ARTICLE
Journal of Cellular Biochemistry 105:688–698 (2008)

Journal of Cellular Biochemistry

Natural Programmed Cell Death in *T. cruzi* Epimastigotes Maintained in Axenic Cultures

V. Jimenez,[1] R. Paredes,[1,2] M.A. Sosa,[3] and N. Galanti[1]*

[1]*Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile*

[2]*Laboratorio de Salud de Ecosistemas, Escuela de Medicina Veterinaria, Facultad de Ecología y Recursos Naturales, Universidad Andrés Bello, Santiago, Chile*

[3]*Instituto de Histología y Embriología, Facultad de Ciencias Medicas, Universidad Nacional de Cuyo, Mendoza, Argentina*

ABSTRACT

Trypanosoma cruzi, a parasitic protozoan, is the agent of Chagas' disease or American trypanosomiasis, an endemic pathology in Latin America, affecting up to 18 million people, with high public health costs. Programmed cell death (PCD) has many functions in development and tissue remodeling in metazoans. In protozoa, it could represent concomitant or alternative mechanisms for clonal selection, immune response evasion, and population size regulation. In this work, we describe the natural occurrence of PCD in *T. cruzi* epimastigotes during the stationary phase of growth in axenic culture or under nutrient deprivation. Thus, we have observed phosphatidylserine externalization, cellular volume decrease, caspase-like protein activity, and DNA fragmentation. Additionally, serum deprivation also induces autophagic characteristics such as monodansylcadaverine-labeled vesicles accumulation and redistribution of proteins homologous to Atg8. In agreement with our results, apoptosis may play an important role in parasite survival. Then, identification and modulation of molecular targets inducing programmed cell death in *T. cruzi* may lead to new potential therapeutic approaches for Chagas' disease. J. Cell. Biochem. 105: 688–698, 2008.

KEY WORDS: APOPTOSIS; AUTOPHAGY; *Trypanosoma cruzi*; STARVATION

Programmed cells death (PCD) is a regulated process allowing organisms to eliminate cells, avoiding an inflammatory response [Hengartner, 2000]. Although this process was described in the 1970s [Kerr et al., 1972], during the last years it has received more attention due to its role in tumor cell elimination and, by default, in carcinogenesis [Dirsch et al., 2001]. PCD includes alternative forms of death with diverse phenotypic characteristics [Sperandio et al., 2000; Assuncao and Linden, 2004; Sperandio et al., 2004; Kroemer and Martin, 2005], mostly related to pluricellular organisms and conserved from *C. elegans* to *H. sapiens* [Danial and Korsmeyer, 2004]. However, in the last 10 years, growing evidence supporting its presence in unicellular eukaryotes has been provided [Ameisen, 1996; Arnoult et al., 2001, 2002; Das et al., 2001; Al-Olayan et al., 2002; Figarella et al., 2005]. In protozoa PCD may play a role in the regulation of parasite number in biological vectors and in definitive host tissues [Welburn et al., 1989; Maudlin and Welburn, 1994], in the maintenance of clonality [Welburn et al., 1997], as a mechanism of immunomodulation [Freire-de-Lima et al., 2000] and of parasite differentiation [Ameisen et al., 1995].

Trypanosoma cruzi, a parasitic protozoan, the agent of Chagas' disease, an endemic pathology in Latin America, infects more than 18 million people [WHO Expert Committee, 2002]. It presents an indirect life cycle, affecting man as well as domestic and wild mammals, its definitive hosts. In these hosts, the parasite develops in two forms: the circulating non-replicative trypomastigotes, infective to mammals and the amastigotes, intracellular and replicative forms. Chagas' disease is mainly transmitted by *Reduvidae* hematophagous insects. There, the epimastigotes, replicative extracellular forms of the parasite, are present. This cellular form may be maintained in axenic cultures.

We propose here that cell volume reduction, extracellular phosphatidylserine exposure, caspase-like protein activity and internucleosomal DNA fragmentation, all of them apoptosis markers, are present at the stationary growth phase of *T. cruzi*

Grant sponsor: Bicentenary Research Projects; Grant numbers: ACT29 and Red 07; Grant sponsor: CONICYT-Chile.
*Correspondence to: N. Galanti, Programa de Biología Celular y Molecular, Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Independencia 1027, Santiago, Chile. E-mail: ngalanti@med.uchile.cl
Received 10 April 2008; Accepted 19 June 2008 • DOI 10.1002/jcb.21864 • 2008 Wiley-Liss, Inc.
Published online 30 July 2008 in Wiley InterScience (www.interscience.wiley.com).

epimastigotes. We also propose that these markers may be mimicked by serum deprivation. In addition, we also advance the possibility that autophagy may explain at least a percentage of the cell death observed under nutrient deprivation. These *T. cruzi* features indicate possible interesting targets for the development of new therapeutic approaches to control Chagas' disease.

