Programmed cell death in *Trypanosoma cruzi* induced by *Bothrops jararaca* venom

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Cells die through a programmed process or accidental death, know as apoptosis or necrosis, respectively. Bothrops jararaca *is a snake whose venom inhibits the growth of* Trypanosoma cruzi *epimastigote forms causing mitochondrion swelling and cell death. The aim of the present work was to determine the type of death induced in epimastigotes of* T. cruzi *by this venom. Parasite growth was inhibited after venom treatment, and 50% growth inhibition was obtained with 10 µg/ml. Ultrastructural observations confirmed mitochondrion swelling and kinetoplast disorganization. Furthermore, cytoplasmic condensation, loss of mitochondrion membrane potential, time-dependent increase in phosphatidylserine exposure at the outer leaflet plasma membrane followed by permeabilization, activation of caspase like protein and DNA fragmentation were observed in epimastigotes throughout a 24 h period of venom treatment. Taken together, these results indicate that the stress induced in epimastigote by this venom, triggers a programmed cell death process, similar to metazoan apoptosis, which leads to parasite death.*

Key words: *Trypanosoma cruzi* - programmed cell death -*Bothrops jararaca* - snake venom

In multicellular organisms, programmed cell death (PCD), also known as apoptosis, is important to control cell number for proper development and tissue homeostasis, removal of unwanted cells and functional control of the immune, haemopoietic and nervous systems (Welburn et al. 1997). The process of PCD is activated by genetically controlled cell suicide machinery (Ameisen 1996). In vertebrate cells, PCD can be activated by external stimuli (ethanol, reactive oxygen species, and receptor ligands) and internal processes (mitotic catastrophe, replication failures, developmental programmed cell death) (Fröhlich & Madeo 2000). PCD is also a common response to cell stress caused by different toxins (Vaux 2002). Independent of the stimulus, PCD usually involves alteration in the mitochondrial membrane permeability, caspase activation, phosphatidylserine (PS) exposure, nuclear and cytoplasmic condensation, DNA fragmentation and breakage of the cell into apoptotic bodies, which are engulfed by the surrounding cells (Vaux & Strasser 1996). PCD is a process found in virtually all nucleated metazoan cells, and it has been recently associated with several species of unicellular eukaryotes, notably kinetoplastids (Ameisen et al. 1995, Welburn et al. 1996, Moreira et al. 1996, Ridgley et al. 1999, Arnoult et al. 2002, Lee et al. 2002, Zangger & Xu 2002), yeast (Madeo et al. 1999), apicomplexans (Picot et al. 1997, Peng et al. 2003), and amitochondrion parasites (Chose et al. 2002, Mariante et al. 2003).

Snake venoms have been identified as the richest source of enzymes among poisonous animal, displaying a variety of biological activities (Tan & Ponnudurai 1992). *Bothrops jararaca* is a snake of the viperidae family, largely distributed in Brazil and responsible for 90% of the ophidian envenomations in humans (Bochner & Struchiner 2003). *Trypanosoma cruzi* is the agent of Chagas disease affecting 16-18 million people in Latin America. We have previously demonstrated that *B. jararaca* venom inhibited the growth of epimastigote forms of *T. cruzi*, causing mitochondrion swelling and kinetoplast disorganization (Gonçalves et al. 2002). Because of the ultrastructural alteration observed in the mitochondrion after venom treatment it was suggested that impairment of this organelle was probably responsible for growth inhibition (Gonçalves et al. 2002). However, how epimastigotes were demising was not analyzed. Thus, the aim of the present work was to identify if PCD or necrosis was being induced in *T. cruzi* epimastigote forms during *B. jararaca* venom treatment.

MATERIALS AND METHODS

Parasite and venom - Epimastigote forms of *T. cruzi* (Y strain) were maintained at 28°C by weekly transfers in liver infusion tryptose (LIT) medium (Camargo 1964) supplemented with 10% fetal bovine serum (FBS). Four-day-old cultured epimastigote forms (mid-log phase) were used for the experiments.

