Hydrogen peroxide induces apoptosis-like death in *Leishmania donovani* promastigotes

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SUMMARY

*Leishmania donovani* promastigotes introduced into the bloodstream by sandfly vectors, are exposed to reactive oxygen species like H$_2$O$_2$ during phagocytosis by the host macrophages. H$_2$O$_2$ can induce promastigote death, but the mechanism of induction of this death is not known. Studies presented in this paper demonstrate that exposure to 4 mM H$_2$O$_2$ results in a pattern of promastigote death that shares many features with metazoan apoptosis. Motility and cell survival in these parasites show a gradual decline with increasing doses of H$_2$O$_2$. Features common to metazoan apoptosis, such as nuclear condensation, DNA fragmentation with accompanying DNA ladder formation and loss of cell volume, are observed after exposure to 4 mM H$_2$O$_2$. Within 30 minutes of the exposure, there is a significant increase in the ability of the cell lysates to cleave the fluorogenic tetrapeptide acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin, which is a substrate for the CED-3/CPP32 group of proteases. Pretreatment of cells with a specific inhibitor of CED-3/CPP32 group of proteases, Z-DEVD-FMK, reduces the number of cells showing apoptosis-like features, prevents DNA breakage and inhibits cleavage of a PARP-like protein. Activation of the caspase-like proteases is followed at 2 hours by the cleavage of a poly(ADP)ribose-polymerase-like protein and a reduction in intracellular glutathione concentration. DNA breakdown as detected by TdT labelling of cells and agarose gel electrophoresis is visible at 6 hours. Taken together, the above data show for the first time that there is a distinct pathway for apoptosis-like death in *L. donovani*.

Key words: *Leishmania*, Cell death, Apoptosis, DNA fragmentation

INTRODUCTION

*Leishmania* spp. are causative agents of a parasitic infection that manifests itself in a variety of clinical forms depending on the species of *Leishmania* and the immunological status of the host (Liew and O’Donnell, 1993). *Leishmania donovani* is the etiological agent of Kala-azar, a chronic and often fatal form of human visceral leishmaniasis (Chang, 1983). The life cycle of this parasite includes flagellated extracellular promastigotes in the gut of the insect vector and a non-flagellated amastigote form that resides within the macrophages of their mammalian host (Alexander and Russell, 1992; Desjardins, and Descoteaux, 1998). Survival within the insect vector and the mammalian macrophages requires careful control of the population of the parasite, and this control is exerted through cell death (Welburn et al., 1997; Welburn and Maudlin, 1997). Recently, a form of cell death resembling metazoan apoptosis has been reported in several parasitic protozoans (Welburn et al., 1999; Moreira et al., 1996), and differential expression of genes during ConA-induced death has been elegantly shown by Welburn and co-workers in *Trypanosoma brucei* (Welburn et al., 1999). However, in spite of these findings, the physiological mechanisms leading to cell death in such organisms remain to be characterised.

Apoptosis is used during development and morphogenesis to control cell number and as a defensive strategy to remove infected, mutated or damaged cells. This type of death is triggered through a controlled programme that is associated with distinctive morphological changes including membrane blebbing, cytoplasmic and nuclear condensation, and chromatin aggregation with accompanying DNA breakage (Vaux and Strasser, 1996). H$_2$O$_2$ can precipitate apoptosis in mammalian and yeast cells (Li et al., 2000; Vollgraf et al., 1999; Clement and Pervaiz, 1999; Madeo et al., 1999), but it is not known whether it can bring about apoptosis-like death in *L. donovani*, although it is well established that H$_2$O$_2$ can kill both the promastigote and amastigote forms of this parasite. The promastigotes may die if they are exposed to H$_2$O$_2$ either during phagocytosis (Channon and Blackwell, 1985a; Channon and Blackwell, 1985b; Hammoda et al., 1996) or if H$_2$O$_2$ is added exogenously in vitro (Murray and Nathan, 1988). Most importantly, reactive oxygen species, including H$_2$O$_2$, generated by antiparasitic agents or macrophages can kill the intracellular parasites (Nabi, 1984; Mauel et al., 1984) and are therefore important regulators of protozoal infection (Solbach and Laskay, 2000; Schirmer et al., 1987). In the light of the above observations, which implicate H$_2$O$_2$ as a physiological regulator of *L. donovani* infection, it was of interest to use H$_2$O$_2$-induced death of these parasites in vitro as a model to study the death-associated phenotype and to identify possible biochemical pathways.
The investigations presented in this communication demonstrate that, upon exposure to suitable doses of H$_2$O$_2$, *L. donovani* promastigotes express several markers common to metazoan apoptosis, including nuclear condensation, accumulation of intracellular calcium, activation of caspase-like proteases, decrease in intracellular glutathione (GSH) content, fragmentation of cellular DNA, formation of DNA ladders, cleavage of a poly(ADP)ribose polymerase (PARP)-like protein and loss of cell volume.