MATERIALS AND METHODS

REAGENTS

Culture media (GIBCO), hemin, actinomycin D and buffer reagents (Sigma), antibodies against caspase 8 and caspase 3, annexin-V FITC and propidium iodide (BD Pharmingen); TUNEL Kit and CaspACE assay system (Promega), DAPI (Pierce), fluorescent secondary antibodies (Invitrogen); anti-rabbit secondary antibody conjugated to horseradish peroxidase (Jackson Immunochemicals); ECL (Amersham) and SDS–PAGE reagents (Bio-Rad).

PARASITES CULTURE

T. cruzi epimastigotes (Tulahuen and MF strains) were grown at 28°C in Diamond's liquid medium [Diamond, 1968] (0.106 M NaCl, 29 mM KH_2PO_4, 23 mM K_2HPO_4, 12.5 g/L tryptose, 12.5 g/L tryptone, and 12.5 g/L yeast extract, pH 7.2) supplemented with 10% fetal calf serum (FCS), 7.5 μM hemin, and antibiotics (penicillin 75 U/ml–streptomycin 75 μg/ml).

QUANTIFICATION OF PARASITE PROLIFERATION

Parasites were counted in a Neubauer chamber, diluted 1:100 v/v in phosphate buffer saline (PBS) containing 10% formalin.

CELL VOLUME DETERMINATION

Epimastigotes at different growth stages, were collected by centrifugation at 1,000*g*, washed twice in PBS, resuspended in PBS to 100,000 parasites/ml and analyzed by fluorescence-activated cell sorting (FACS; Becton Dickinson & Co., NJ). Density plots of forward (FSC) versus side (SSC) scatter represent the acquisition of 10,000 events. Histograms and analysis were performed in WinMDI 2.8 software.

PS EXPOSURE

Exposed phosphatidylserine (PS) was detected using Annexin-V FITC (BD Pharmingen). Parasites were collected by centrifugation at 1,000*g*, washed twice in PBS and resuspended in 100 μl of binding buffer (140 mM NaCl, 5 mM $CaCl_2$, 10 mM HEPES-Na pH 7.4). Annexin-V FITC and propidium iodide were added at the final concentration indicated by manufacturer. Fluorescence was measured by FACS analysis in a FACSort apparatus (Becton Dickinson & Co.). Analysis was performed in WinMDI 2.8 software considering as apoptotic those cells stained by annexin-V (propidium iodide positive or negative) and, as necrotic, those only positive to red stain.

DNA FRAGMENTATION

TUNEL assay. We analyzed DNA double-strand ruptures in situ by TUNEL (Terminal deoxynucleotidyl transferase-mediated dUTP Nick End Labeling assay) according to manufacturer's protocol (DeadEnd Fluorometric TUNEL system, Promega). Briefly, parasites in different stages of growth were collected by centrifugation at 1,000*g*, washed twice in PBS and resuspended in the same buffer. Once placed on a slide and dried at room temperature, the cells were fixed with 4% *p*-formaldehyde and washed in PBS. After permeabilization with 0.2% Triton X-100, the cells were incubated with reaction mix containing dUTP-FITC (Fluorescein isothiocyanate). Nuclei were counterstaining with DAPI (4′,6-diamidin-2-phenylindol) (1 μg/ml) or propidium iodide (1 μg/ml). Fluorescence was observed in a Nikon Eclipse E400 microscopy and pictures were captured with a Nikon Coolpix 4500 digital camera. Results were quantified counting about 200 cells in duplicate from three independent experiments. Apoptotic index was obtained from the ratio between TUNEL positive cells and total cells.

DNA isolation and agarose electrophoresis. Parasites were collected by centrifugation, washed in PBS and resuspended in lysis buffer (1% SDS, 10 mM Tris–HCl pH 8.0). Suspension was incubated at room temperature in the presence of 300 μg/ml proteinase-K until pellet was dissolved completely. RNAase A was added at 200 μg/ml and incubated 1 h at 37°C with gentle agitation. DNA was extracted with phenol–chloroform–isoamylic alcohol mix (25:24:1 v/v). After centrifugation, the upper phase was separated and precipitated overnight at −20°C with 3 M sodium acetate and 100% ethanol. The pellet was washed with 70% ethanol and resuspended in 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris. DNA concentration was estimated at 260/280 nm. DNA was electrophoresed in 1.8% agarose gel containing ethidium bromide (0.5 μg/ml).

CASPASE-LIKE DETECTION

Western blotting. *T. cruzi* epimastigotes were lysed in buffer containing 1% Triton X-100, 5 μg/ml Aprotinin, 2 mM EDTA, 5 mM phenylmethylsulphonylfluoride (PMSF), 2.5 mM N-tosyl-L-lysine-chloromethyl ketone (TLCK) and 10 mM Tris–HCl pH 8.0. Total proteins, measured by the bicinconinic acid method (BCA protein assay, Pierce), were separated by electrophoresis in SDS–polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane (Bio-Rad), blocked overnight with 3% serum bovine albumin in PBS and incubated with heterologous antibodies against caspase 3 (BD Pharmingen 552785) for 2 h. Reactive proteins were visualized by secondary antibodies conjugated to horseradish peroxidase (Jackson Immunochemicals 111-035-144) and ECL Western blotting (Amersham, Biosciences) in Bio-Max film (Kodak). HeLa cells treated with 2 μg/ml of actinomycin as apoptotic inducer were used as positive control.

