting procedures (Bello *et al.*, 1994; Papadopolou *et al.*, 1994b, 1996; Mottram *et al.*, 1996).

In this work, we have generated double and single TR disruption mutants for *L. donovani* and *L. major*, but were unable to obtain TR null mutants. Instead, the TR locus became trisomic (Figure 1). Recombinant parasites possessing only one TR allele produce less mRNA (Figure

3A) which correlates with lower TR activity when compared with wild-type levels (Table I). Despite a marked decrease in TR activity in the mutants, we found no differences in trypanothione levels between wild-type and mutants (Table I), and this is in accordance with previous results reported by Kelly *et al.* (1993). Reduced TR activity does not affect the growth of *Leishmania* in culture media, nor does it influence the transformation to amastigotes in vitro (not shown). The loss of one TR allele by gene disruption is not compensated by either an increase in TR copy number or RNA production (Figure 3A). These observations are in agreement with two alternative approaches to modulating TR activity that involve either the extrachromosomal expression of dominant-negative TR mutants of *T. cruzi* (Borges *et al.*, 1995) in *Leishmania* cells (J. Tovar, M.L. Cunningham and A.H. Fairlamb, unpublished) or the homologous overexpression of anti-sense RNA for TR in *T. cruzi* (Tovar and Fairlamb, 1996). In both of these approaches, no increase in TR copy number or levels of TR mRNA from the endogenous gene was observed.

Interestingly, *Leishmania* possessing only one wild-type TR allele showed a dramatic decrease in their capacity to survive within human macrophages. Indeed, 72 h following infection either with the *L. donovani* or *L. major* TR-targeted mutants, the number of amastigotes inside human macrophages decreased by 17- and 5-fold, respectively

(Figures 4 and 5). This decreased survival inside macrophages may be explained as follows. In standard culture medium, one *TR* allele is sufficient to maintain wild-type levels of trypanothione. However, in the case where the demand for trypanothione is high, such as during infection of the macrophage where reactive oxygen metabolites are produced, the capacity to regenerate reduced trypanothione via *TR* needs to be of wild-type levels since many of the steps of the antioxidant defence system involve trypanothione (Henderson *et al.*, 1987; Fairlamb, 1989). If *TR* activity levels drop, as is the case for the *TR* mutants, decreased intracellular survival will occur as a result of a less efficient defence against the oxidative stress. This model is consistent with our observations that *TR* mutants grow well inside a murine macrophage cell line incapable of producing reactive oxygen derivatives, whereas they are eliminated rapidly inside human macrophages that produce high amounts of superoxide anion (Table II). This, we believe, is a formal proof that one of *TR*'s physiological roles in the parasite is to combat oxidative stress inside macrophages. The ability of the *TR* gene alone to restore the mutant infectivity phenotype (Figure 4) strongly supports that *TR* is indeed involved in defence against oxidative damage. Moreover, as we never observed any recombination/repair event of the *hyg* disruption in *L.donovani* *TR/TR::hyg* transfected with a *TR*-expressing plasmid (not shown), we can conclude that we are clearly complementing the *TR* disruption rather than any putative neighbouring gene.

In view of the claimed pivotal role of *TR* in the defence against oxidative stress in *Leishmania*, our inability to generate *TR* null mutants was not overwhelmingly surprising. Indeed, following successful integration of both *hyg*- and *neo*-selectable markers into the two existing *TR* genomic alleles by gene disruption, a third allelic locus was created not only in species pathogenic to humans but also in the lizard parasite *L.tarentolae* (unpublished data). The genomic rearrangements leading to formation of a third locus for *TR* have been studied by hybridization experiments. In *L.donovani* *TR/TR::hyg/TR::neo* mutant, the third *TR* copy arose via translocation of a large segment of DNA to a new chromosome. The exact region within the *TR* locus translocated from the 520 to the 1200 kb chromosome is unknown. Our results demonstrated that homologous sequences, most likely of small size (based on the hybridization signal intensity), are present both in the vicinity of the 5'-flanking sequences of the *TR* locus and on a 1200 kb chromosome (Figure 2C). Indeed, the rearrangement point located upstream of the *TR* gene has been mapped to a region lying within a 3.1 kb *SacII* fragment (Figure 1B) and it is likely that generation of the third *TR* allele may have arisen by homologous recombination through these sequences. Several of the genomic rearrangements observed in *Leishmania* are indeed mediated by homologous recombination at the level of repeated sequences (Ouellette *et al.*, 1991; Grondin *et al.*, 1993). Since the downstream rearrangement point has not yet been identified, at this stage we cannot exclude the possibility that the whole region from the *TR* gene to the telomeric end has been translocated into a larger chromosome. However, this seems unlikely because we have mapped the *TR* gene at 380 kb from the telomere (S.Tamar and B.Papadopolou, unpublished), yet we

