ACCELERATED PUBLICATION

Role of superoxide dismutase in survival of *Leishmania* within the macrophage

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Intracellular parasitic protozoans of the genus *Leishmania* depend for their survival on the elaboration of enzymic and other mechanisms for evading toxic free-radical damage inflicted by their phagocytic macrophage host. One such mechanism may involve superoxide dismutase (SOD), which detoxifies reactive superoxide radicals produced by activated macrophages, but the role of this enzyme in parasite survival has not yet been demonstrated. We have cloned a SOD gene from *L. tropica* and generated SOD-deficient parasites by expressing the corresponding antisense RNA from an episomal vector. Such parasites have enhanced sensitivity to menadione and hydrogen peroxide in axenic culture, and a markedly reduced survival in mouse macrophages. These results indicate that SOD is a major determinant of intracellular survival of *Leishmania*.

Key words: antisense RNA, *Leishmania*, macrophage, superoxide dismutase.

INTRODUCTION

Kinetoplastid protozoa of the genus *Leishmania* have a digenetic life cycle comprising an extracellular promastigote stage in the insect vector and an intracellular stage in the macrophages of the infected host [1]. Upon infection, macrophages undergo a respiratory burst, producing reactive oxygen intermediates such as H_2O_2, $OH^{\bullet}$ radical, superoxide anion ($O_2^{\bullet -}$) and peroxynitrate as a part of an oxygen-dependent mechanism to destroy invading micro-organisms [2]. Two key macrophage enzymes responsible for generating these species, namely phagocyte oxidase and inducible nitric oxide synthase, are together critically important for host antimicrobial defence, as shown by the massive infections that occur in mice that are genetically deficient in both of these enzymes [3].

Conversely, intracellular pathogens may have evolved enzymic mechanisms to detoxify the reactive oxygen species. In various systems, four enzymes have been implicated in antioxidant defence: catalase, glutathione peroxidase, superoxide dismutase (SOD) and peroxidoxins. The first two enzymes have not been found in *Leishmania*. However, a high level of SOD is present in promastigotes [4]. Two types of SOD gene, SOD-A and SOD-B, have been cloned from *L. chagasi* [5]. SOD-A is a single-copy gene present on a small chromosome, whereas SOD-B is multicopy and resides on a large chromosome. The two genes differ from each other in primary sequence, particularly in the presence of an N-terminal extension encoded by the SOD-A gene ([5] and see Figure 1). The localization and individual functions of these two isoforms are unknown. The *L. chagasi* SOD gene complements an *Escherichia coli sod*$^-$ mutant, rendering it relatively resistant to paraquat, an O_2^- generator [5]. Additionally, the *in vitro* growth of *L. chagasi* promastigotes transfected with the SOD-A or -B gene is more resistant than wild-type to sodium nitroprusside or paraquat [5]. However, such studies do not address the question of whether SOD-mediated $O_2^{\bullet -}$ scavenging is a major determinant of parasite survival within macrophages. Recently, genes for peroxidoxin, which acts upon H_2O_2, alkyl peroxides and peroxynitrate, have been isolated from two different *Leishmania* species [6,7], suggesting peroxide detoxification as an alternative, or additional, defence mechanism.

In the present study, we have cloned a fragment of a *L. tropica* SOD (LtSOD) gene and inserted it in antisense orientation in an expression vector. Promastigotes of *L. tropica*, as well as *L. donovani*, transformed with this construct show reduced levels of SOD mRNA, protein and functional SOD enzyme. The SOD-deficient parasites are more sensitive than wild-type to $O_2^{\bullet -}$ generators, as well as H_2O_2, in axenic culture. Importantly, SOD deficiency results in a markedly reduced survival of *L. donovani* amastigotes in mouse macrophages.

EXPERIMENTAL

Parasites

Promastigotes of *L. tropica* strain UR6 and *L. donovani* strain AG83 were cultured at 22 °C on solid blood-agar medium [8] and medium M199 (Gibco) with 10% (v/v) fetal calf serum respectively.

Cloning of LtSOD cDNA

Polyadenylated [poly(A)$^+$] RNA from *L. tropica* promastigotes was used for 3′ rapid amplification of cDNA ends (RACE).