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Lyophilized *B. jararaca* venom was purchased from the Instituto Butantan (São Paulo, Brazil). Venom stock solutions (2.5 mg/ml) were prepared in 20 mM Tris, 300 mM NaCl, pH 8.0, esterilized in a 0.22 µm filter (Millipore) and kept at 4°C. Crude venom solutions were diluted in the cell culture medium before use.

Treatment of cultured parasites - *T. cruzi* epimastigotes were seeded (1.5 x 10^6 cell/ml) into flasks (final volume 4 ml) containing medium supplemented with 0 (control), 5, 10, 25 or 50 µg/ml of venom. Parasite growth was monitored, up to 7 days post-seeding, by the counting of formalin fixed parasites in a hemacytometer chamber daily. The inhibitory $K_{0.5}$ (50% growth inhibition) was estimated by plotting the parasite numbers from day 4 as a percentage over venom concentration; cultures without any venom were used as 100% growth. For most of the following characterizations, parasites were treated with double of the amount of the inhibitory $K_{0.5}$ of the venom (20 µg/ml) for a maximum of 24 h. For mitocondrial membrane potential and PS exposure assays cells were treated at different time points, as below. And for detection of caspase-3 like activity cells were treated with 10 µg/ml for 24 h. Control parasites were cultured in parallel without venom and analyzed at the same time.

Cell viability and PS exposure assays - Epimastigotes were treated with venom and the viability assay was performed in a flow cytometer (Coulter Elite, SP) with propidium iodide (PI) (Sigma) as a vital stain. Cells were washed with phosphate buffer saline (PBS) and 1.5 x 10^6 cells in 500 µl of PBS were incubated with 10 µg/ml of PI for 30 min and analyzed. For PS exposure assay, epimastigotes were treated with venom for 4, 8, and 24 h. PS exposure on the outer leaflet of the plasma membrane was measured by incubating cells with 1 µg/ml of annexin V-FITC (Santa Cruz Biotechnology Inc., US) for 20 min in a buffer containing 140 mM of sodium chloride, 10 mM of HEPES, 2.5 mM calcium chloride at pH 7.4. Viability was also assayed in parallel using PI as before. Cells were analyzed in a flow cytometer. Data, histograms and dot plot were obtained using WinMDI 2.8 software.

Transmission electron microscopy - Epimastigotes treated with venom were washed three times with PBS, fixed for 2 h with 2.5% glutaraldehyde, 2% recently prepared formaldehyde in 0.1 M phosphate buffer, pH 7.2. Cells were washed in the same buffer, post-fixed for 1 h with 1% osmium tetroxide in 0.1 M phosphate buffer, dehydrated in graded acetone and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate and lead citrate, and observed in a ZEISS EM900 transmission electron microscope.

Mitochondrion membrane potential (Dy_m) analysis - Alterations in Dy_m were analyzed in epimastigotes treated for 4 h with venom. After treatment, cells were centrifuged at 1500 g for 10 min and adhered to cover slips coated with poly-L-lysine. Cells attached to cover slips were incubated in LIT medium containing 50 µg/ml of rhodamine 123 during 20 min, washed and observed in a laser scan confocal microscopy Zeiss SM 410 using a 488 nm and 543 nm argon laser.

Detection of caspase-3 like activity - The CM1 antibody used was a gift from Dr Marlene Benchimol (Universidade Santa Úrsula, Brazil) and Dr Robert C Armstrong (Idun Pharmaceuticals, US), and a detailed description was provided by Namura et al. (1998). Treated epimastigotes were immunolabeled following a modification of the method used by Mariante et al. (2003). Cells were fixed for 30 min in 3% recently prepared formaldehyde in PBS, washed, incubated for 10 min in 50 mM amonium chloride in PBS, washed and attached to cover slips coated with poly-L-lysine. Cells were washed and incubated for 10 min in a blocking buffer containing 2% bovine serum albumin, 0.2% non-fat milk powder and 0.8% Triton X-100 in PBS at room temperature. After that, cells were incubated overnight in CM1 primary antibody diluted (1:5000) in the blocking buffer at 4°C. Cells were washed with PBS, incubated for 10 min in the blocking buffer and further incubated for 40 min with a FITC labeled goat anti-rabbit IgG (Sigma) diluted 1:100 at 4°C. Cells were washed with PBS, mounted in N-propyl-gallate and observed in the Confocal microscope.