observed no change in the size of the 1200 kb chromosome by CHEF between the wild-type and the mutant in which the rearrangement took place (Figure 2C).

Regardless of the exact mechanism leading to partial trisomy at the *TR* locus, it is different from the sole example described previously in a virulent *L.major* where the chromosome number was increased to keep one *dhfr-ts* allele intact when attempting to generate a dihydrofolate reductase-thymidylate synthase null mutant (Cruz *et al.*, 1993). These authors have proposed that such alterations in the genome could be a diagnostic criterion for essentiality of gene function. If this supposition is correct, then the *TR* gene is likely to be essential for parasite survival under conditions not involving exogenous oxidant stress.

We used several approaches to try to determine whether our inability to obtain a *TR* null mutant was related to the fact that the *TR* gene is essential. All attempts were inconclusive, but one will be discussed. Vectors carrying inserts of different sizes expressing the *TR* gene were transfected into *L.donovani* *TR/TR::hyg* clones. These transfectants and the *TR/TR::hyg* clone were subjected to a stepwise increase in hygromycin B concentration. This approach, first used in mammalian cells (Mortensen *et al.*, 1992) and more recently in *Leishmania* (Gueiros-Filho and Beverley, 1996), can lead to loss of heterozygosity. In three independent clones containing the *TR* plasmids and selected for 8-fold more resistance to hygromycin B, we were indeed capable of disrupting a second (rearranged) *TR* allele with the *hyg* gene, but not of engineering a *TR* null mutant because a wild-type copy was detected in these cells despite the presence of the plasmid. This would suggest that a gene (or sequence) near to *TR* is essential and that double disruption of the *TR* gene by *hyg* and *neo* would be detrimental to its expression in such a way that the region becomes trisomic. However, when the *TR/TR::hyg* mutant was selected for 10-fold more resistance to hygromycin B in the absence of the *TR* plasmid, we did not observe any loss of heterozygosity or genomic rearrangements. This suggests that *TR* itself is also refractory to change and is likely to be an important gene. Our present working hypothesis is that both *TR* and a nearby gene or sequence are important for parasite survival. More experiments are required, however, to draw firm conclusions on this question.

Genes, such as *TR*, that are required for parasite intracellular survival should constitute interesting targets to inactivate in order to generate a live vaccine. There are no reliable vaccines against trypanosomatid infections and, in many cases, treatment with drugs currently available is unsatisfactory due to toxicity or limited efficacy (Ouellette and Papadopolou, 1993). Approaches to developing live vaccines for leishmaniasis are now theoretically feasible and, recently, a *L.major* *dhfr-ts* null mutant was used successfully in vaccination of mice (Titus *et al.*, 1995). The *TR* mutants we have generated in this study indeed showed an attenuated capacity to survive the oxidative stress in macrophages. Despite their interesting phenotype, these mutants probably could not be used for vaccination, given the possibility of reversion *in vivo* due to the presence of one intact *TR* copy. However, it could be possible to use this mutation in combination with others in order to obtain stable attenuated strains useful for vaccine development.