**MATERIALS AND METHODS**

**Cells**
Promastigotes of *L. donovani* (UR6) were obtained from the Cell Biology Laboratory, National Institute of Immunology, New Delhi, India. For experimental purposes, cells were harvested from 3-day-old blood agar slants by scraping into phosphate-buffered saline (PBS; 10 mM, pH 7.2).

**Reagents**
Monoclonal antibody against PARP was purchased from Pharmingen (San Diego, CA). Goat anti-rabbit IgG conjugated to horseradish peroxidase was purchased from Jackson Immunoresearch (West Grove, PA). All other chemicals, unless attributed explicitly, were purchased from Sigma Chemical Company (St Louis, MO). Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling (TUNEL) kit was from Promega (Madison, WI). Proteinase K was from Roche Molecular Biochemicals GmbH (Mannheim, Germany). Caspase assay kits were from the BIORAD laboratories (Hercules, CA). Bicinchoninic Protein Assay Reagents A and B were purchased from Pierce (Rockford, IL). ABC staining kit was from Vector Laboratories Inc. (Burlingame, CA).

**Promastigote culture and cell treatments**
*L. donovani* promastigotes were cultured in blood agar as described previously (Sengupta et al., 1999). Briefly, the cells were routinely maintained on solid blood agar slants containing 1% glucose, 5.2% brain heart infusion agar extract and rabbit blood (6% v/v) with gentamycin at a final concentration of 1-1.5 mg ml$^{-1}$ of medium at 25°C. For experimental purposes, cells were recovered from the blood agar culture in medium 199 supplemented with 10% foetal calf serum, 25°C. For experimental purposes, cells were harvested from 3-day-old blood agar slants by scraping into phosphate-buffered saline (PBS; 10 mM, pH 7.2).

**Detection of mode of cell death**
The percentages of viable, necrotic and apoptotic cells were assessed in the preparations after a culture period of 2, 4 and 6 hours. Cells were exposed to the DNA binding dyes Hoechst 33342 (HO) (10 μg ml$^{-1}$) and PI as described previously (Morana et al., 1996). The characteristic nuclear phenotype exhibited by apoptotic cells (condensation of genomic DNA) was used to distinguish between normal and apoptotic cells. Viable or necrotic cells were identified by nuclei with dull blue or red fluorescence, respectively. Apoptotic cells were detected by their condensed nuclei that exhibited a bright blue fluorescence. In early apoptosis, only HO will reach the nuclear material, whereas, in the later phase, PI will penetrate the cells also. Cells from five microscopic fields (magnification ×200) were counted and a minimum of 200 cells were observed per field and classified as follows: (i) live cells (light blue chromatin); (ii) membrane-intact apoptotic cells (bright blue chromatin); and (iii) necrotic cells (dense bright red nuclei). All these studies were carried out blindly where samples were coded prior to counting.

**Assay of caspase activity**
Cell lysates (200 μg protein) were incubated with caspase buffer (50 mM HEPES, pH 7.4; 100 mM NaCl; 10% sucrose; 1 mM EDTA, 0.1% CHAPS and 100 mM DTT) containing 100 μM fluorogenic peptide substrates, acetyl-Asp-Glu-Val-Asp-7-amino-4-trifluoromethyl coumarin (Ac-DEVD-AFC), acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Ac-LEHD-AFC) and acetyl-Tyr-Glu-Asp-7-amino-4-trifluoromethyl coumarin (Ac-WEHD-AFC) at 37°C. Apopain from a BIORAD caspase-3 assay kit was used as a positive control. AFC release was measured with the help of a Perkin-Elmer LS-5B luminescence spectrometer (Perkin-Elmer, Norwalk, CT) at excitation wave length of 390-400 nm and emission wavelength of 510-550 nm. Appropriate inhibitor (Z-DEVDFMK) was used for the assay of CED-3/CPP32 group of proteases.

**Preparation of cell lysates**
Treated and untreated cells were suspended in cell extraction buffer (20 mM HEPES-KOH, 10 mM KCl, 1.5 mM MgCl$_2$, 1 mM EDTA, 1 mM DTT, 100 μM PMSF, 10 μg ml$^{-1}$ leupeptin and 2 μg ml$^{-1}$ aprotinin), and lysed by nitrogen cavitation in a cell disruption chamber (Parr Instruments, UK) at 750 ps (Earnshaw et al., 1985). The lysate was centrifuged at 100,000 g for 1 hour and the supernatant

Frozen at –70°C. Protein was estimated by bicinchoninic protein assay reagent. For western blots, treated and untreated cells were directly lysed in sample buffer (0.12 M Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, 10% 2-mercaptoethanol).

