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Glutathione and the redox control system trypanothione/trypanothione reductase are involved in the protection of *Leishmania* spp. against nitrosothiol-induced cytotoxicity

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Abstract

Glutathione is the major intracellular antioxidant thiol protecting mammalian cells against oxidative stress induced by oxygen- and nitrogen-derived reactive species. In trypanosomes and leishmanias, trypanothione plays a central role in parasite protection against mammalian host defence systems by recycling trypanothione disulphide by the enzyme trypanothione reductase. Although Kinetoplastida parasites lack glutathione reductase, they maintain significant levels of glutathione. The aim of this study was to use *Leishmania donovani trypanothione reductase* gene mutant clones and different *Leishmania* species to examine the role of these two individual thiol systems in the protection mechanism against S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP), a nitrogen-derived reactive species donor. We found that the resistance to SNAP of different species of *Leishmania* was inversely correlated with their glutathione concentration but not with their total low-molecular weight thiol content (about 0.18 nmol/10^7 parasites, regardless *Leishmania* species). The glutathione concentration in *L. amazonensis*, *L. donovani*, *L. major*, and *L. braziliensis* were 0.12, 0.10, 0.08, and 0.04 nmol/10^7 parasites, respectively. *L. amazonensis*, that have a higher level of glutathione, were less susceptible to SNAP (30 and 100 µM). The IC_{50} values of SNAP determined to *L. amazonensis*, *L. donovani*, *L. major*, and *L. braziliensis* were 207.8, 188.5, 160.9, and 83 µM, respectively. We also observed that *L. donovani* mutants carrying only one *trypanothione reductase* allele had a decreased capacity to survive (~40%) in the presence of SNAP (30-150 µM). In conclusion, the present data suggest that both antioxidant systems, glutathione and trypanothione/trypanothione reductase, participate in protection of *Leishmania* against the toxic effect of nitrogen-derived reactive species.

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Key words
- *Leishmania*
- Glutathione
- Trypanothione
- Trypanothione reductase
- Nitric oxide
- Free radicals

Introduction

The trypanosomatids, members of the order Kinetoplastida, include parasitic protozoa of importance to public health such as *Leishmania* spp. *Leishmania* cause a spectrum of diseases ranging from self-healing ulcers to disseminated and often fatal infections, depending on the species involved and the host's immune response. Adequate vaccines against trypanosomatid infections have yet to be developed, and drugs currently available for chemotherapeutic intervention are mostly unsatisfactory mainly because of their lack of specificity, toxicity to humans, and, in many cases, developed parasite resistance (1). Thus, one of the priorities in tropical medicine research has been the identification and characterisation of parasite-specific biomolecules, which play relevant physiological roles and thus might be exploited as selective targets.

Among many other metabolic distinctions, trypanosomatids maintain their intracellular redox balance by a mechanism that is different from that of their insect vectors and mammalian hosts. They lack glutathione reductase, which in nearly all other organisms is responsible for the maintenance of an intracellular thiol-reducing environment, and thus for the reduction of disulphides, detoxification of peroxides and synthesis of DNA precursors (2). Instead, they possess a unique system using trypanothione [$T(SH)_2$] that is the major reduced thiol of Kinetoplastida parasites (3) and comprises a spermidine moiety linked to two glutathione molecules (2). Together with three thiol-redox proteins, trypanothione reductase (TryR), tryparedoxin and tryparedoxin peroxidase (4,5), $T(SH)_2$ is thought to provide defence against oxidants, certain heavy metals (6) and xenobiotics (3). Thus, TryR has a vital physiological role in maintaining $T(SH)_2$ redox, particularly within the highly oxidative intracellular environment of the host cells which is generated during the antimicrobial defence response.

In murine leishmaniasis, nitric oxide (NO) plays a crucial role in the killing of parasites both *in vitro* (7) and *in vivo* (8,9). *In vitro* macrophage microbicidal activity correlates with NO production, and both *in vivo* and *in vitro* microbicidal activities are completely inhibited by the NO synthase inhibitor L-arginine analogue N^G-monomethyl-L-arginine (L-NMMA) but not by its enantiomer D-NMMA (8). In addition, the NO donors, S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) and 3-morpholino-sydnonimine hydrochloride are able to kill *Leishmania* parasites in a cell-free model system (10).