Detection of caspase 3-like activity. Parasites were collected by centrifugation, washed twice in PBS and resuspended in lysis buffer. Homogenate was centrifuged at 16,000*g* for 10 min at 4°C. The assay was performed in the presence of the pan-caspase inhibitor Z-VAD-FMK (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethyl ketone). Briefly, the reaction mixture contained about 1 mg of soluble

protein, 64 μl of caspase assay buffer, 4 μl of dimethyl sulfoxide (DMSO), 20 μl of ditiotreitol (DTT) (100 mM) and 4 μl of colorimetric substrate (10 mM Ac-DEVD-pNA: acetyl-Asp-Glu-Val-Asp *p*-nitroanilide). Upon cleavage by caspase-3, pNA produces a yellow color that was measured at 405 nm. Samples extracts from *T. cruzi* epimastigotes at exponential and stationary phase of growth were co-incubated with caspase-3 inhibitor Z-VAD-FMK (final concentration of 150 μM). After 12 h incubation, the difference in absorbance between the samples with and without caspase 3-inhibitor was considered caspase 3 activity, expressed as specific activity (pmol pNA/mg protein/h). Results correspond to three experiments in duplicate.

AUTOPHAGY SEARCH

Monodansylcadaverine (MDC) labeling. The assay was performed according to Munafo and Colombo [2001]. Briefly, after autophagy induction by serum deprivation, parasites were incubated with 0.05 mM MDC in PBS at 28°C for 1 h. Cells were washed twice in PBS and lysed in 10 mM Tris–HCl pH 8.0 containing 1% SDS. MDC stain was measured by fluorescent photometry (excitation wavelength 380 nm, emission filter 525 nm). To normalize the measurements, fluorescence of total DNA was quantified previously staining with ethidium bromide (0.2 μM final concentration) with an excitation wavelength of 530 nm and an emission filter of 590 nm. Results were expressed as specific activity (percentage respect to the control). Parasites were observed in a fluorescence microscopy Nikon Eclipse E400 and images were captured with a Nikon Coolpix 4500 digital camera.

LC3 immunolocalization. After 5 days of treatment, *T. cruzi* epimastigotes were collected by centrifugation 10 min at 1,000*g*, washed in PBS and placed on a slide pre-treated with silane. Parasites were fixed with 4% *p*-formaldehyde, washed twice in PBS and treated overnight with blocking solution containing 3% calf serum, 1% serum bovine albumin and 0.1% saponin. Then, the parasites were incubated in the presence of heterologous polyclonal antibodies against rat LC3, kindly provided by Dr. Masahiro Shibata (Department of Cell Biology & Neuroscience, Osaka University). As a detection immune probe, affinity purified polyclonal rabbit immunoglobulins, conjugated with Alexa fluor 488, were used. Cells were analyzed by fluorescence microscopy in a Nikon Eclipse E400.

Statistics. Results are expressed as mean ± SEM from n independent experiments. Normal distribution of data and significant differences were probed by ANOVA with Tukey post-test and Bonferroni correction for multiple conditions. Paired conditions were analyzed by Student's *t*-test.

RESULTS

EARLY MARKERS FOR PCD IN EPIMASTIGOTES AT THE STATIONARY PHASE OF GROWTH

One of the apoptotic hallmarks is the cell shrinkage that occurs during the early phase of this process. When the volume of an epimastigote population in axenic culture was analyzed by FACS, a marked reduction in the average cell size was found during the transition from the exponential to the stationary phase of growth (Fig. 1A,B). Compared with the mean cell size observed at 4 days of culture, this shrinkage is statistically significant at 7 days of culture that corresponds to the inflexion point between the exponential and the stationary growth phases, reaching a 50% reduction at 14 days (Fig. 1C).

In order to confirm whether volume reduction was related to cell death, the exposition of phosphatidylserine at the cell surface was analyzed at different stages of the proliferative curve. A significant increase in the percentage of parasites positive to annexin V at days 7 and 14 of culture was observed (Fig. 2A,B). These parasites were negative to propidium iodide, showing plasma membrane integrity and excluding necrosis as a mechanism of cell death.

DNA FRAGMENTATION AND MORPHOLOGICAL FEATURES OF APOPTOSIS

Programmed cell death through apoptotic pathways is characterized by morphological and biochemical changes that result from proteases and nucleases activity. Laddering of DNA in oligonucleosomal fractions is one of the final steps in the apoptotic process, together with chromatin condensation, nuclear picnosis and the formation of apoptotic bodies. We then asked whether these characteristics are present in *T. cruzi* epimastigotes at the stationary phase of growth.