In situ nick-end labeling - Treated epimastigotes were washed with PBS, fixed with 3% recently prepared formaldehyde in PBS and attached to cover slips coated with poly-L-lysine. In situ DNA nick-end labeling was performed using a DNA fragmentation detection kit (FragEL, Oncogene) according to manufacturer's specifications.

RESULTS

The effect of crude venom treatment on epimastigotes was dose-dependent. No growth was detected when parasites were treated with 25 or 50 µg/ml of venom (Fig. 1). The inhibitory $K_{0.5}$ (50% growth inhibition) at 4th day was 10 µg/ml (Fig. 1, inset).

Flow cytometry analysis of epimastigotes treated with venom showed cytoplasmic condensation (Fig. 2A) and 97% of dead cells indicated by propidium iodide staining

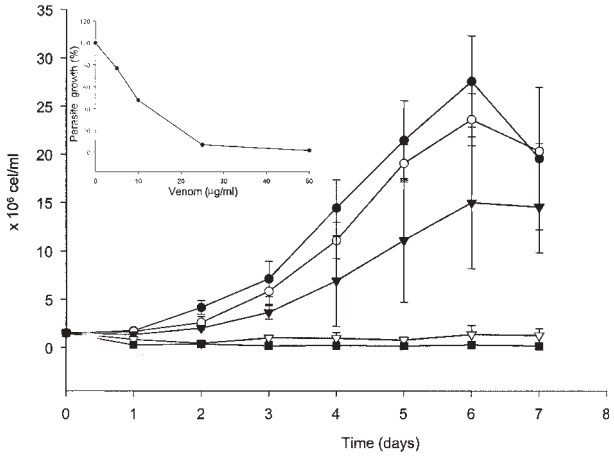

Fig. 1: growth inhibition of *Trypanosoma cruzi* epimastigote forms by *Bothrops jararaca* venom. Cells were cultured for 7 days with increasing venom concentrations (● 0; ○ 5; ▼ 10; ▽ 25; ■ 100 µg/ml) and counted daily. The inhibitory $K_{0.5}$ (50% growth inhibition) was estimated by plotting the parasite numbers from day 4 as a percentage over venom concentration (inset). The bars indicate standard deviation of four experiments.

(Fig. 2B). Only 3% of the cell population of untreated parasites showed cell death.

Ultrastructural observations of untreated epimastigote showed all the typical ultrastructural characteristics of the trypanosomatids. The flagellar pocket and the flagellum close to the kinetoplast (large condensed mitochondrion DNA), and a normal mitochondrion next to the cell periphery were seen (Fig. 3A). Epimastigotes treated with venom presented mitochondrion swelling and kinetoplast disorganization (Fig. 3B). Treated parasites also exhibited a rounded shape instead of the normal elongated appearance of untreated cells (Fig. 3B). Margination and condensation of nuclear chromatin was rarely observed after venom treatment (not shown).

Due to the swelling of the mitochondria observed after treatment of parasites with venom, alterations in the Dy_m were analyzed. After incubation with rhodamine 123, untreated epimastigotes showed intense labeling of the mitochondrion as expected (Fig. 4A, B). Parasites treated with venom showed a decrease in fluorescence, indicating lost of Dy_m (Fig. 4C, D).

PS exposure in the outer leaflet of the plasma membrane is a common feature of cells undergoing apoptosis. Using annexin V-FITC and PI, PS exposure and membrane permeability of parasites treated with venom were simultaneously accessed. Untreated parasites were annexin V and PI negative (Fig. 5A). After 4 h of venom treatment, 7% of the parasite population exposed PS and exhibited no plasma membrane permeability (Fig. 5B). After 8 h of treatment, the population exposing PS and preserving membrane integrity increased to 21%. However, another 26% of treated cells exposed PS and were also positive for PI (Fig. 5C). After 24 h, 94% of the treated parasite population was PI positive and 50% of this population exposed PS (Fig. 5D).