We have reported that glutathione is involved in the protection of mammalian macrophages against the cytotoxic effects of NO. Furthermore, despite evidence that glutathione in *Leishmania* appears not to be the major antioxidant, we have demonstrated that it protects *L. major* from the toxic effects of NO (11).

In the present study, we have extended these observations by comparing the glutathione levels and SNAP sensitivity of different *Leishmania* species: *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*. Our results demonstrate that the sensitivity of distinct species of *Leishmania* to SNAP is inversely correlated with their glutathione concentration. When we extended our investigation to the role of TryR in the protection against SNAP using mutants of *L. donovani* for the TryR gene (*tryR*, formerly *tryA*) (12), we found that, compared to control parasites (*tryR* genotype^{+}/$^{+}$/$^{+}$), a double mutant clone (*tryR* genotype^{-}/$^{-}$/$^{+}$) was significantly more sensitive to the cytotoxic effect of SNAP. Overall, these results demonstrate that glutathione as well as the $T(SH)_2$/TryR redox system are essential protective components against NO cytotoxicity in *Leishmania.*

Material and Methods

Leishmania strains and culture conditions

The *Leishmania* species used in this study

were *L. braziliensis* (MHOM/BR/75/M2904), *L. amazonensis* (MPRO/BR/72/M1841-LV-79), *L. major* (LV-39, clone 5-Rho-SU/59/P), and *L. donovani* (clone LV9-3 from MHOM/ET/67/HU3). The *L. donovani* clones used were: wild-type LV9-3, which possesses three copies of the *tryR* (formerly *tryA*) gene ($tryR^{+/+/+}$) and the mutants of TryR: clones H2-$tryR^{-/+/+}$ (LV9-3 submitted to single replacement), clone HB3-$tryR^{-/-/+}$ (LV9-3 submitted to double replacement (12) and clone HB3-pTTcTR (Tovar J and Fairlamb AH, unpublished results) that is identical to clone HB3 ($tryR^{-/-/+}$) but harbours plasmid pTTcTR (13). Promastigote forms of all *Leishmania* species were grown in M199 medium supplemented with 40 mM HEPES, pH 7.4, 0.1 mM adenine, 7.7 mM hemin, 10% (v/v) heat-inactivated foetal calf serum, 50 U/mL penicillin, and 50 µg/mL streptomycin. Cultures were incubated at 26ºC, and cells were kept at densities ranging between 5 x 10^5 and 3 x 10^7 parasites/mL. Transfectants were cultured in the presence of selective drugs (12). The mutant H2-$tryR^{-/+/+}$ was cultured in the presence of 16 µg/mL hygromycin B, the clone HB3-$tryR^{-/-/+}$ in the presence of 16 µg/mL hygromycin B plus 2.5 µg /mL phleomycin, and the HB3-$tryR^{-/-/+}$ clone plus episomal pTTcTR in the presence of 16 µg/mL hygromycin B, 25 µg/mL G418 and 2.5 µg/mL phleomycin.

Growth curves

Promastigotes of wild-type or *L. donovani* mutants were cultured in M199 medium prepared as described previously (14). Cell density in the inoculum was 1 x 10^5/mL. Viability was evaluated from motility and cell density was determined daily using a hemocytometer.

Glutathione and non-protein low-molecular weight thiol measurement

Non-protein low-molecular weight thiols and glutathione (reduced plus disulphide forms) were measured in lysates of promastigote forms (stationary phase) of different *Leishmania* species (*L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*) including the different clones of *L. donovani*. Low-molecular weight thiols were measured using Ellman's reagent (15). To measure soluble thiols the samples were deproteinised with 1% sulfosalicylic acid in the presence of 5 mM EDTA. The concentration of SH groups was calculated from a standard curve of 0.01 to 2 nmol cysteine. Glutathione levels were measured by the glutathione reductase enzyme recycling method (16). These assays were adapted for use in a microtitre plate using a microplate spectrophotometer system spectra MAX 250 (Molecular Devices, Union City, CA, USA). Cells were lysed by the addition of 100 µL 1 mM EDTA to each well and freezing immediately. Following thawing, plates were shaken for 30 s and then sonicated for 60 s. Assays were carried out immediately (17). Although this assay provides a measure of both oxidised and reduced glutathione, in non-oxidative stress equilibrium, the cellular condition under which we have measured glutathione, the thiol content is 95-99% reduced glutathione.