**Cell viability and motility assays**
For membrane permeability assay as viability test, the cells were treated with 10 μg ml$^{-1}$ of live cell impermeant dye propidium iodide (PI) and cell counts were made under a Nikon Optiphot fluorescence microscope at different time points. Total cells versus labelled and unlabelled cells were calculated and data expressed as percentage viability. Cell motility was checked using a Neubauer haemocytometer at different time points after exposure to H$_2$O$_2$ and the number of motile cells versus the total number of cells was calculated and expressed as percentage motile cells. Counts were done on coded samples to avoid bias.

**Cellular and nuclear morphology**
To observe changes in cell morphology, cells were examined under a phase contrast microscope. For recording alterations in nuclear morphology, treated and untreated cells were fixed with 2% paraformaldehyde and incubated with 0.2% Triton X-100 for 1 minute for permeabilization, washed with PBS and incubated with PI (10 μg ml$^{-1}$) for 2 minutes. Subsequently, observations were made with a confocal laser scanning microscope (LSM 510, Carl Zeiss, Jena, Germany). It should be noted that at least 100 cells per group with identical morphology were recorded for each condition. Examinations were carried out on coded samples to avoid bias.

**Materials and methods**

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![Image](image-url)
performed using an apoptosis detection system as described previously (Rao and Shaha, 2000). Briefly, H2O2-treated promastigotes were harvested at different time points, fixed in 4% formaldehyde and coated onto poly-L-lysine covered slides. Permeabilisation was done with 0.2% (v/v) Triton X-100 and equilibration buffer (200 mM potassium cacodylate, 25 mM Tris-HCl, 0.2 mM DTT, 0.25 mg mL−1 BSA, 2.5 mM cobalt chloride) for 10 minutes at room temperature followed by incubation with TdT buffer containing nucleotide mix (50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris-HCl, 1 mM EDTA, pH 7.6) for 1 hour at 37°C. The samples were counterstained with 10 μg mL−1 PI and visualised under a Nikon Optiphot fluorescence microscope. At least 400 cells of four independent experiments were counted. All counts were carried out on coded samples.

DNA fragmentation assay by agarose gel electrophoresis
To determine the sizes of DNA fragments generated during cell death, total cellular DNA was isolated by a previously described procedure (Sambrook et al., 1989) and analysed by agarose gel electrophoresis. Briefly, pellets of 107 promastigotes were treated with sarkosyl detergent lysis buffer (50 mM Tris, 10 mM EDTA, 0.5% w/v sodium-N-lauryl sarcosine, pH 7.5) and proteinase K (15.6 mg mL−1), vortexed and allowed to digest overnight at 50°C. RNase A (0.3 mg mL−1) treatment was given for 1 hour at 37°C. The lysates were then extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 16,000g for 5 minutes. The upper phase was treated with 3 M sodium acetate and 100% ethanol for overnight at −20°C. The sample was centrifuged at 16,000 g for 10 minutes, the supernatant removed and 0.5 ml of 70% ethanol added. DNA was solubilized in Tris/EDTA (10 mM/1 mM) buffer and quantitated spectrophotometrically at 260/280 nm. Total DNA was mixed with tracking dye and loaded on 1% agarose gels containing ethidium bromide. Gels were run for 2.5 hours at 50 V.

Measurement of GSH and calcium
GSH levels were measured as described previously (Sies and Akerboom, 1984). Briefly, treated and untreated L. donovani promastigotes were lysed with 10% TCA and spun at 300 g. Glutathione peroxidase (0.1 unit) and cumene hydroperoxide (0.1 mM) was added to the supernatant, and incubated for 10 minutes. Subsequently, 1 unit of glutathione reductase and 3 mM of NADPH were added and the reduction of NADPH was measured at 340 nm at different time points. Level of GSH was calculated from the amount of NADP formed.

Calcium levels were measured according to a method described previously (Ruben et al., 1991) using FURA-2AM and measurements were done using a Perkin-Elmer spectrofluorimeter at 340 nm excitation and 510 nm emission wavelengths. Minimum fluorescence signal was obtained by lysing the cells with 0.1% digitonin followed by addition of 4 mM EGTA; maximum fluorescence was obtained upon addition of 10 mM Ca2+.

SDS-PAGE and western blot analysis
Cell lysate proteins were separated by electrophoresis on 12.5% polyacrylamide gels (Laemmli, 1970) and subjected to western blot analysis as described previously (Aravinda et al., 1995). Anti-PARP antibodies (purified mouse anti-human PARP) diluted to 1:1000 and goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:5,000 dilution were used. Reactive bands were visualised by using an ABC staining kit.