Abbreviations used: LtSOD, *Leishmania tropica* SOD; PARP, poly(ADP-ribose) polymerase; PFGE, pulsed field gel electrophoresis; poly(A)$^+$, polyadenylated; RACE, rapid amplification of cDNA ends; SOD, superoxide dismutase.

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The nucleotide sequence data reported will appear in DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY161306.

```
            1                                                    50
     Ltropica ..........  ..........  ..........  ..........  ..........
   LchagasiA MFRRVSMKAA  TATAPVGFAF  LCYHTLPLLR  YPAELPTLGF  NYKDGIQPVM
   LchagasiB ..........  ..........  ..........  MPFAVQPLPY  PHDALASKGM
  Ahydrophila ..........  ..........  ..........  MAFELPALPY  AINALEPH.I
HinfluenzaeMn ..........  ..........  ..........  MSYTLPELGY  AYNALEPH.F

            51                                                   100
     Ltropica ..........  ...RTYVEKL  NQLIE.GTPL  YGMQLDALIV  KSADD...SQ
   LchagasiA SSRQLELHYK  KHHSAYVDKL  NTL...GKGC  EGKTIEEIIL  ATSGT...TE
   LchagasiB SKEQVTFHHE  KHHKGYAVKL  NAAAESNSGL  ASKSLVDII.  ....K...SE
  Ahydrophila SQETLEYHHG  KHHNTYVVNL  NNLVP.GTEF  EGKSLEEII.  ....K...TS
HinfluenzaeMn DAQTMEIHHS  KHHQAYVNNA  NAALEGLPAE  LVEMYPGHLI  SNLDKIPAEK
                        *
            101                                                  150
     Ltropica LKGVYNNAAQ  HYNHSFFWKC  IQP.YGSNIP  PDLSAAVSAQ  YGSVEKFEKQ
   LchagasiA SKVMNNQAAQ  HFNHSFFWKC  LSP.GGKKIP  KTLENAIANE  FGSVDDFTVS
   LchagasiB KGPAFNCAAQ  IFNHDFFWRC  LSREAGGEPS  GPLASAIVDS  FGTFASFKKE
  Ahydrophila TGGIFNNAAQ  IWNHTFYWHC  LSPNGGGEPT  GALADAITKA  FGSFAEFKDA
HinfluenzaeMn RGALRNNAGG  HTNHSLFWKS  LKK..GTTLQ  GALKDAIERD  FGSVDAFKAE
                          *
            151                                                  200
     Ltropica FITAAQNLFG  SGWVYWVYDK  RAGAFDILSY  GNAGCPLTNY  EYT.....PL
   LchagasiA FQQAGVNNFG  SGWTWLCVDP  RTKELRIDNT  SNAGCPLTSG  LR......PI
   LchagasiB FTDAPNGHFG  SGWAWLVKDK  SSGKLKVLQT  HDAGCPLTEP  NLV.....PM
  Ahydrophila FTKSAIGNFG  SSWTWLVK.K  ADGSLAIVNT  SNAGCPLTEA  GTT.....PL
HinfluenzaeMn FEKAAATRFG  SGWAWLVL.T  AEGKLAVVST  ANQDNPLMGK  EVAGCEGFPL

            201                                                  250
     Ltropica LCVDVWEHAY  YIDYENKRPE  YLSKYFD.VV  DWHWAERHWK  RATDQEYHEM
   LchagasiA FTADVGEHAY  YKDFENRPRD  YLKELWQ.IV  DWEFVCQMYE  KATK......
   LchagasiB LTCDIWEHAY  YIDYRNDRAS  YVNAFWN.MV  DWDFASSQL.  ..........
  Ahydrophila LTVDLWEHAY  YIDFRNLRPK  YMETFWT.LV  NWEFVAKNLA  A.........
HinfluenzaeMn LGLDVWEHAY  YLKFQNRRPD  YIKEFWN.VV  NWDFVAERFE  QKNSTL....
                 *    *

            251
     Ltropica KFW
   LchagasiA ...
   LchagasiB ...
  Ahydrophila ...
HinfluenzaeMn ...
```

Figure 1 Partial sequence of SOD from *L. tropica*

Comparison of amino acid sequences of FeSODs from *L. tropica* (Ltropica; GenBank® accession number AY161306), *L. chagasi* FeSODA (LchagasiA; accession number AF003964), *L. chagasi* FeSODB (LchagasiB; accession number AF003963), *Aeromonas hydrophila* (Ahydrophila; accession number AF317227) and MnSOD from *Haemophilus influenzae* (HinfluenzaeMn; accession number X73832). Gaps were introduced in the sequences to maximize the similarities and compensate for different chain lengths. * Denotes residues involved in metal binding. The residues that are primary candidates for distinguishing FeSODs from MnSODs [15] are in **bold** type. The five conserved regions of SODs [16] are indicated by solid horizontal bars. The conserved SOD signature motif is boxed.