Cytotoxic effect of S-nitroso-*N*-acetyl-D,L-penicillamine on *Leishmania* viability

The direct cytotoxic effect of the nitrosothiol SNAP on *Leishmania* species was measured. Briefly, parasites (3 x 10^6/well) were incubated in M199 medium supplemented with 10% heat-inactivated foetal calf serum in the presence or absence of SNAP (30-1000 µM) for 12 h. The incubation medium contains L-cystine which allows the membrane transport of SNAP into cells (18). Parasites were then pulsed with 1 µCi/well [^{3}H]-thymidine, and the incorporation of radioactivity by viable parasites was determined after 24 h in a ß-counter (11). The

50% inhibitory concentration (IC_{50}) values for each *Leishmania* species were determined using Sigma-Plot software, Version 5.0.

Statistical analysis

Data are reported as means ± SEM and statistical significance (P < 0.05) was assessed by ANOVA followed by Bonferroni's *t*-test.

Results

Non-protein low-molecular weight thiol and glutathione concentration in different *Leishmania* species

Glutathione concentrations of different species of *Leishmania* (*L. amazonensis*, *L. braziliensis*, *L. major*, and *L. donovani*) are shown in Figure 1. Statistically significant differences in glutathione levels were observed between all *Leishmania* species, with promastigotes of *L. braziliensis* having the lowest level. Glutathione concentration was *L. amazonensis* > *L. donovani* > *L. major* > *L. braziliensis*. No significant differences in the non-protein low-molecular weight thiol levels were detected between different *Leishmania* species (Figure 2).

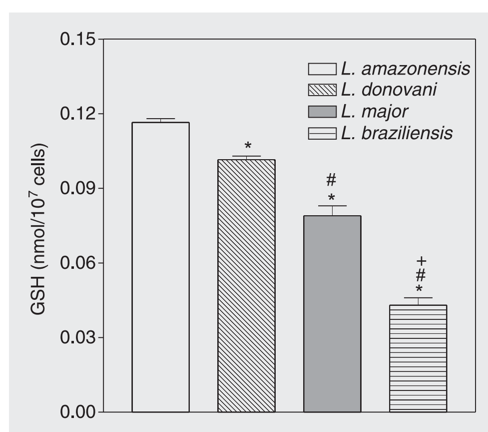

Figure 1. Intracellular glutathione (GSH) concentrations in different *Leishmania* species. Total glutathione (GSH + oxidized glutathione (GSSG)) was measured in lysates of 1 x 10^7 promastigotes of different *Leishmania* species (*L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*). Data are reported as means ± SEM of four replicate cultures and are representative of three experiments. *P < 0.05 compared with *L. amazonensis*; #P < 0.05 compared with *L. donovani*; +P < 0.05 compared with *L. major* (ANOVA followed by Bonferroni's *t*-test).

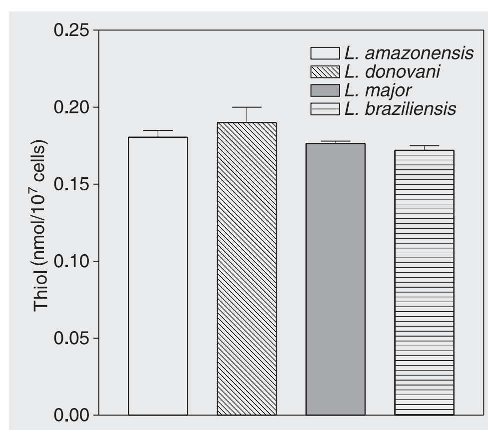

Figure 2. Concentrations of non-protein low-molecular weight thiols in different *Leishmania* species. Total non-protein thiols (glutathione, $T(SH)_2$, cysteine, and ovothiol) were measured in lysates of 1 x 10^7 promastigotes of *Leishmania* species (*L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*). Data are reported as means ± SEM of four replicate cultures and are representative of three experiments.

Cytotoxic effect of S-nitroso-*N*-acetyl-D, L-penicillamine on different *Leishmania* species

To ascertain whether the glutathione levels in the different *Leishmania* species (Figure 1) correlate with their sensitivity to reactive nitrogen species, we investigated the cellular viability of *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major* after treatment with SNAP. The addition of a SNAP directly to promastigotes of different species of *Leishmania* resulted in dose-dependent parasite killing (Figure 3). The sensitivity of *Leishmania* species to SNAP correlated inversely with the glutathione levels. *L. amazonensis*, that has a higher glutathione concentration, was more resistant to the cytotoxic effect of SNAP at concentrations of 30 and 100 µM, with an IC_{50} of 207.8 (200.2-215.7 µM). In contrast, *L. braziliensis*, that shows lower levels of glutathione, was more susceptible to the toxic effect of SNAP (IC_{50} of 83 (79.7-85.5 µM)). The IC_{50} values determined for *L. donovani* and *L. major* were 188.5 and 160.9 µM, respectively.