Materials and methods

DNA constructs

Part of the coding region for *L. donovani* TR (nucleotides 153–1406; Taylor *et al.*, 1994) was amplified by PCR using degenerate oligonucleotide primers. The full primer sequences used were 5'-TGC GTT AAC GTC(G) GGT(C) TGC GTC(G) CC-3' and 5'-GCA GAG CTC CTC G(C)GC C(G)GA G(C)GT C(G)GG-3' containing the cloning sites *HpaI* and *SacI* (underlined). The same primers were used to amplify part of the TR gene from *L. major* total DNA. The *HpaI*–*SacI* PCR products (1250 bp for *L. donovani* and ~1350–1400 bp for *L. major*) were subcloned into the *SmaI*–*SacI* sites of pSP72 vector (Promega). To make the TR gene knockout constructs, *neo* and *hyg* cassettes (Papadopolou *et al.*, 1994a) were introduced as *SmaI*–*EcoRV* fragments into the unique *BalI* site of the TR gene from both species. Constructs were linearized by digestion with *BamHI*–*EcoRV* and purified by electrophoresis on LMP agarose (Gibco-BRL) before transfection. A cosmid clone (Ldi E7-2) containing the TR locus was isolated by screening an *L. donovani* infantum genomic cosmid library made in the vector CL-hyg (Ryan *et al.*, 1993) and gridded on high density filters with an *L. donovani* TR-specific probe. A 2.2 kb *SphI*–*SphI* fragment containing the TR gene coding sequence and 650 bp of upstream sequences was subcloned into pGEM7-*neo* expression vector (Papadopolou *et al.*, 1992) and transfected into *L. donovani* TR/TR::hyg single mutant strain to test for the specificity of the TR mutant phenotype.

Cell culture and transfections

Leishmania major MHOM/IL/67/JERICO II (50122) and *L. donovani* MHOM/IN/80/DD8 (50212) strains were obtained from ATCC. Both strains were grown in SDM-79 medium (Brun and Schönenberger, 1979) supplemented with 10% fetal bovine serum (FBS) (Multicell, Wisent Inc.) and 5 µg/ml of haemin. Two to 3 µg of linearized DNA from the targeting constructs were transfected into *L. donovani* promastigotes by electroporation as described previously (Papadopolou *et al.*, 1992). Conditions for *L. major* transfections were as described by Descoteaux *et al.* (1994). Transfectants were selected with 40 µg/ml of G418 (Geneticin, Gibco-BRL) or 160 µg/ml of hygromycin B (CalBiochem) on SDM-agar plates (1%). The mouse monocyte–macrophage-like cell line J774A.1 obtained from ATCC (TIB-67) was cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% FBS. Human peripheral blood monocytes were isolated from heparinized venous blood supplied by the Canadian Red Cross. Cells were centrifuged over a Ficoll-Paque gradient (Pharmacia) as previously described (Olivier *et al.*, 1992). After several washes, cells were resuspended in RPMI 1640 medium (Gibco-BRL) containing 10% human serum (Gibco-BRL). In order to differentiate monocytes into macrophages, 3×10^6 peripheral blood leucocytes, counted using trypan blue, were adhered and cultured for 5 days at 37°C in a humidified atmosphere of 5% CO₂/95% air. To transform *L. donovani* promastigotes into amastigotes *in vitro*, the parasites were grown up to stationary phase at room temperature in SDM medium in which HEPES was replaced by MES to adjust the pH to 5.0, and then switched overnight at 37°C in 5% CO₂ atmosphere (Charest and Matlashewski, 1994).

In vitro macrophage infection

The capacity of the *Leishmania* TR mutants to infect murine and human macrophages *in vitro* was tested in comparison with a control *Leishmania-neo* transfectant, as follows. Macrophages from murine and human origins were seeded (200 µl/well, 5×10^4 cells/ml) into 8-well chamber slides, and were infected with recombinant parasites, at a parasite to cell ratio of 20:1 for a period of 6 h. Following this incubation, the non-engulfed parasites were removed by 3–5 washes with warm medium and chambers were replenished with 500 µl of fresh culture medium. The level of infection was determined at 6, 24, 48 and 72 h by optical microscopy examination following Diff Quick staining of cell preparations (Muyombwe *et al.*, 1997). Values are expressed either as the percentage of macrophages that were infected by *Leishmania* or as the total number of amastigotes within 100 macrophage cells.