Caspase-3 activity is a hallmark of cells dying by PCD. By immunofluorescence it was possible to detect a caspase-like protein in treated epimastigotes. Untreated parasites presented an elongated morphology and did not label for the CM1 antibody (Fig. 6A, B). Treated epimastigotes presented round (cytoplasmic shrinkage) and elongated forms (Fig. 6C). Only the rounded shape parasites had caspase-like protein activity (Fig. 6D).

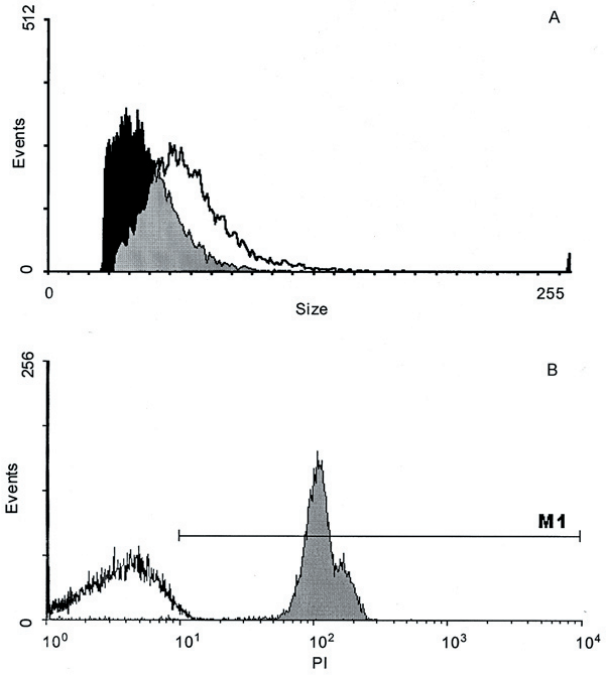

Fig. 2: cytoplasmic condensation and viability of *Trypanosoma cruzi* epimastigote forms after *Bothrops jararaca* venom treatment. Parasites were treated for 24 h with 20 µg/ml of venom. Untreated (white area) and treated parasites (gray area) can be distinguished in the overlay histograms; parasite size (A) and propidium iodide (PI) intercalation (B) are shown. Note the smaller size and higher PI positive fluorescence (97% as defined by the M1 region) in parasites treated with venom. This experiment is representative of five performed.

DNA fragmentation is characteristic of cells in the PCD process. Thus, DNA fragmentation was analyzed with a nick-end labeling detection system. Untreated parasites exhibited no DNA fragmentation and normal epimastigote morphology (Fig. 7A). DNA fragmentation was observed in epimastigotes treated with venom (Fig. 7B). Again it was confirmed that venom treatment induced cytoplasmic shrinkage. In some slides labeling in two regions of the cell cytoplasm was observed (not shown).

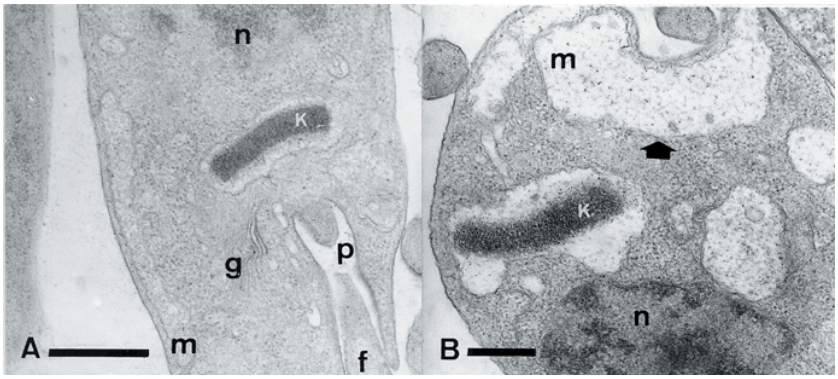

Fig. 3: transmission electron microscope images of *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom. Parasites were incubated for 24 h with 20 µg/ml of venom. A: untreated parasite showing mitochondrion (m), Golgi apparatus (g), kinetoplast (k), flagellar pocket (p), flagellum (f) and nucleus (n); B: treated parasite exhibiting a rounded shape, mitochondrion swelling (arrow), and kinetoplast disorganization. Bars = 0.5 µm. This experiment is representative of three performed.