As shown in Figure 3A,a,b, at 15 days of culture apoptotic bodies positive to TUNEL assay were observed. Similarly, picnotic nuclei strongly stained by this technique were also evident (Fig. 3A,c,d), revealing DNA fragmentation in these parasites.

In order to establish the kinetics of DNA fragmentation throughout the growth curve, TUNEL assay was performed in epimastigotes from days 1 to 60 of culture. As the cells leave the log phase and enter the stationary phase of growth, the percentage of TUNEL positive cells increases, becoming significant at 13th day (Fig. 3B). Concomitantly, at day 15 DNA laddering was observed (Fig. 3C), being more evident from 30 days on. This result is in agreement with the apoptotic index obtained by TUNEL at the same times (Fig. 3D), dying cells increasing from 24.8% at day 15 to 48% at day 30. Thereafter, the apoptotic index did not increase (Fig. 3D), probably because a maximal death rate was reached.

SERUM DEPRIVATION INDUCES PCD DURING THE EXPONENTIAL PHASE OF GROWTH

We evaluated whether the reduction or the absence of FCS is a sufficient stimulus to induce apoptosis in epimastigotes during the exponential phase of growth. As expected, parasites maintained in 0.5% or in the absence of FCS show a proliferative index significantly lower that in the presence of 10% FCS. Linear regression analysis for the growth curves in the absence of FCS versus 10% FCS indicates a significantly non-zero slope ($F = 25.57$ P-value $= 0.037$; Fig. 4A). When DNA fragmentation was analyzed under these culture conditions, an increase in TUNEL positive parasites was observed (from 2.7 ± 0.7% in 10% FCS to 14.2 ± 0.8% in 0% FCS, Fig. 4B). However, morphological alterations were not as evident as those observed in parasites at the stationary phase of growth (Figs. 3A and 4C).