Briefly, 1 μg of poly(A)$^+$ RNA was used to prime the synthesis of cDNA by Superscript II reverse transcriptase (GIBCO) using oligo(dT)$_{17}$-anchor primer GCTCTAGATCTCGAGCGT$_{17}$ at 42 °C for 90 min. One-tenth of the cDNA reaction mix was used for PCR with the forward gene-specific primer 5′-GCGGATCCGCCGACACCCGCACCTAC-3′ and the reverse anchor primer 5′-GCTCTAGATCTCGAGCGT-3′. The PCR products were run on native PAGE (5% gel), eluted and ligated into pUC19 plasmid vector to yield pLtSOD.

Plasmids

The antisense construct pASLtSOD was derived from the expression vector pRH2 (kindly provided by Dr Andre Schneider, Department of Biology/Zoology, University of Fribourg, Fribourg, Switzerland). This plasmid contains a hygromycin-resistance gene under the control of the *Leishmania* poly(ADP-ribose) polymerase (PARP) promoter, followed by a minicircle sequence that allows autonomous replication. The PARP promoter fragment was subcloned into plasmid pBluescript to yield pBS-PARP. The *Not*I site of pLtSOD was repaired and the insert subcloned into pBSPARP between the *Eco*RI and *Sal*I sites, resulting in antisense orientation of LtSOD downstream of the PARP promoter. The *Not*I–*Sal*I fragment of the resulting plasmid was next subcloned between the corresponding sites of pRH2, giving rise to pASLtSOD. The control plasmid pAS contains all the sequences of pASLtSOD except the SOD gene.