Effect of S-nitroso-*N*-acetyl-D,L-penicillamine on the viability of *Leishmania donovani tryR* gene mutants

To ascertain whether the $T(SH)_2$/TryR antioxidant system is involved in the protection of *Leishmania* against nitrogen-derived reactive species, we investigated the effect

of SNAP on the viability of targeted *L. donovani* TryR mutants that have been generated by gene disruption. Wild-type *L. donovani* (LV9-3, genotype of *tryR*$^{+/+/+}$) and clones submitted to a single (clone H2 genotype of *tryR*$^{-/+/+}$) or double (clone HB3 genotype of *tryR*$^{-/-/+}$) experiment for the replacement of the *tryR* locus were utilised. It is important to mention that a null mutant has not yet been obtained for *tryR* (12,19). The addition of SNAP directly to promastigotes of different *L. donovani* clones resulted in partial parasite killing (Figure 4). Recombinant HB3 possessing only one *tryR* allele was more sensitive to the toxic effect of SNAP at concentrations of 30-150 µM compared with recombinant H2 or *L. donovani* parental clone (LV9-3). The sensitivity to high concentration of SNAP (300 µM) was unchanged. To demonstrate that the decrease in the sensitivity was solely due to the disruption of the *tryR* gene, we tested the viability of clone HB3 (*tryR*$^{-/-/+}$) supplemented with the plasmid pTTcTR that harbours a functional *T. cruzi tryR* gene (13). This recombinant strain, whose levels of TryR are higher than those of wild-type parasites, regained wild-type levels of resistance to SNAP-generated stress (Figure 4).

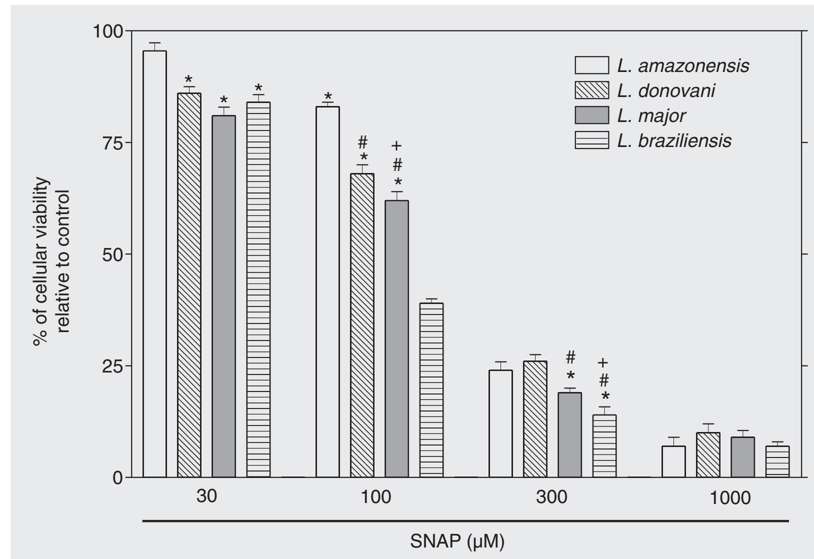

Figure 3. Cytotoxic effect of S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on different *Leishmania* species. Promastigote forms of *L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major* (3 x 10^6 cells/well) were incubated in M199 medium (control) or M199 plus SNAP (30-1000 µM). The cultures were pulsed with ^{3}[H]-thymidine 12 h after SNAP treatment. *Leishmania* survival was determined after 24 h of further culture by the ability of residual live parasites to incorporate ^{3}[H]-thymidine. Data are reported are means ± SEM of four replicates and are representative of three experiments. Statistically significant different with *P < 0.05 compared with *L. am*azonensis, #P < 0.05 compared with *L. donovani*, and +P < 0.05 compared with *L. major* (ANOVA followed by Bonferroni's *t*-test).