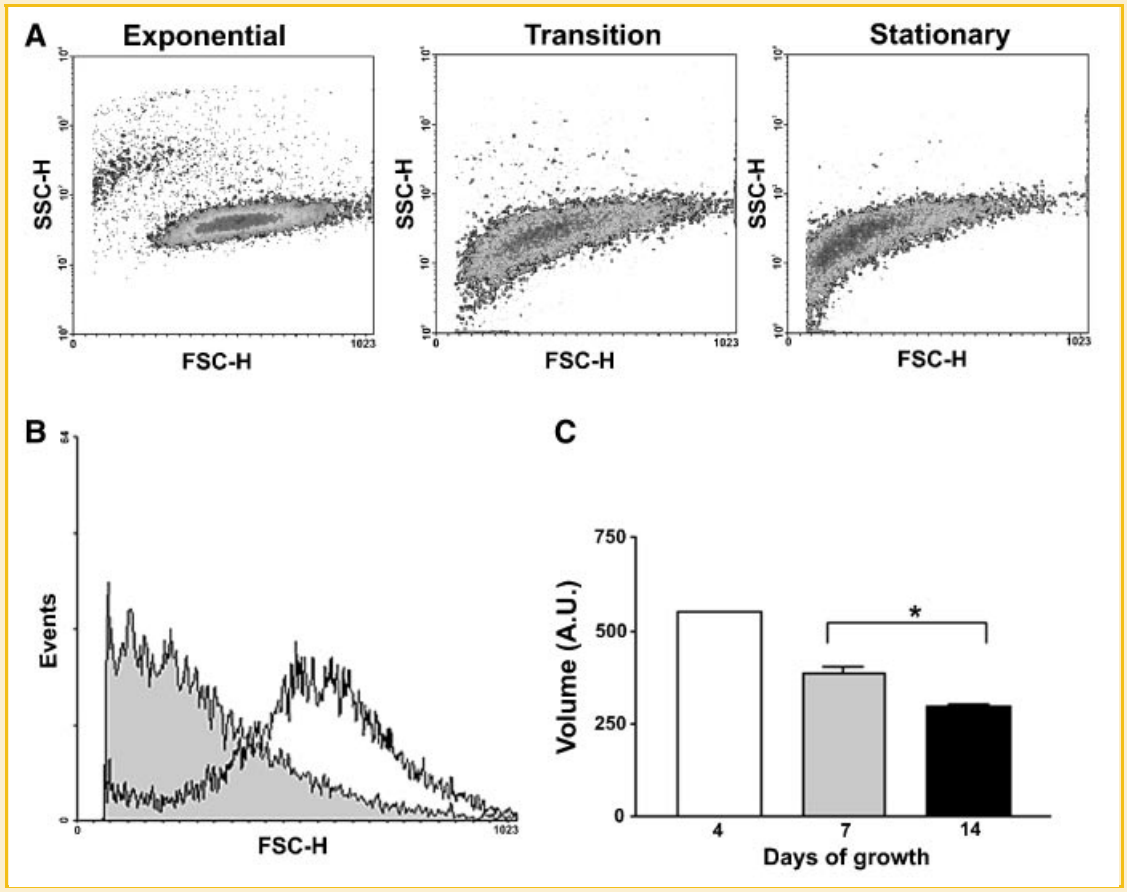

Fig. 1. FACS analysis of *T. cruzi* epimastigotes cell volume populations. Forward light scatter (FSC-H) was considered as function of cell size and side light scatter (SSC-H) as result of cell granularity. A: Density plots for FSC versus SSC in *T. cruzi* epimastigotes at 4, 7, and 14 days of culture. B: Representative FACS histogram showing FSC-H of *T. cruzi* epimastigotes at exponential (black line) and stationary (gray full histogram) growth phases. C: Cell volume estimation (in arbitrary units) in a *T. cruzi* epimastigotes population at different days of growth. Asterisks indicate significant differences between conditions analyzed by ANOVA (*) $P < 0.001$.

These results suggest that *T. cruzi* epimastigotes activate PCD during the stationary phase of growth in axenic cultures and that it can be induced by serum deprivation.

INDUCIBLE PCD BY SERUM DEPRIVATION IN *T. cruzi* EPIMASTIGOTES PRESENTS AN AUTOPHAGIC COMPONENT

The maintenance of both cell morphology (Fig. 4C) and plasma membrane integrity, evaluated by propidium iodide exclusion (data not shown), suggests that necrosis is not involved as a mechanism of death in these parasites. To assess whether an autophagic component is involved in serum deprived *T. cruzi* epimastigotes, parasites were treated with MDC, a fluorescent probe that accumulates in autophagic vacuoles [Biederbick et al., 1995]. As shown in Figure 5A, parasites maintained during 5 days in the absence of serum presented rounded structures that are stained by MDC (black and white arrows). When the MDC signal was measured against total DNA ethidium bromide fluorescence, in parasites maintained in 10% FCS or in the absence of serum (Fig. 5B), a significant increase of MDC staining was observed in the parasites cultured without FCS. This effect can be partially prevented in parasites incubated 24 h with wortmannin (0.2 μM), a potent PI3-kinase inhibitor (Fig. 5B). This enzyme is part of the signaling pathway involved in autophagy regulation [Blommaart et al., 1997], suggesting that increase in the number of MDC-marked vacuoles may be related to this cell death pattern.

On the other hand, parasites cultured in the absence of serum and treated with cycloheximide show a significant decrease in MDC specific activity indicating that protein synthesis is required for autophagic-like vacuole formation (Fig. 5B).

In parasites maintained 5 days in the presence of 10% FCS, the autophagosomal marker LC3 (mammalian microtubule-associated protein light chain 3) was not detected by an immunocytochemical reaction using a polyclonal heterologous antibody (Fig. 5C,a–c). However, parasites grown in total absence of FCS show a strong reaction against this protein, with a punctuated pattern of cytoplasmic and perinuclear distribution (Fig. 5C,g–i).

Accumulation of anti LC3-labeled vesicles can be prevented by adding 0.2 μM wortmannin to the culture medium 24 h before immunolocalization, again suggesting that the autophagic process induced by starvation is regulated by PI3-kinases (Fig. 6C,d–f). Probably, autophagy is one of the processes that operate in parasites during stationary phase of growth, contributing to the increase in mortality by apoptotic-like cell death observed at latter days of culture.