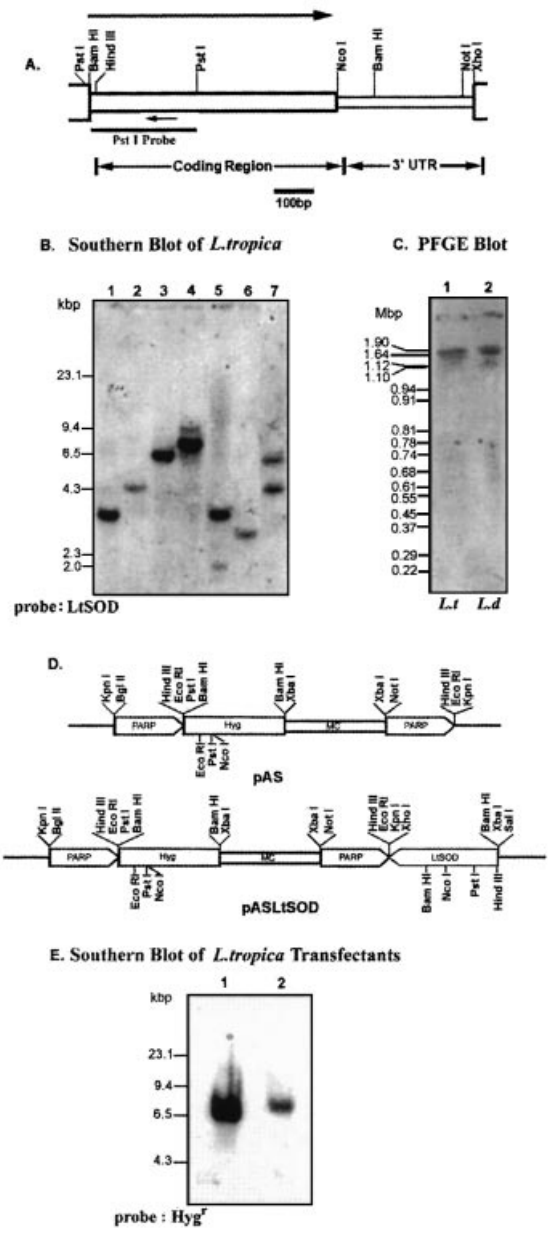

Figure 2 Genomic organization of LtSOD

(**A**) Restriction map of pLtSOD cDNA insert. The smaller arrow represents the position of the reverse primer used for primer extension analysis and the larger arrow indicates the 5′ → 3′ direction of the gene. (**B**) Southern blot of *L. tropica* genomic DNA probed with the *Pst*I fragment of LtSOD cDNA and digested with *Bam*HI (lane 1), *Pst*I (lane 2), *Nco*I (lane 3), *Xho*I (lane 4), *Bam*HI plus *Hind*III (lane 5), *Pst*I plus *Bam*HI (lane 6) and *Pst*I plus *Nco*I (lane 7). (**C**) PFGE blot of *L. tropica* (lane 1) and *L. donovani* (lane 2) genomic DNA probed with LtSOD cDNA. (**D**) Restriction maps of the SOD antisense knockout vector (pASLtSOD) and the control vector (pAS). (**E**) Southern blot of *L. tropica* genomic DNA transfected with pASLtSOD (lane 1) and pAs vector (lane 2) digested with *Bgl*II and probed with a hygromycin-resistance (Hyg^r) gene fragment. The lengths of size standards are indicated on the left.

Transfection of *Leishmania*

Promastigotes were grown to a density of (2–4) × 10^7 cells/ml and transfected with 30 μg of pAS or pASLtSOD by electroporation in a Gene Pulser apparatus (Bio-Rad), under conditions described previously [9], using a capacitance of 500 μF and a pulse of 2250 V/cm. Transfectants were allowed to recover in drug-free

medium for 24 h and then selected for resistance to hygromycin B at 10 μg/ml. Transfectants were sub-cultured every 4–5 days in presence of antibiotic. Freshly transformed *L. donovani* promastigotes (two passages) were used for infectivity assays.

Nucleic acid analyses

The pLtSOD insert was sequenced from both ends by an ABI Prism automated DNA sequencer, and the sequence was submitted to DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession number AY161306. BLAST analysis was performed against the *L. major* sequence database (http:/www.ebi.ac.uk/parasite/leish.html). The conserved domain and putative secondary structure were identified by a BLAST search of the NCBI databases and by the Predict-Protein program (http://cubic.bioc.columbia.edu). Southern blotting, hybridization and pulsed field gel electrophoresis (PFGE) analysis were carried out as described previously [10]. For primer extension assays, the 5′ ^{32}P-labelled primer CCCAAGCTTATGTTGCTGCCGTACGGCTGG, complementary to positions 145–165 of the LtSOD ORF (see Figure 2), was annealed with 2 μg of total RNA and the hybrid extended using Superscript II RT (GIBCO) at 42 °C for 90 min. Northern blotting and hybridization was done as described previously [11].

Protein analyses

The LtSOD insert was subcloned into bacterial expression vector pGEX4T-1 (Amersham Pharmacia) and expressed in *E. coli* BL 21 at 22 °C overnight. Recombinant GST-fusion protein was isolated using Bulk GST purification module (Pharmacia). For expression as N-terminal His_6-tagged protein, LtSOD insert was subcloned at the *Bam*HI site of pQE30 (Qiagen) and purified using a Ni^{2+}-nitrilotriacetate column. Polyclonal antibody against the His_6-tagged protein was raised in rabbits and purified by adsorption on immobilized recombinant protein. Promastigotes were subjected to hypotonic lysis [12] and centrifuged at 10000 ***g*** for 10 min. The supernatant was centrifuged at 100000 ***g*** for 60 min to yield the cytosolic (soluble) and microsomal (particulate) fractions. Western blot analysis was performed with affinity-purified rabbit IgG antibody raised against His_6-tagged LtSOD protein at 1:50 dilution. The secondary antibody used was peroxidase-conjugated goat anti-rabbit IgG (Sigma) at 1:1000 dilution. The blots were developed with 4-chloro-1-naphthol as the substrate.

SOD activity assay

Cytosolic fractions were resolved by native PAGE (8 % gel). The gel was stained for SOD activity by the method of Beauchamp and Fridovich [13]. Briefly, this assay depends on the ability of SOD to inhibit the reduction of Nitro Blue Tetrazolium to blue-coloured formazan by $O_2^{\bullet-}$ generated by reoxidation of photochemically reduced riboflavin. Thus regions containing active SOD appear colourless against a blue background. The band intensity (determined by densitometry) is roughly proportional to the amount of SOD protein [13].