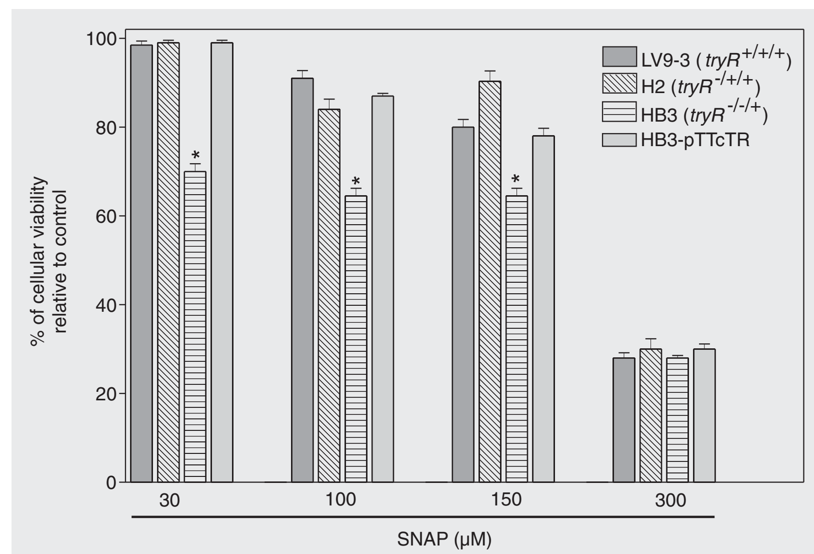

Figure 4. Cytotoxic effect of S-nitroso-*N*-acetyl-D,L-penicillamine (SNAP) on *L. donovani* viability. Effects of the number of *tryR* allele promastigotes (3 x 10^6) of wild-type *L. donovani* (LV9-3: *tryR*$^{+/+/+}$) and clones H2 (*tryR*$^{-/+/+}$), HB3 (*tryR*$^{-/-/+}$) and HB3-pTTcTR (*tryR*$^{-/-/+}$ plus pTEXTcTR episomic) were incubated in M199 medium (control) or M199 plus SNAP (30-300 µM) for 12 h. *Leishmania* survival was determined after 24 h of further culture by the ability of residual live parasites to incorporate ^{3}[H]-thymidine. Data are reported as means ± SEM of four replicates and are representative of three experiments. *P < 0.05 compared with the parental clone (LV9-3; ANOVA followed by Bonferroni's *t*-test).

Glutathione concentration in *Leishmania donovani* TryR mutants

Our previous results (11) and the data presented here suggest that glutathione is involved in the protection of *Leishmania* against the toxic effects of SNAP. As TryR is the enzyme that maintains $T(SH)_2$ in its reduced form and plays a central role in oxidant detoxification through the enzymatic regeneration of the thiol pool, we examined whether the effect of the loss of the *tryR* copy in *L. donovani* mutant clones changed the glutathione levels. No significant differences in glutathione were detected between the wild-type and single- or double-targeted *L. donovani tryR* mutants or with the HB3-

pTTcTR transfected clone (Figure 5).

Growth of *Leishmania donovani* clones

Growth curves of both wild-type (LV9-3) and *L. donovani* mutants (clones H2-*tryR*$^{-/+/+}$, HB3-*tryR*$^{-/-/+}$ and HB3-pTTcTR) show the typical log and stationary growth phases. A similar pattern of continual growth was observed for all transfectants (Figure 6).

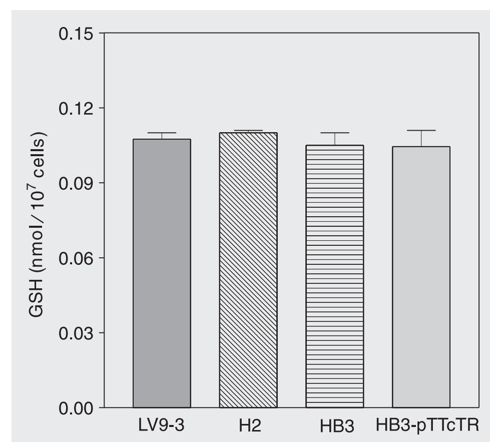

Figure 5. Intracellular glutathione (GSH) levels in *L. donovani* trypanothione reductase (*tryR*) mutants. Total glutathione (GSH + oxidized glutathione (GSSG)) was measured in lysates of 1 x 10^7 promastigotes of wild-type *L. donovani* (LV9-3: *tryR*$^{+/+/+}$) and clones H2 (*tryR*$^{-/+/+}$), HB3 (*tryR*$^{-/-/+}$), and HB3-pTTcTR (*tryR*$^{-/-/+}$ plus pTEXTcTR episomic). Data are reported as means ± SEM of four replicate cultures and are representative of three experiments.