Statistical analyses
An unpaired two-tailed Student’s t test using T-EASE software (Version 2.0; Institute for Scientific Information®, Philadelphia, PA) was used for statistical analyses. Data sets were said to be significantly different for P<0.01.

RESULTS

H2O2 treatment reduces promastigote motility and changes cell permeability
We first investigated the cytotoxic effects of H2O2 on L. donovani promastigotes. As they are freely motile cells, changes in motility was chosen as a visually recognisable energy-dependent process that can be studied as an indicator of cell change. Treatment with H2O2 at final concentrations ranging from 0.1 mM to 8 mM resulted in a dose-dependent inhibition of cell motility (Fig. 1A). At a dose of 8 mM H2O2, motility was inhibited in 80% of cells within the first hour, whereas, by comparison, similar percentages of inhibition was achieved only at 3 hours with 4 mM H2O2. Parallel tests done with cells not treated with H2O2, treated with lower doses or preincubated with catalase demonstrated no change in motility (Fig. 1A).

Fig. 1. Effects of H2O2 on the motility and viability of L. donovani promastigotes. (A) Percentage motility of cells after exposure to 0.1–8 mM doses of H2O2. (B) Percentage viability of cells after exposure to 0.1–8 mM doses of H2O2. Symbols: X, control without H2O2; +, 0.1 mM H2O2; ▲, 1 mM H2O2; ▼, 4 mM H2O2; ◆, 8 mM H2O2; ●, 8 mM H2O2 + catalase; ■, 4 mM H2O2 + catalase. The data represents mean ± s.e.m. of four experiments.
As changes in cell motility reflect alterations in cellular metabolism, the effect of the treatments on cell survival was studied by measuring disruption of membrane integrity using the live-cell-impermeant dye PI. As shown in Fig. 1B, with 8 mM H₂O₂, 85% of cells were dead by 2 hours whereas treatment with 4 mM H₂O₂ led to ~23% cell death by 6 hours. During the initial 1 hour of exposure to 4 mM H₂O₂, when ~25% of cells became immotile (Fig. 1A), 99% cells still had intact membranes (Fig. 1B). Pretreatment of cells with 100 IU ml⁻¹ catalase could completely abrogate the effect of H₂O₂ on both motility and cell viability.

Therefore, it is clear from the motility and cell survival data that L. donovani promastigotes are sensitive to the degree of stress induced by the different doses of H₂O₂. Loss of motility did not coincide with cell death as membranes were intact even in immotile cells. Because catalase was able to inhibit changes in both motility and viability, it is clear that H₂O₂ was the source of stress that caused metabolic changes within the cells.

H₂O₂ induces morphological changes and precipitates apoptosis-like death

Having established that certain doses of H₂O₂ can generate sufficient oxidative stress in L. donovani promastigotes to bring about cell death, we sought to establish the type of death that the different doses precipitated. Exposure to H₂O₂ triggers apoptosis in numerous mammalian cells and yeast (Vollgraf et al., 1999; Clement and Pervaiz, 1999; Madeo et al., 1999), and the process of apoptosis is accompanied by marked morphological changes, whatever the cause (Vaux and Strasser, 1996). A visual inspection of changes in the morphology of the L. donovani promastigotes at 6 and 12 hours after H₂O₂-induced stress at different doses observed by phase contrast microscopy revealed that, with 4 mM H₂O₂, cell shrinkage started at around 6 hours in ~20% of cells, whereas 80% maintained their elongated form (Fig. 2Ab). By 12 hours, 99% of the cells showed cytoplasmic condensation (Fig. 2Ac) in comparison with untreated cells (Fig. 2Aa). Dual staining with HO 33342 and PI, which identify the nuclei of live and dead cells, respectively, showed that the number of cells undergoing apoptosis-like death became higher with the increase in the time of exposure to 4 mM H₂O₂ (Fig. 2B). Catalase was able to inhibit the increase in the number of apoptotic cells. Treatment with 8 mM H₂O₂ did not lead to an increase in the number of apoptotic cells. Death induced by 8 mM H₂O₂ showed signs of necrosis as cell swelling and rupture was visible. Because 4 mM H₂O₂ was precipitating apoptosis in a significant number of cells, this dose was chosen for further study.

In order to morphologically identify the nuclear changes brought about by H₂O₂ treatment, a confocal laser scanning microscope was used to visualise the nuclei. As shown in Fig. 2C, clear nuclear material was visible in most cells from the control group (Fig. 2Ca), whereas nuclear condensation was very apparent after 4 hours in treated cells (Fig. 2Cb), and this was followed by nuclear breakdown at 6 hours (Fig. 2Cc).