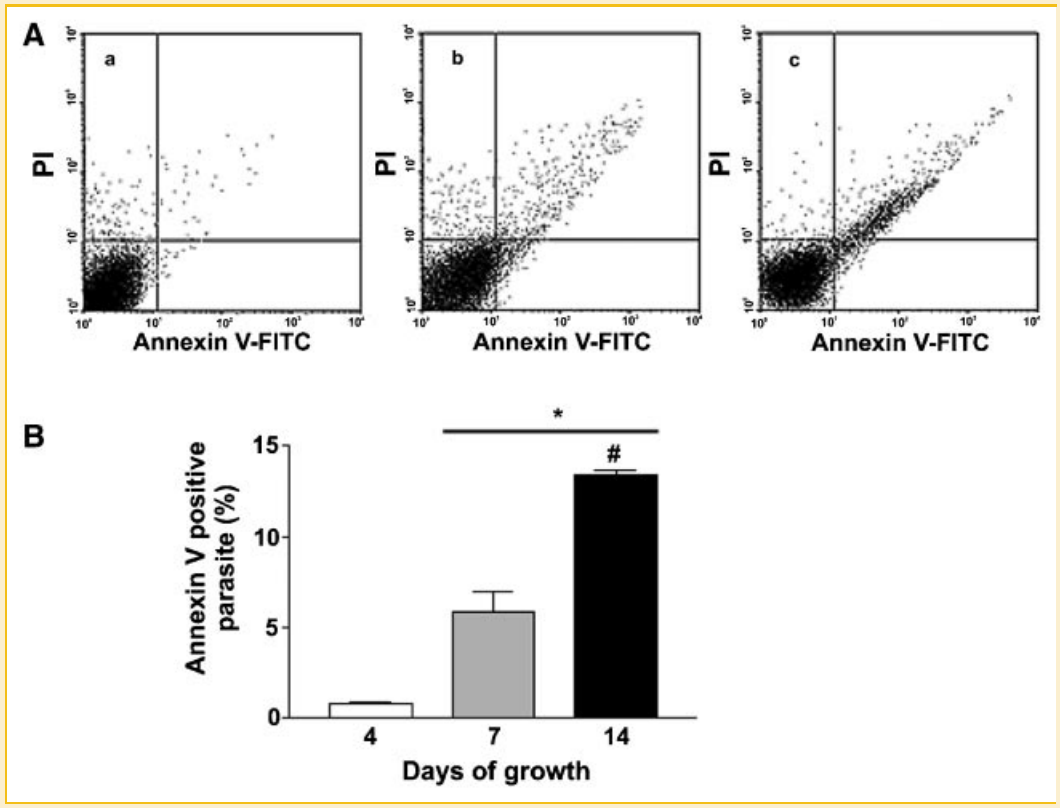

Fig. 2. Analysis of phosphatidylserine (PS) extracellular exposure. A: Representative dot plot of FACS analysis for PS exposure, measured by double staining with Annexin V-FITC and propidium iodide (PI) in *T. cruzi* epimastigotes at 4 (a), 7 (b), and 14 (c) days of growth. B: Quantitative analysis of PS exposure from two independent experiments (in triplicate). * Indicates significant differences between PS labeling at 4, 7, and 14 days of culture ($P < 0.001$, ANOVA). # Indicates statistical differences in the percentages of PS extracellular exposure obtained at 7 and 14 days of growth ($P < 0.001$, ANOVA).

ACTIVITY OF CASPASE-LIKE PROTEINS IN *T. cruzi* EPIMASTIGOTES

Caspase-like activity in parasites at exponential and stationary phase of growth was measured using Ac-DEVD-pNA, a caspase-3 specific substrate. As shown in Figure 6A, at the stationary phase of growth, caspase 3-like activity increases, suggesting that this type of cysteine-protease could be involved in apoptosis-like cell death observed in the parasites.

The presence of caspase 3-like proteins was confirmed by Western blot using a polyclonal heterologous antibodies against human procaspase 3 (Fig. 6B), both in *T. cruzi* Tulahuen and MF strains, presenting the expected molecular mass for *T. cruzi* metacaspase 3 [Kosec et al., 2006]. In addition, two reactive bands whose molecular mass correspond to those predicted for the active fragments of this protein (11 and 17 kDa) were also detected.

Using heterologous polyclonal antibodies against human procaspases 3 and 8, a positive reaction was obtained by immunocytochemistry in *T. cruzi* epimastigotes. The localization of these proteins, with a punctuate pattern of distribution, was restricted to the cytoplasm and excluded completely from nuclei and kinetoplast, as was confirmed by confocal microscopy (data not shown). Immunoreactivity against caspase-like proteins 3 and 8 can also be detected in trypomastigotes pointing to the presence of these enzymes in all cellular forms of the parasite and suggesting that caspase-like proteins could be involved in cell death pathways.

DISCUSSION

Though morphological features of apoptosis are frequently absent in unicellular organisms, there are evidences of PCD in Actinomycetes, Cyanobacteria, yeasts and several protozoa including trypanosomatids [Frohlich and Madeo, 2000; Koonin and Aravind, 2002; Debrabant et al., 2003]. Similarly to *C. elegans*, in protozoa the apoptotic machinery is simpler that in vertebrates, although it maintains the functional homology of proteins involved [Lettre and Hengartner, 2006].

In this work we describe classic parameters associated to apoptosis in *T. cruzi*, similar to those described in pluricellular organisms. Thus, isotonic volume reduction, PS externalization and internucleosomal DNA fragmentation, accompanied by nuclear picnosis and apoptotic bodies' formation were observed in epimastigotes at the stationary phase of growth.

Indeed, significant increases in the number of apoptotic cells were observed at the stationary phase of growth in axenic cultures in diverse cellular species, including trypanosomatids. PCD would contribute to regulate the size of the parasite population, facing restrictive conditions such as medium acidification and the consumption of nutrients, as has been reported in other unicellular organisms [Billaut-Mulot et al., 1996; Chou and Yung, 2001; Lee et al., 2002; Zangger et al., 2002; Mousavi and Robson, 2003].