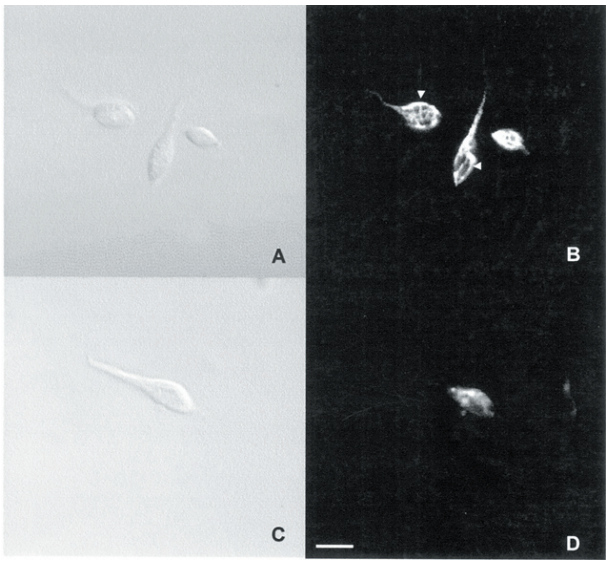

Fig. 4: *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom show reduction in the mitochondrion membrane potential (Dy_m). Parasites were treated with 20 µg/ml of venom for 4 h and incubated with rhodamine 123. A: differential interference contrast (DIC) image of untreated parasites; B: confocal laser scanning microscopy (CLSM) image of untreated parasites showing mitochondrion labeling with rhodamine 123 (arrows); C: DIC image of treated parasite; D: CLSM image of treated parasite. Note the decrease in fluorescence of rhodamine 123 labeling in treated parasites indicating loss of $\Delta\psi_m$. Bars = 5 µm. This experiment is representative of two performed.

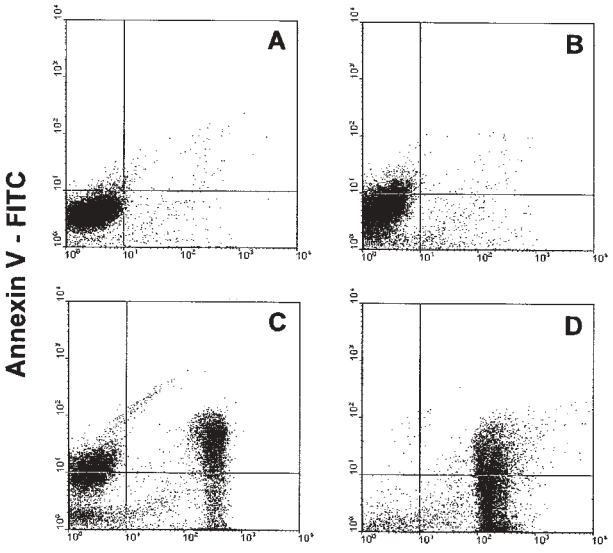

Fig. 5: time-dependent increase in phosphatidylserine (PS) exposure by *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom. Parasites were treated for 4, 8, and 24 h with 20 µg/ml of venom and incubated with 10 µg of propidium iodide (PI) and 1 µg/ml of annexin V-FITC. A: untreated parasites did not expose PS and were negative for PI; B: after 4 h of treatment, 7% of the parasites exposed PS and were negative for PI; C: after 8 h, 21% of the parasites exposed PS and were negative for PI, 26% were positive for both markers and 21% were PI positive only; D: parasites treated for 24 h exhibited 94% of the parasite population PI positive. This experiment is representative of three performed.