Changes in morphology like loss of cell volume and nuclear alterations like condensation and fragmentation of nuclear material indicated that 4 mM H₂O₂ could precipitate a type of death in L. donovani promastigotes that shares many characteristics with apoptotic death of the metazoans. Because death induced by the higher dose (8 mM H₂O₂) showed features of necrotic death, it is apparent that the cells could express either an apoptotic-like or a necrotic phenotype depending on the extent of oxidative stress.

H₂O₂ treatment results in the fragmentation of DNA

To further characterise the changes occurring in the nuclear...
material, TUNEL staining was used to detect the free ends of DNA after breakage. As shown in Fig. 3, there were significantly fewer TdT labelled nuclei in the control groups (Fig. 3A,C,E) compared with treated groups at 6 hours, where nuclei fluoresced brightly indicating fragmentation of DNA (Fig. 3F,F1). After 2 hours of exposure to H$_2$O$_2$, only the kinetoplast DNA stained (Fig. 3D,D1), compared with no staining in the controls (Fig. 3C). Cell counts showed that at 6 hours, 87% of cells were labelled by TdT, compared with 20% observed at 2 hours (Fig. 4A). It is important to note that, at 6 hours, when most of the nuclei were staining for TdT, >75% cells had intact membranes. Tests for another hallmark of apoptosis, the internucleosomal degradation of genomic DNA, showed that the experimental group had nucleosome-sized DNA fragments, giving a DNA-ladder-like pattern identified by agarose gel electrophoresis of DNA from treated cells (Fig. 4B, lane d). The ladder pattern was not as clear as metazoan DNA ladders and showed some degree of smearing.

Fig. 3. DNA fragmentation in *L. donovani* promastigotes with or without H$_2$O$_2$ treatment in culture as detected by TUNEL staining. (A) At 1 hour without H$_2$O$_2$ exposure. (B) At 1 hour with H$_2$O$_2$ treatment. (C) At 2 hours without H$_2$O$_2$ treatment. (D) At 2 hours after H$_2$O$_2$ treatment. (E) At 6 hours without H$_2$O$_2$ treatment. (F) At 6 hours after H$_2$O$_2$ treatment. (B1-F1) Scan-enhanced close ups of promastigotes with H$_2$O$_2$ treatment for 1 hour (B1), after 2 hours exposure to H$_2$O$_2$ (D1; notice the kinetoplast (k) staining) and after 6 hours exposure to H$_2$O$_2$ (F1; notice both kinetoplast and nuclei (n) staining). Bar, 10 μm.

Fig. 4. DNA breakdown in *L. donovani* promastigotes after H$_2$O$_2$ exposure and effects of a caspase inhibitor on DNA breakage. (A) Number of cells positive for TUNEL staining at various hours after H$_2$O$_2$ treatment. Symbols: ●, 4 mM H$_2$O$_2$ treatment; ■, control with no treatment. Data are mean ± s.e.m. of four experiments. (B) DNA profile in agarose gels from treated and untreated promastigotes. (a) Without H$_2$O$_2$ exposure. (b) After 2 hours of 4 mM H$_2$O$_2$ treatment. (c) After 4 hours of 4 mM H$_2$O$_2$ exposure. (d) After 6 hours of 4 mM H$_2$O$_2$ treatment. (C) DNA breakage and the number of TdT labelled cells after inhibition of caspase activity by Z-DEVD-FMK. Symbols: ■, 4 mM H$_2$O$_2$; ▲, 4 mM H$_2$O$_2$ + 1 μM inhibitor; ▼, 4 mM H$_2$O$_2$ + 10 μM inhibitor; ●, control without treatment. Inset: DNA gel showing (a) marker, (b) DNA of cells pretreated with 1 μM inhibitor prior to exposure to 4 mM H$_2$O$_2$ and (c) DNA of cells treated with 4 mM H$_2$O$_2$. Data are mean ± s.e.m. of four experiments. (D) TdT labelled cells at 6 hours under different treatments. (a) Phase contrast of the same field as (b). (b) TdT labelled cells in 4 mM H$_2$O$_2$-treated group. (c) Phase contrast of same field as (d). (d) TdT labelled cells in group pretreated with Z-DEVD-FMK (1 μM) prior to exposure to H$_2$O$_2$. (e) Phase contrast of (f). (f) TdT labelled cells in the control group. Results are representative of three experiments. Bar, 100 μm.
It is evident, therefore, that DNA fragmentation occurs in the nuclei during H₂O₂-induced cell death before membrane integrity is compromised, showing that the death process observed is not necrotic. Because DNA fragmentation and laddering are features of metazoan apoptosis, it is clear that *L. donovani* promastigotes share these two very prominent features with the multicellular organisms.