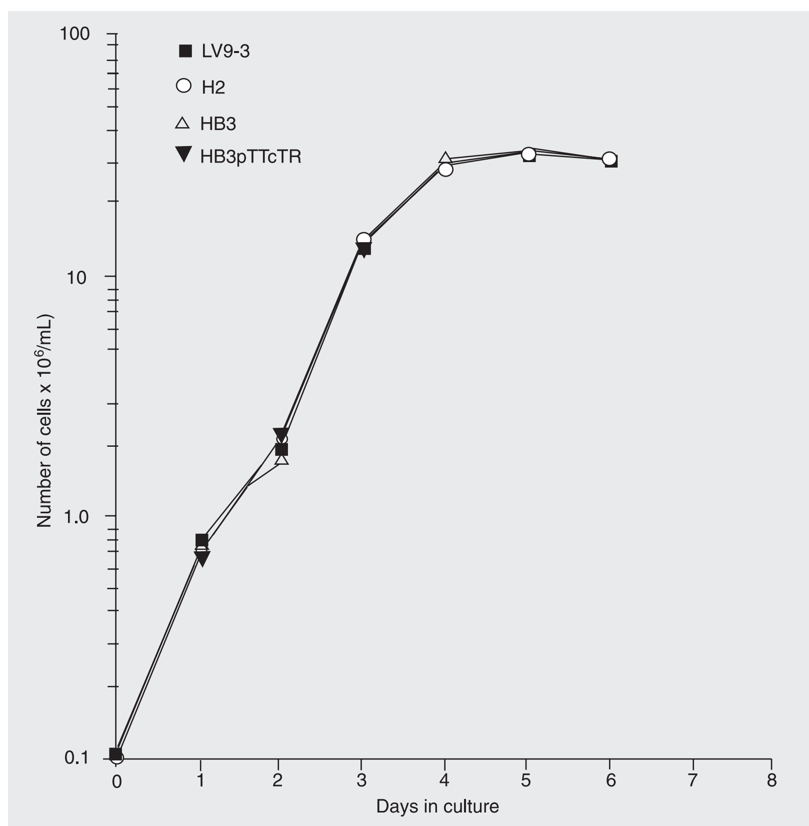

Figure 6. Growth curve of wild-type *L. donovani* (LV9-3: *tryR*$^{+/+/+}$) and clones H2 (*tryR*$^{-/+/+}$), HB3 (*tryR*$^{-/-/+}$), and HB3-pTTcTR (*tryR*$^{-/-/+}$ plus pTEXTcTR episomic). Each point represents the average of counts from two cultures, initially inoculated with 1 x 10^5 organisms/mL in M199 medium.

Discussion

The results presented in this study suggest that both glutathione and $T(SH)_2$/TryR antioxidant systems are important components of the protective mechanisms of *Leishmania* against the cytotoxic effects of nitrogen-derived reactive species. This conclusion is supported by the following observations: a) the sensitivity of distinct species of *Leishmania* (*L. amazonensis*, *L. braziliensis*, *L. donovani*, and *L. major*) to SNAP, a nitrogen-derived reactive species donor, correlated inversely with their cellular glutathione levels but not with their total non-protein low-molecular weight thiol content; b) mutants of *L. donovani* possessing only one wild-type *tryR* allele, *tryR*$^{-/-/+}$ (submitted to a double replacement of the *tryR* gene locus), had a decreased capacity to survive in the presence of SNAP. Nevertheless, under normal conditions of *in vitro* growth, the *L. donovani* wild-type and recombinant mutants have similar levels of glutathione.

Our data are consistent with Lemesre et al. (20) who demonstrated that amastigote forms of *L. amazonensis*, which have higher levels of glutathione, are particularly resistant to NO killing. It is recognised that similar molecular targets of NO are present in microorganisms and in mammalian cells (21,22) and, it has been demonstrated that glutathione protects several mammalian cells, including macrophages, against the oxidative stress induced by oxygen (23) and nitrogen-derived oxidants (11,24,25). Thus, the different intracellular glutathione levels may explain the large differences in susceptibility to SNAP among different species of *Leishmania*. The observation that the levels of non-protein thiols were similar in all *Leishmania* species analysed supports this con-

clusion. Furthermore, it has been demonstrated that buthionine sulfoximine, an inhibitor of glutathione synthesis, protects mice from *Trypanosoma brucei* infections by lowering the parasites' glutathione and protein thiol levels (26). In addition, conjugation of electrophilic drugs with glutathione via glutathione S-transferase has been shown to be an important detoxification system in *T. cruzi* epimastigotes (27).