Growth analysis

The *L. tropica* pAS or pASLtSOD transfectants were seeded at 2×10^6 cells/ml and allowed to recover overnight in M199 medium containing 10 μg/ml hygromycin. Increasing concentrations of freshly prepared menadione (0.1 to 2 μM) and H_2O_2 (10 to 100 μM) were added to 2 ml aliquots of the cells grown overnight, and the parasite count was taken after 72 h.

Macrophage infectivity assay

Macrophages were isolated from BALB/c mice by peritoneal lavage with chilled RPMI 1640 media (Gibco) containing 10 % fetal calf serum and allowed to adhere on coverslips at 5×10^5 cells/coverslip overnight at 37 °C under CO_2/O_2 (1:19). Non-adherent macrophages were removed by washing with PBS and incubated with transfectant parasites at a multiplicity of infection of 10:1 for 3 h. Unbound parasites were removed by washing with PBS and fresh RPMI 1640 medium added to each coverslip followed by incubation at 37 °C for 48 h. Fresh medium was added every 24 h. The coverslips were stained with Giemsa stain immediately after initial binding (zero time) and subsequently every 24 h. Cells were counted at $\times$1000 magnification. *P* values were determined by two-tailed Student's *t* test.

RESULTS AND DISCUSSION

Cloning of the SOD gene

A 0.9-kb fragment of the LtSOD gene, containing an open reading frame of 179 amino acids terminating in a stop codon, followed by 360 bp of 3′-untranslated sequence, was recovered using 3′ RACE (Figure 1). The coding region is 44 % identical at the amino acid level with the SOD-A sequence of *L. chagasi*, less similar (38 % identity) with the SOD-B sequence from the same organism, and 98 % identical to the putative SOD-A gene of *L. major* on chromosome 32, but lacks an N-terminal 14-amino-acid stretch present in the latter. The sequence contains the SOD signature motif DVWEHAYY, all residues considered to be diagnostic of FeSOD (as opposed to MnSOD), and two of the three histidine residues and the asparagine residue thought to be involved in metal binding. Moreover, the sequence could be almost perfectly aligned with the conserved three-dimensional structure of the C-terminal domain (Pfam02777; Protein Families Database of Alignments and HMMs, http://www.sanger.ac.uk/Software/Pfam/index.shtml) of the Fe/Mn SOD family.

Organization of the SOD gene

Southern blot analysis of genomic DNA digested with single restriction enzymes and the 250-bp *Pst*I fragment of clone pLtSOD yielded single hybridizing bands (Figure 2). For example, *Bam*HI and *Pst*I yielded 3.5 and 4.5 kb bands respectively, whereas the *Bam*HI–*Pst*I double digest gave a *Bam*HI–*Pst*I fragment of 3 kb, as expected from the presence of an internal *Bam*HI site 0.5 kb away from the *Pst*I site. Taken together, these and other data (S. Ghosh, S. Goswami and S. Adhya, unpublished work) suggested the presence of a single or low copy number gene. Quantitative dotblot analysis (results not shown) indicated two or three copies per haploid genome. The SOD probe hybridized to a 1.6 Mb *L. tropica* chromosome resolved by PFGE, and more weakly to a 1.3 Mb band (Figure 2). The chromosomal localization is similar to that observed for the SOD-A gene of *L. major*, but different from that of *L. chagasi*, which contains a single SOD-A gene on a small chromosome [5].

Expression of SOD in *L. tropica* and *L. donovani*

The pLtSOD open reading frame was expressed as a 22-kDa protein in *E. coli* using both glutathione S-transferase and polyhistidine-tagged vectors, and purified using the appropriate affinity columns. The purified protein has little or no enzymic activity (results not shown), probably due to the lack of an essential histidine residue in the truncated N-terminus (Figure 1). Affinity-purified polyclonal antibody against the expressed protein reacted with a major 22-kDa band on Western blots of