**Caspase-like proteases are involved in H₂O₂-induced death**

It is known that, in order to bring about an organised form of death, certain cysteine proteases, designated caspases in metazoans, break down specific substrates. To discover whether some caspase-like proteases were involved in the observed apoptosis-like death of the *L. donovani* promastigotes, a cell-permeable caspase inhibitor that can inhibit CED-3/CPP32 family of caspases, Z-DEVD-FMK (1 μM and 10 μM), was used to preincubate cells prior to exposure to H₂O₂. This treatment reduced the number of TdT labelled cells that was around 90% in the H₂O₂ treated group and was less than 25% in the groups pretreated with Z-DEVD-FMK (Fig. 4C,D). Breakage of DNA did not occur in the inhibitor treated groups, although DNA breakage was apparent in the H₂O₂-treated groups (Fig. 4C, inset). Furthermore, there was a decrease in the number of cells with apoptosis-like morphology when cells were pretreated with the caspase inhibitor compared with the H₂O₂-treated group (Fig. 5A). There was no significant difference in effects between the 1 μM and 10 μM doses of the inhibitor.

To further substantiate the existence of caspases, activity assays were carried out with substrates for both the CED-3/CPP32 and the ICE families of proteases. The activities measured in terms of liberation of 7-amino-4-trifluoromethyl coumarin showed an increase in caspase activity at 30 minutes after H₂O₂ treatment with the substrate Ac-DEVD-AFC (Fig. 5B), which is a substrate for CED-3/CPP32 group of proteases. No increase in activity was detected with substrates Ac-LEHD-AFC and Ac-WEHD-AFC, which are substrates for caspase 9 and caspases 1, 4 and 5, respectively. The same cell lysates were assayed in the presence of 1 μM and 10 μM Z-DEVD-FMK and a significant inhibition of the activity was obtained showing specificity of the reaction (Fig. 5B).

Caspases 3 and 7 can cleave PARP when they are activated. Using anti-PARP antibodies, western blots of lysates of the cells exposed for different periods of time to H₂O₂ were checked for PARP-like protein cleavage. A distinct cleavage of a PARP-like protein was observed over a period of time (Fig. 5C). There was a gradual decrease in the concentration of the uncleaved protein (78 kDa), which generated a 63-kDa cleaved fragment with increasing time of exposure. Fig. 5D shows that cleavage of this PARP-like protein did not occur when cells were pretreated with the caspase inhibitor Z-DEVD-FMK.

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**Fig. 5.** Caspase activity and effects of inhibition of caspase activity. (A) Number of cells undergoing apoptosis-like death under different treatments. Symbols: ■, treated with 4 mM H₂O₂; cells preincubated with CED-3/CPP32 group of protease inhibitor Z-DEVD-FMK (▲, 1 μM; ●, 10 μM; ▼, 25 μM) and 4 mM H₂O₂; ○, control without any treatment. (B) Caspase activity in cell lysates with or without inhibitor treatment. Cell lysates were from *L. donovani* promastigotes at 30 minutes after exposure to 4 mM H₂O₂. Z-DEVD-FMK was used as a caspase-like protein inhibitor. Results are mean ± s.e.m. of three experiments. (C) Time course of PARP-like protein cleavage during H₂O₂-induced apoptosis in *L. donovani* promastigotes shown on western blots with anti-PARP antibody at different time points after H₂O₂ induced stress. Lanes: m, marker; 1, 0 hour; 2, 1 hour; 3, 2 hours; 4, 4 hours; 5, 6 hours. (D) PARP-like protein cleavage induced by H₂O₂ in the presence of a caspase inhibitor at 4 hours of treatment. Lanes: 1, with 4 mM H₂O₂ treatment; 2, control without treatment; 3, cells preincubated with 1 μg of Z-DEVD-FMK prior to exposure to 4 mM H₂O₂. Result is representative of three experiments.
Table 1. Level of intracellular GSH in Leishmania donovani promastigotes after exposure to 4 mM H2O2

<table>
<thead>
<tr>
<th>Hour</th>
<th>GSH (mM/10^7 cells) ± s.e.m.</th>
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<tr>
<td>0</td>
<td>0.48±0.02</td>
</tr>
<tr>
<td>2</td>
<td>0.27±0.01</td>
</tr>
<tr>
<td>4</td>
<td>0.17±0.01</td>
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<tr>
<td>6</td>
<td>0.07±0.008</td>
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Data are mean ± s.e.m. of three experiments (0 vs. 2, P<0.003; 0 vs. 4 and 6, P<0.001).

Taken together, the above data suggest that there is an activation of caspase-like proteases in the L. donovani promastigotes when they are exposed to H2O2. The activation is further confirmed by the cleavage of a PARP-like protein, which is very similar to post-caspase activation in mammalian apoptosis.