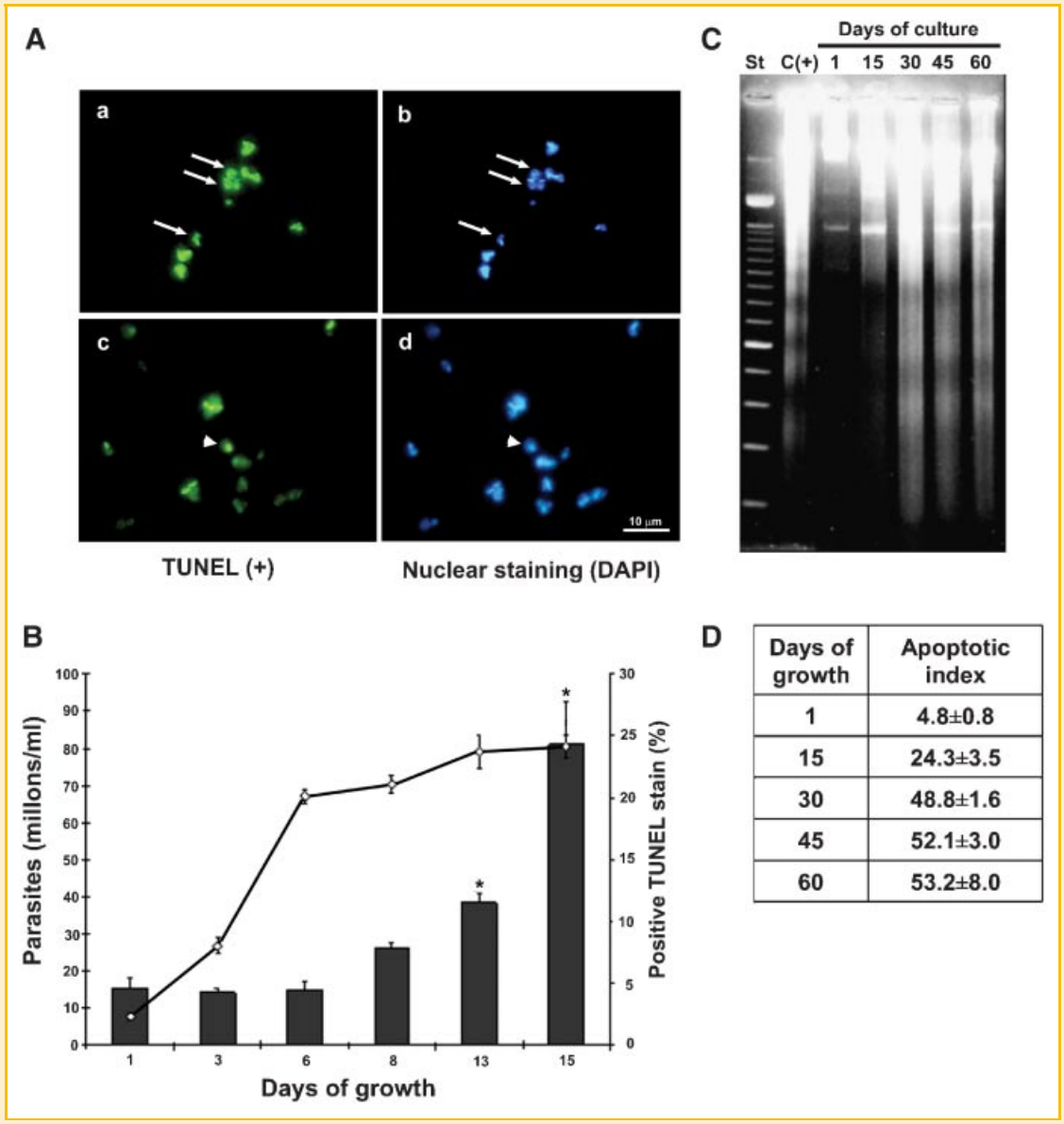

Fig. 3. DNA fragmentation in epimastigotes at different stages of growth. A: TUNEL assay of parasites at 15 days of growth. a,b: Apoptotic bodies TUNEL positive (white arrows) and DAPI, respectively. c,d: Picnotic nuclei TUNEL positive (white head arrow) and DAPI staining. B: Cell proliferation (black line) and TUNEL positive cells (columns) at different points of growth curve. * Statistical differences of TUNEL positive parasites evaluated at 13 days of growth versus previous days ($P < 0.01$; ANOVA). ** Statistical differences between TUNEL positive cells at 15th and previous days of culture ($P < 0.01$; ANOVA). C: DNA laddering agarose gel electrophoresis. St: size marker 100 bp (Invitrogen), C(+): DNA from Jurkat cells at the stationary phase of growth. D: Apoptotic indexes at indicated days of growth. Values correspond to mean ± SEM from two independent experiments. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

Changes in cellular volume associated to apoptosis were related to ion movement through the plasma membrane under isotonic conditions [Gomez-Angelats et al., 2000; Razik and Cidlowski, 2002; Bortner and Cidlowski, 2004]. We have detected a significant reduction in the cell volume average, measured in the total parasite population, during the transition from the log to the stationary phase of growth in *T. cruzi* (Fig. 1). This phenomenon is not due to an increase in trypomastigotes which are slender than epimastigotes, because in the Tulahuen strain the percentage of spontaneous transformation in axenic culture is less than 5%. In addition, the population became more heterogeneous at the stationary phase of growth, with the appearance of spheroid forms that have lost motility, as observed by light microscopy (data not shown).