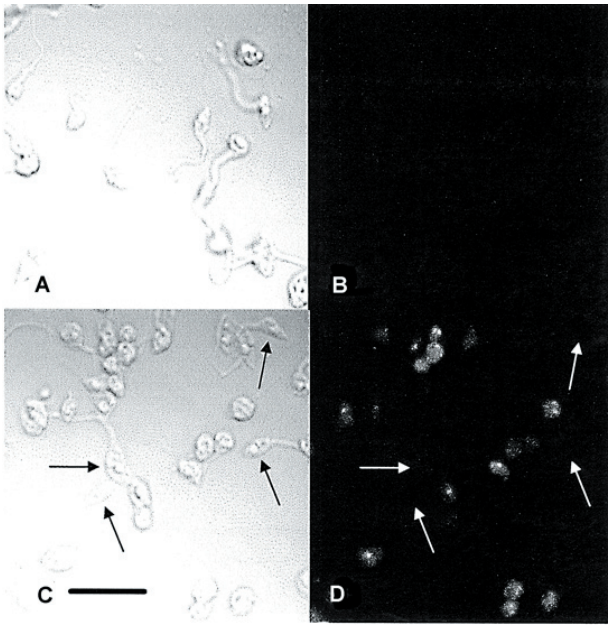

Fig. 6: *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom exhibited caspase-like activity. Parasites were treated for 24 h with 10 µg/ml of venom. Cells were labeled with CM1 as in materials and methods. A: differential interference contrast (DIC) image of untreated parasites; B: confocal laser scanning microscopy (CLSM) image of untreated parasites, no labeling can be observed; C: DIC image of treated parasite; D: CLSM image of treated parasite. Note that round cells are labeled and that elongated epimastigotes are not (arrows). Bar = 10 µm. This experiment is representative of two performed.

Fig. 7: *Trypanosoma cruzi* epimastigote forms treated with *Bothrops jararaca* venom exhibited DNA fragmentation. Parasites were treated for 24 h with 20 µg/ml of venom. In situ nick-end labeling of cells was performed using a DNA fragmentation detection kit (Oncogene). A: untreated parasites. Bar 7 mm; B: treated parasites are labeled indicating DNA fragmentation (arrows). Note the cytoplasmic condensation of cells. Bar = 17 µm. This experiment is representative of three performed.

DISCUSSION

In a previous work we have shown that epimastigote forms of *T. cruzi* had their growth arrested after *B. jararaca* venom treatment (Gonçalves et al. 2002). Here we confirmed that this treatment induced growth arrest and mitochondrion alteration. Furthermore, venom treatment caused cytoplasmic condensation, loss of mitochondrion membrane potential, exposure of PS on the outer leaflet of the plasma membrane, caspase 3 like protein activation

and DNA fragmentation. Taken together, these results strongly suggest that this venom induces death in epimastigote forms by triggering PCD with features of metazoan apoptosis.

The induction of apoptosis in other cell types by snake venom treatment has already been described. Venom of *Crotalus viridis* (Suzuki et al. 1997) and other hemorragic snake venoms (Araki et al. 1993) induce PCD in vascular endothelial cells, resulting in vascular malfunctions and alteration of the haemostatic process. Venoms are a complex mixture with different biological effects, thus we can only speculate on the component(s) responsible for the induction of PCD in venom treated *T. cruzi* epimastigotes. However, Tempone et al. (2001) have shown that the venom of *Bothrops moojeni* presents an L-amino acid oxidase responsible for growth inhibition of different *Leishmania* species. L-amino acid oxidase uses amino acids as substrate producing H_2O_2, which is a known inducer of PCD in metazoans (Hockenbery et al. 1993, Clement & Pervaiz 1999) and also in unicellular organisms (Madeo et al. 1999, Ridgley et al. 1999, Mariante et al. 2002). The venom used in this work is from a snake of the same genus used in Tempone's work, thus we are now trying to determine if the oxidases from the same family could be responsible for triggering PCD in epimastigotes due to H_2O_2 production.