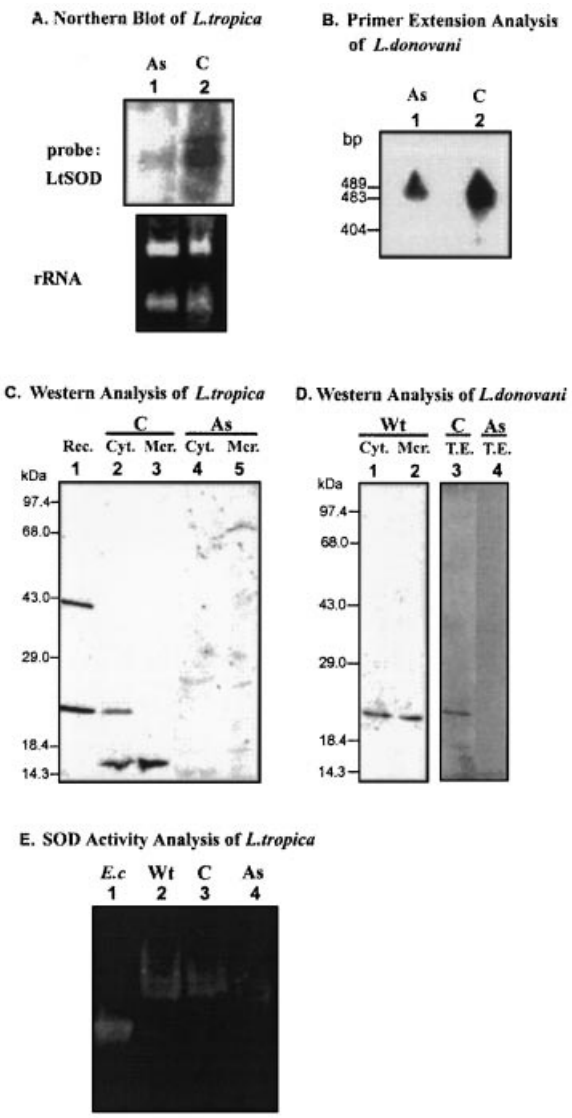

Figure 3 Inhibition of SOD expression in antisense-transfected *Leishmania*

(**A**) Upper panel: Northern blot of *L. tropica* pASLtSOD transfectant (lane 1) and pAS transfectant (lane 2) probed with pLtSOD insert. Lower panel: ethidium bromide staining of total RNA showing the rRNA species. (**B**) Primer extension analysis of *L. donovani* pASLtSOD transfectants (lane 1) and pAS transfectants (lane 2). (**C**) Western blot of *L. tropica* transfectants probed with anti LtSOD IgG. The cytosolic fractions of the pAS (lane 2) and pASLtSOD (lane 4) transfectants are compared with the microsomal fractions of pAS (lane 3) and pASLtSOD (lane 5) transfectants. Recombinant His_6–LtSOD and GST–LtSOD proteins are shown in lane 1. (**D**) Western blot of *L. donovani* parasites probed with anti-LtSOD IgG. Cytosolic and microsomal fractions of the untransfectant parasites are shown in lanes 1 and 2 respectively. Total parasite extract of the pAS transfectants (lane 3) and pASLtSOD transfectants (lane 4) are shown. (**E**) *In situ* gel assay of SOD activity. Cytosolic extracts of the wild-type (lane 2), pAS transfectants (lane 3) and pASLtSOD transfectants (lane 4) are compared with *E. coli* FeSOD (lane 1) as a control. Molecular-mass markers are indicated on the left.

cytosolic fractions of both *L. tropica* and *L. donovani* promastigotes (Figures 3C and 3D). The same 22-kDa band is present in the microsomal fraction of *L. donovani*, but not of *L. tropica*. Additionally, the cytosolic and microsomal fractions from *L. tropica*, but not *L. donovani*, promastigotes, contain a cross-reacting species of about 15 kDa (Figure 3C). Since activity gels (see below) failed to reveal a similar sized enzyme, the 15-kDa band could represent a proteolytic cleavage product of native SOD, but its function remains unknown. Indeed, incubation of [^{35}S]methionine-labelled expressed LtSOD protein with a promastigote cytosolic extract resulted in proteolysis with generation of a 15-kDa species (results not shown). These results reveal an unexpected degree of complexity in the subcellular localization and protease cleavage pattern of SOD in different *Leishmania* species.