Intracellular GSH levels decrease after H2O2 treatment

GSH is a cellular thiol that is an important antioxidant by virtue of its involvement with products of lipid peroxidation and of taking part in reactions catalysed by glutathione peroxidase and glutathione transferase. GSH levels were measured because H2O2 generates products that are detoxified by actions of cellular thiols and the defensive enzymes. The intracellular level of GSH was found to decrease, reaching half the normal levels at 2 hours and declining to negligible levels at 6 hours (Table 1). The lowering of GSH level indicates its increased usage owing to severe oxidative stress in the cell.

DISCUSSION

In this study, we attempted to answer three questions. First, whether H2O2-induced death in L. donovani promastigotes is dependent on the dose of oxidative stress, and if so whether there is a phenotypic variance in the type of death that occur at the different doses. Second, whether this process resembles metazoan apoptosis. Third, whether this death induced by H2O2 occur via a death machinery.

Higher concentrations of H2O2 increased the loss of motility as well as cell death, suggesting that the degree of oxidative stress that the cells are exposed to is very important for the ability of the cell to survive. Similar phenomenon with a superoxide generation system has been observed in Trypanosoma brucei brucei (Ridgley et al., 1999). It is our observation that L. donovani promastigotes undergo a necrosis-like death with doses above 4 mM H2O2, whereas 4 mM H2O2 and lower doses precipitate an apoptosis-like death that shares many characteristics with metazoan apoptosis. It is known that, in metazoans, the response to oxidative stress varies in different cell types and, with a higher degree of oxidative stress, necrosis is promoted (Palomba et al., 1999).

In multicellular organisms, apoptosis is characterised by two prominent changes in the nucleus: (1) destruction of normal structural organisation of the nucleus owing to collapse of the chromatin into condensed electron-dense masses; and (2) fragmentation of nuclear DNA into oligonucleosomal fragments. Our observations of the condensation of nuclear material followed by DNA fragmentation into oligonucleosomal fragments that occur prior to permeabilisation of membranes in the promastigotes under oxidative stress show that these parasites share two very prominent features of metazoan apoptosis. We encountered certain amount of smearing in the DNA gels, which is not uncommon because DNA smearing in the absence of DNA ladders during apoptosis is known in yeast (Madeo et al., 1999) and some metazoan cell types (Oberhammer et al., 1993).

Interestingly, TUNEL labelling of kinetoplast DNA observed at 2 hours may suggest breakage of the kinetoplast DNA prior to nuclear DNA breakdown that is in contradiction to the report by Ridgley et al., in which TUNEL labelling of mitochondrial DNA in untreated cells was observed (Ridgley et al., 1999). Recently, it has been shown that induction of topoisomerase II causes breakdown of mitochondrial DNA leading to cell death in L. donovani promastigotes (Mitra, 2000). Thus, the breakdown of mitochondrial DNA at 2 hours may indicate an important step in the apoptosis-like death process in L. donovani. Breakdown of PARP, a DNA repair enzyme that catalyses the poly(ADP-ribo)sylation of various nuclear proteins with NAD as substrate (D’Amours et al., 1999) is a hallmark of metazoan apoptosis. We demonstrate cleavage of a PARP-like protein using a mammalian anti-PARP antibody, but the molecular weight of the native protein is ~78 kDa, and the breakdown product is 63 kDa, which differs from the size of mammalian PARP. It will be interesting to probe further into the identity of this PARP-like protein in L. donovani to identify unique forms, as inhibition of activity of this protein leads to cell death (Pieper et al., 1999) and could provide a target for developing novel therapeutic approaches.

PARP is cleaved by cysteine proteases called caspases in metazoans (Kaufman et al., 1993; Pieper et al., 1999). If there is a PARP-like protein cleavage then there should arguably be activation of proteases in L. donovani. We show the activation of caspase-like proteins using substrates for the CED-3/CPP32 group of proteases, an activation that was quick and occurred at 30 minutes after H2O2 treatment, followed by PARP-like-protein cleavage at 2 hours. The role of caspase-like proteins was further substantiated by the lower rate of cell death when cells were pretreated with an inhibitor of CED-3/CPP32 group of proteases prior to exposure to H2O2. CED-3 proteases have been described in Caenorhabditis elegans (Hengartner, 1999), with homologues described in mammals (Cohen, 1997), but these proteins have not been described in the unicellular eukaryotes. Consequently, the identification of caspase-like activity in these parasites is very important as it indicates the existence of a pathway for apoptosis-like death. This observation raises questions about the pathway of protease activation. Unpublished data* from this laboratory show that mitochondrial membrane potential is lost upon treatment with H2O2, but there is no release of cytochrome c from the mitochondria in response to H2O2 treatment (M.D. et al., unpublished). This is consistent with our observations in the present study that no caspase-9-like protein activation is present.