Measurement of superoxide production in macrophage cells

Superoxide (O₂⁻) was measured by the reduction of ferricytochrome *c* (Sigma type III) as described (Olivier *et al.*, 1992). Approximately 10⁶ adherent macrophages either from the murine cell line J774 or from human origin were incubated in RPMI medium containing 1 mg/ml of ferricytochrome *c* and then infected with 2×10^7 *Leishmania* (ratio 20 parasites/cell) for 2, 4 and 6 h at 37°C. As a control, macrophage cells

were stimulated with 100 ng/ml of phorbol 12-myristate 13-acetate (PMA) (Sigma). Quantitation of reactive oxygen intermediates on cell supernatants was done spectrophotometrically at 550 nm. Results were expressed in nmol/h/mg cell protein.

Trypanothione reductase and thiol measurement

Preparation of cell-free extracts to measure the TR enzymatic activities in wild-type and TR-disrupted transfectants was as described previously (Tovar and Fairlamb, 1996). TR activity was assayed spectrophotometrically by monitoring trypanothione-dependent oxidation of NADPH at 340 nm (Shames *et al.*, 1986). Alanine aminotransferase activity was measured as an internal control to correct for any variability in the extraction procedure (Tovar and Fairlamb, 1996). One unit of activity (U) is defined as the amount of enzyme required to catalyse the conversion of 1 µmol of NADPH to NADP⁺ per min at 27°C. Thiol content was determined by fluorescence detection following pre-column derivatization with monobromobimane and separation by HPLC. Thiol content was determined relative to authentic standards. Exponential phase cells (typically 1×10^7 cells/ml) were harvested and washed, and 1×10^8 cells were derivatized as previously described (Shim and Fairlamb, 1988). Analyses were performed in duplicate or quadruplicate for three independent experiments.

DNA manipulations

Total genomic DNA from *Leishmania* was prepared as described (Bernards *et al.*, 1981), digested with *SacI*, *XhoI* or *EcoRI*, resolved on 0.7% agarose gels and transferred to nylon membranes (Hybond-N, Amersham). Total RNA from *L. donovani* wild-type and TR mutants from both promastigote and amastigote stages were prepared using Trizol (Gibco BRL). Southern and Northern blots, hybridizations and washings were done following standard procedures (Sambrook *et al.*, 1989). Intact chromosomes were prepared from *Leishmania* promastigotes harvested during mid- to late log phase, washed and lysed *in situ* in 1% low melting agarose plugs as previously described (Van der Ploeg *et al.*, 1984). The blocks were loaded directly into the wells of 1% agarose gel and sealed in place. *Leishmania* chromosomes were separated by pulsed-field gel electrophoresis using a Bio-Rad CHEF-DR III apparatus at 5 V/cm, 120° separation angle and switch times varying from 35 to 120 for 30 h and transferred to nylon membranes for Southern blot hybridization. Blots were hybridized with radiolabelled probes prepared by randomly primed synthesis with Klenow DNA polymerase and [α -³²P]dATP. The TR probes used here correspond to PCR-generated fragments from the two species covering a large part of the gene-coding region. The other probes used correspond to *hyg*- and *neo*-coding sequences also made by PCR, to the α -tubulin gene of *T. brucei* (Thomashow *et al.*, 1983) and to the A2 amastigote-specific gene (Charest and Matlashewski, 1994). Probe 1 corresponds to a 4.1 kb *Clal*–*EcoRI* fragment containing a large part of the TR gene and upstream sequences, and probe 2 is a 23 kb *EcoRI*–*EcoRI* fragment located 9 kb downstream of the TR gene (see Figure 2A). These two probes were generated by digestions of the Ldi E7-2 cosmid.

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