The mechanism by which glutathione is implicated in the protection of trypanosomatid protozoa against the cytotoxic effects of nitrogen-derived oxidants is not clear. However, there is evidence that biological NO-derived oxidants such as nitrogen dioxide and carbonate radical anion dioxide promote cell injury (28-30). These events depend on the intracellular concentration of low-molecular weight thiols such as glutathione (24,31,32). Furthermore, glutathione and protein-cysteines are the preferred targets of NO-derived oxidants being oxidised to the respective thyil radicals (24,33).

$T(SH)_2$, a glutathione and spermidine conjugate, is one of the unique features of Kinetoplastida. In trypanosomatids, the existence of TryR instead of glutathione reductase suggested that the thiol pool in these organisms is dependent on TryR (3). Thus, TryR has long been regarded as a target molecule for selective attack of the trypanosomatids. Our data demonstrate that the *L. donovani* recombinant HB3 clone possessing only one wild-type *tryR* allele, retaining only one-third of its original TryR activity (12), has a partially decreased capacity to survive in the presence of SNAP. Furthermore, this mutant and the *L. donovani* mutants *TR/TR::hyg* and *TR/TR::hyg/TR::neo*, which also carry a single *tryR* allele, have been shown to be impaired in their ability to survive inside cytokine-activated mouse and human macrophages (12,19). Significantly, when the HB3 clone was electroporated with the plasmid pTTcTR, this recombinant strain (HB3-pTTcTR) regained its ability to resist SNAP cytotoxicity to approximately wild-type levels. It is important to note that the HB3 mutant clone possesses only one *tryR* allele, whilst the parental clone (LV9-3) possesses three allelic copies of the gene (12). Despite this, the parental and all mutant lines used in the present study proliferated at equivalent rates under the conditions used.

The fact that the *tryR* mutant HB3 showed only a partial decrease in the survival rate (~40%) when incubated with low doses of SNAP suggests that the remaining TryR activity present in this clone may still provide important antioxidant protection. This finding is consistent with the observation that *Leishmania* cells that overexpress a trans-dominant mutant version of *tryR* and as a result retain only about 15% of TryR activity, are not significantly impaired in their ability to metabolise hydrogen peroxide *in vitro* and are able to maintain wild-type levels of thiols under normal conditions of growth (34). It is possible that GSH may also contribute significantly to the residual antioxidant activity observed in clone HB3, as discussed above. In this context, we observed that all *tryR* mutant clones, despite having a reduction in TryR activities, present similar glutathione levels compared to *L. donovani* wild-type cells. This finding is in accordance with previous data demonstrating that *L. donovani* wild-type and *tryR* mutants with low levels of TryR catalytic activity did not differ in their levels of $T(SH)_2$, glutathionyl-spermidine or glutathione (19, 35). Levels of reduced thiols (e.g., GSH, $T(SH)_2$) may only become limiting under conditions of oxidative stress in the context of a compromised $T(SH)_2$/TryR antioxidant system (12,19). An intriguing aspect of the *L. donovani* mutants that needs further investigation is that, in contrast to wild-type *L. donovani* (Figure 3), their sensitivity to SNAP was not dose-dependent.

Trypanosomatids also produce significant amounts of ovothiol A (36) which has recently been shown to catalytically acceler-

ate nitrosothiol, both GSNO and $T(SNO)_2$, decomposition to NO (37). The rate-limiting step in this reaction is the reduction of oxidised ovothiol which in *Leishmania* is mediated indirectly via trypanothione/trypanothione reductase, as is also the case for oxidized glutathione (GSSG) (5). Thus, we cannot exclude the possibility that the difference in cytotoxic potency of SNAP observed here between different *tryR* clones of *Leishmania* is dependent on variable ovothiol A concentrations and hence on nitrogen-free radical production.

The present data suggest that both antioxidant systems, glutathione and $T(SH)_2$/TryR, are involved in protecting *Leishmania* against the toxic effect of nitrogen-derived reactive species.

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