Generation of SOD-deficient strains

To study the physiological function of SOD, we used the antisense approach to generate SOD-deficient *Leishmania*. The LtSOD insert was cloned in inverted orientation downstream of a PARP promoter (a strong RNA polymerase I promoter active in *Leishmania*) in the expression vector pAS. The resultant plasmid, pASLtSOD, or the parental vector pAS, was electroporated into promastigotes and hygromycin-resistant transformants selected. Genomic Southern blots showed the presence of intact 6.3 kb pAS and 7.2 kb pASLtSOD plasmids in the two transformants (Figure 2). The copy number of the empty vector pAS was approx. 5-fold higher than that of the SOD antisense plasmid, probably reflecting counterselection of a lower copy number by the lethal effect of SOD depletion (see below).

The effect of antisense expression on cellular SOD levels was monitored in several ways. Northern blot analysis showed a 3.5-fold reduction in the 1.2-kb SOD mRNA in pASLtSOD transformants compared with the empty vector control (Figure 3A). A second mRNA species of approx. 1.3 kb present in control parasites was undetectable in the antisense transformants. A similar reduction (approx. 3-fold) was observed by the primer extension assay (Figure 3B). Western blot analysis using antibody against the expressed SOD clone failed to detect significant levels of the native 22-kDa protein, or the 15-kDa derivative, in either the cytosolic or microsomal fraction of the pASLtSOD transformants (Figures 3C and 3D). Finally, an *in situ* gel assay of cytosolic fractions for SOD activity showed the presence of two closely spaced bands in comparable amounts in both wild-type and pAS-transformed promastigotes, but the SOD activity was reduced to one quarter of this level in the pASLtSOD transformants (Figure 3E). Thus antisense expression leads to a significant decline in the amount of enzymically active SOD. The levels of rRNA (Figure 3A) and tubulin were not affected by the antisense expression, and these cells were morphologically indistinguishable from the wild-type strain (results not shown). The fact that antisense expression shuts off expression of the corresponding immunologically reactive gene product, but leaves a residual activity of SOD, may be attributed to the presence of other SOD isoforms (e.g. corresponding to SOD-B of *L. chagasi*) that are not affected by this treatment.

Effect of SOD deficiency on sensitivity to pro-oxidants

One phenotype generally observed for SOD mutations or deficiency is an increased sensitivity of growth to $O_2^{\bullet -}$ generators. In the absence of such reagents, both pAS and pASLtSOD transformants grew at comparable rates (generation time of approx. 20 h in each case). However, in presence of menadione, a known $O_2^{\bullet -}$ generator [14], the growth of the SOD-deficient transformant was inhibited to a greater extent than that of the control, with LD_{50} values of 0.5 and 3.2 μM respectively (Figure 4A). Interestingly, the SOD-deficient cells were also more sensitive to H_2O_2 (LD_{50} reduced from 42 to 10 μM; Figure 4A), which does not generate $O_2^{\bullet -}$, but can react with it to generate the extremely reactive $OH^{\bullet}$ radicals:

$$H_2O_2 + O_2^{\bullet -} \rightarrow OH^{\bullet} + OH^{-} + O_2$$

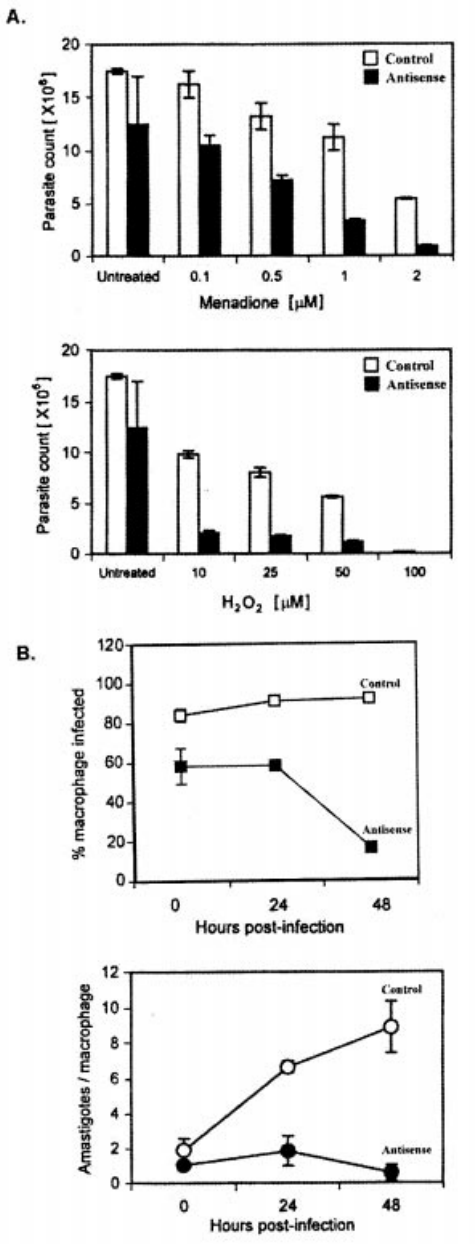

Figure 4 Effect of SOD depletion on growth and macrophage infectivity

(**A**) *L. tropica* transfectants were grown in presence of indicated concentrations of menadione (upper panel) or H_2O_2 (lower panel), and a cell count was taken after 72 h. (**B**) *L. donovani* transfectants were used for macrophage infection assays *in vitro* for 48 h and analysed after Giemsa staining. □, pAS transfectants; ■, pASLtSOD transfectants. Values are expressed as the means ± S.E.M. for at least three independent samples.

Thus enhancement of intracellular $O_2^{\bullet -}$ levels in SOD-deficient cells could lead to greater H_2O_2 lethality through increased $OH^{\bullet}$ generation. It was previously shown [5] that overexpressing SOD in *L. chagasi* promastigotes does not reduce sensitivity to H_2O_2, as expected by this mechanism. It is possible that H_2O_2 toxicity in *L. chagasi* is mediated primarily by an $O_2^{\bullet -}$-independent mechanism, e.g., through Fe^{2+}-mediated disproportionation to $OH^{\bullet}$ and OH^-. It is noteworthy that the LD_{50} for H_2O_2 reported with *L. chagasi* (approx. 250 μM) is significantly different from that observed with *L. tropica* (40 μM), which would be expected if the $OH^{\bullet}$-generating reactions were different in the two species.

Effect of SOD deficiency on survival within macrophages

To assess the effect of SOD depletion on the intracellular growth and survival of *Leishmania*, promastigotes of the pASLtSOD-transformed *L. donovani* strain, and the corresponding empty vector transformed control strain, were used to infect mouse peritoneal macrophages, and the intracellular amastigotes counted at different times of infection. Most of the macrophages were infected by the control strain, the number of infected macrophages remained constant up to at least 48 h post-infection ($92 \pm 1.7\%$), and the average number of amastigotes per macrophage increased over this period (to 9.66 ± 1.45), indicating intracellular multiplication (Figure 4B). In contrast, the number of macrophages containing amastigotes of the SOD-deficient strain declined sharply from $58 \pm 2.1\%$ ($P < 0.0004$) to $16 \pm 1.5\%$ ($P < 0.0001$) between 24 and 48 h post-infection (Figure 4B), although the total number of macrophages remained unaltered, i.e., there was no host cell lysis (results not shown). Correspondingly, the number of amastigotes per macrophage declined from 1.83 ± 0.88 ($P < 0.002$) to 0.58 ± 0.44 ($P < 0.003$) (Figure 4). The numbers of promastigotes initially attached to the macrophage surface were comparable [2.85 ± 0.217 versus 3.33 ± 0.881 ($P > 0.05$) per macrophage] for the two strains. Thus SOD deficiency results in a markedly reduced growth and survival of amastigotes within the macrophage host.

Conclusions

Although the presence of antioxidant defence mechanisms in *Leishmania* has been suspected for some time on the basis of the presence of the appropriate enzymes, this is the first demonstration of the physiological role of such an enzyme in intracellular parasite growth and survival. Our studies further reveal subtle interactions between different pro-oxidants, i.e. H_2O_2 and $O_2^{\bullet -}$ in inhibiting parasite growth, and the possible differences between New World (*L. chagasi*) and Old World (*L. donovani* and *L. tropica*) species in the mechanism of oxidative killing. The susceptibility of the parasite to SOD deficiency suggests this enzyme to be a suitable target for chemotherapy.

This work was supported by a grant from the Department of Biotechnology, Government of India. S. Ghosh and S. Goswami received Senior Research Fellowships from the University Grants Commission and The Council of Scientific and Industrial Research respectively. We thank Andre Schneider for the pRH2 plasmid.

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Received 29 October 2002/11 November 2002; accepted 2 December 2002
Published as BJ Immediate Publication 2 December 2002, DOI 10.1042/BJ20021684