In mammalian cells, a common pro-apoptotic stimulus is the release of cytochrome c from mitochondrion due to the outer membrane permeabilization (Brenner & Kroemer 2000). The kinetoplast disorganization, mitochondrion swelling and loss of its membrane potential seen in epimastigotes after venom treatment suggests that the observed PCD might involve the mitochondrion cell death machinery. This is further confirmed by the activity of caspase-like found in treated epimastigotes. The CM1 antibody is able to recognize the cleavage product of the human or mouse caspase-3 (Namura et al. 1998) and also label *Tritrichomonas foetus* treated with H_2O_2 (Mariante et al. 2003). In this report, epimastigotes were treated with the inhibitory $K_{0.5}$ venom concentration, which was able to maintained some elongated form of the parasites in the period analyzed. Only epimastigotes that suffered cytoplasmic condensation were labeled by the antibody. Thus, this labeling was specific and shows that a caspase-like was being activated after venom treatment. This is another evidence that a PCD process was induced in epimastigotes after venom treatment.

It was observed that PS exposure after venom treatment was time-dependent and was followed by an increase in PI signal indicating membrane permeability. Thus, PCD was being induced after treatment with venom and was followed rapidly by secondary necrosis. This indicates that the venom was extremely toxic and killed epimastigote through a PCD mechanism, probably as a response to cell stress (Vaux 2002) caused by the treatment. DNA fragmentation is a hallmark of apoptosis (Zhang & Xu 2002). In our experiments DNA fragmentation was clearly detected by in situ nick-end labeling further supporting that venom treatment was inducing cell death by an apoptotic mechanism. Furthermore, some cells exhibited two labeled regions. This might indicate that during cell death by venom treatment, nuclei were fragmenting or that kinetoplast could also suffer the action of endonucleases, which would corroborate with the disorganization appearance as seen at the ultrastructural level. Further experiments are necessary to investigate these possibilities.

Multicellular organisms use physiological mechanisms of cell death for development and morphogenesis, to control cell number, as well as a defensive strategy to remove infected, mutated or damaged cells (Vaux & Korsmeyer 1999). Until recently, it was assumed that PCD was a process confined to metazoan organisms (Vaux et al. 1994). However, new findings indicate that unicellular eukaryotes exhibited a type of cell death similar to typical mammalian PCD. PCD has been described in several species including the kinetoplastids *Trypanosoma cruzi* (Welburn et al. 1996), *L. amazonensis* (Moreira et al. 1996) *T. brucei brucei* (Ridgley et al. 1999), *L. donovani* (Lee et al. 2002), *L. mexicana* (Zangger et al. 2002), *L. major* (Arnoult et al. 2002), the amitochondrial parasites *Trichomonas vaginalis* (Chose et al. 2002) and *Tritrichomonas foetus* (Mariante et al. 2003), *Plasmodium falciparum* (Picot et al. 1997), *Toxoplasma gondii* (Peng et al. 2003), yeast (Madeo et al. 1999) and *Dictyostelium* (Cornillon et al. 1994). However, the function of PCD in unicellular organisms is under speculation. It has been proposed that the reason of a PCD pathway in unicellular organisms is to control the cell population. Because these organisms are largely clonal populations, an altruistic mechanism of cell death avoiding uncontrolled growth would be logical (Welburn et al. 1997). The prediction that a single molecular mechanism of PCD emerging in evolution prior to the postulated multiple emergences of multicellularity indicates that some of the molecules involved in the PCD mechanisms of phylogenetically distant organisms might be related (Cornillon et al. 1994).

In conclusion, our results show that *T. cruzi* epimastigote forms exhibited PCD after *B. jararaca* venom treatment. This result is another example that the PCD process occurs in unicellular organism and might be the result of cell stress caused by venom treatment. This finding indicates that pharmacological and immunological manipulation of the PCD process may lead to new therapeutic approaches to chronic parasitic diseases (Barcinski & DosReis 1999). We are now characterizing the component of venom responsible for this effect on parasites.

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