Apoptosis-like death in Leishmania donovani

*Cytochrome c release in the cytosol following oxidative stress was checked by probing western blots of cytosol and mitochondria. These were prepared from cells treated with H2O2 or untreated, or of supernatant and pellet of mitochondrial fractions from untreated cells exposed to H2O2 in vitro. The blots were probed with anti-cytochrome-c antibody (Santa Cruz Biotech, Santa Cruz, CA) at 1:1000 dilution, followed by secondary antibody at 1:5000 dilution. In both cases, reactive bands were visible in the mitochondrial pellet only.
observed with the treated cell lysates analysed at different time points. It will be interesting to investigate proteins other than cytochrome c released from the mitochondria, as is observed in mammals (Susin et al., 1999), that may take part in apoptotic changes introduced in these kinetoplastid parasites.

**Increased calcium accumulation after H$_2$O$_2$ exposure**

There is an increase from 10-100 nmol Ca$^{2+}$ 10$^{-7}$ cells at 30 minutes to 400 nmol Ca$^{2+}$ 10$^{-7}$ cells at 60 minutes. In multicellular organisms, free radical production in conjunction with changes in Ca$^{2+}$ homeostasis is a common feature of programmed cell death (Lipton and Nicotera, 1998). In a related parasite, *Leishmania amazonensis*, a calcium-dependent apoptosis-like death occur when there is a shift in temperature (Moreira, 1996). Loss of cell volume is a major feature of metazoan apoptosis (Gómez-Angelats et al., 2000) and our studies show that this feature is also shared by the *L. donovani* promastigotes when they die via an apoptosis-like mechanism. However, the loss of cell volume that occurs is a slow process in comparison to that occurring in the metazoans.

It is our observation with these parasites that there is an increase in the accumulation of products of lipid peroxidation after H$_2$O$_2$ treatment, and exogenous addition of these products precipitates apoptosis-like death (data not shown). It is noteworthy that there is a temporal relationship between increase in intracellular Ca$^{2+}$, activation of caspase-like proteases, breakdown of a PARP-like protein, nuclear condensation, nuclear breakage and cell volume loss, indicating that they are related to one another. Early biochemical signals like activation of caspase-like proteases, increase in intracellular Ca$^{2+}$, generation of products of lipid peroxidation and GSH depletion show that H$_2$O$_2$ generates a death signalling pathway rather than simply causing DNA breaks and cellular damage. Moreover, DNA fragmentation is visible when membrane permeability is still intact and so this breakage may be interpreted as not being due to necrosis induced by H$_2$O$_2$.

Apoptosis-like death allows constant selection for the fittest cell in the colony, optimal adaptation of the cell number to the environment and tight regulation of cell cycle and cell differentiation. It is generally assumed that a regularised system of cell death in phylogenesis appeared after the onset of multicellularity. However, apoptosis-like death of single-celled organisms can be beneficial to its genetically identical neighbours in certain situations and hence be adaptive. A number of single-celled organisms undergo apoptosis-like death, for example, the slime mould *Dictyostelium discoideum* (Cornillon et al., 1994), intracellular parasites like *Trypanosoma* (Welburn et al., 1997; Welburn and Maudlin, 1997) and yeast (Frohlich and Madeo, 2000). unicellular organisms might have selected for genes of cell suicide as a defence strategy (Vaux and Korsemeyer, 1999).

*L. donovani* populations are largely clonal (Tibayrenc et al., 1991) and it is logical for an altruistic mechanism like apoptosis to promote and maintain genetic stability within the clonal population. A similar scenario is visible within the mammalian immune system, in which cell populations are controlled at the clonal level (Williams, 1994). Because parasites like *L. donovani* or related ones survive within the insect gut or the macrophages, they must have a stringent control to restrict the number individuals, otherwise death of the insect vector or the macrophages may occur prematurely. This control is also required because they are competing for limited resources. To understand the evolution of the process of apoptosis, it is necessary to identify the gene products and biochemical pathways for cell death in lower organisms, from where the metazoan process appears to have evolved. Kinetoplastid parasites, being early in the evolutionary tree, occurring between 1.5 billion and 2.0 billion years ago, it is important that components of the apoptotic pathway be identified to determine where the evolutionary divergence occurred.

Therefore, our studies show for the first time the activation of caspase-like proteins after exposure to oxidative stress in a kinetoplastid parasite that leads to apoptosis-like death. This death share many characteristics with metazoan apoptosis and uses similar biochemical pathways. Thus, this study provides a groundwork for the identification of cell death components in *L. donovani*.

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