We have also observed that parasites expose PS at the external side of the plasma membrane when entering the stationary phase of growth (Fig. 2). PS externalization has been observed in other trypanosomatids [Arnoult et al., 2002; Sudhandiran and Shaha, 2003; Sen et al., 2004; Figarella et al., 2005], but had not been previously described in *T. cruzi* epimastigotes. The exposure of this phospholipid in these organisms could also constitute a mechanism

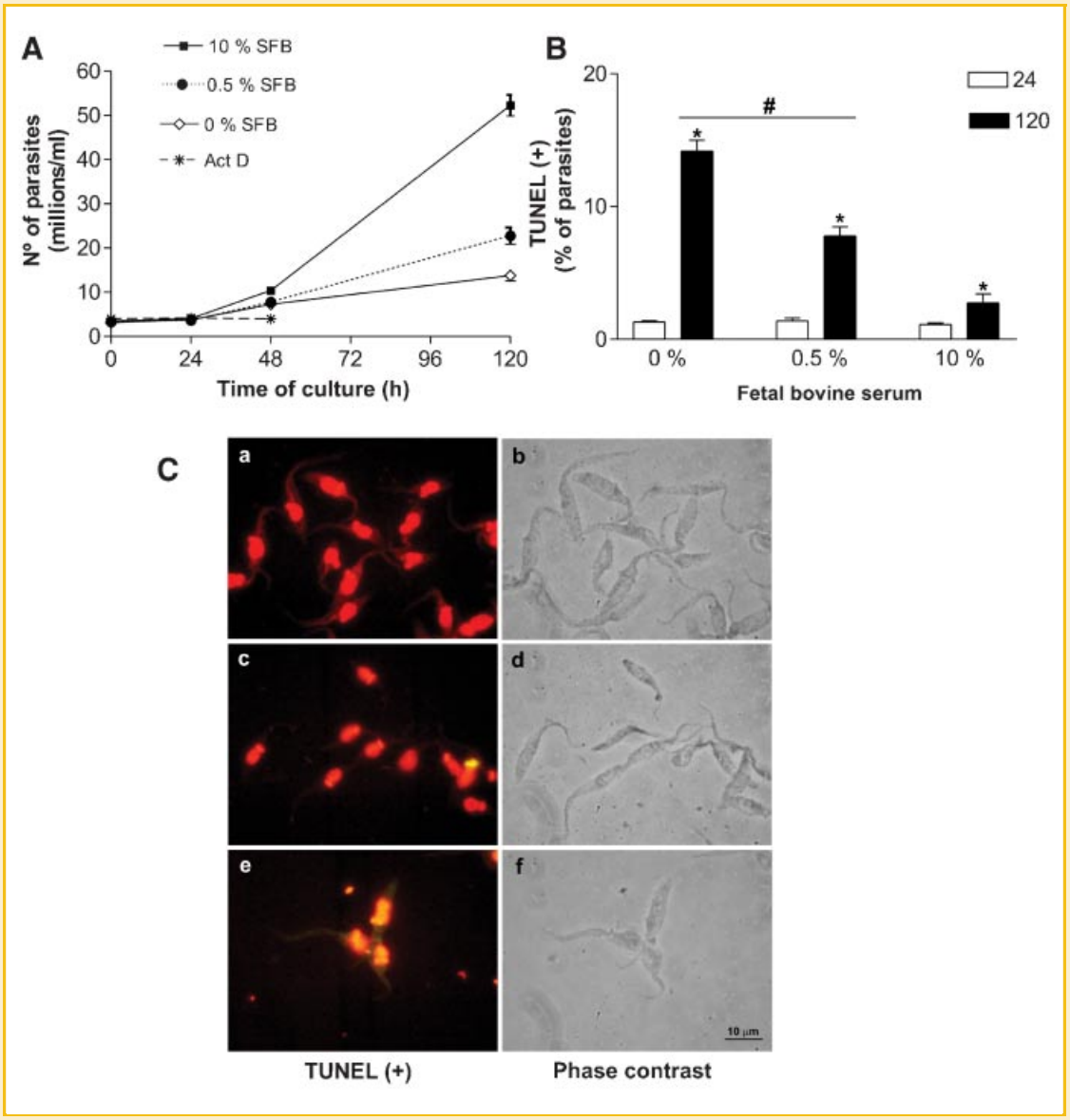

Fig. 4. *T. cruzi* epimastigotes cell death induction by serum deprivation. A: Parasite proliferation under different percentages of FCS. Actinomycin D (10 μg/ml) was added as apoptosis inducer. B: Apoptotic index for *T. cruzi* epimastigotes cultured in the presence or absence of FCS. Values correspond to mean ± SEM from three independent experiments. * Significant differences in the percentage of TUNEL positive cells between 24 and 120 h of culture for each condition ($P < 0.001$, Student's *t*-test). # Statistical differences in apoptotic index at 120 h of growth for parasites cultured with 0% or 0.5% FCS vs. cells grown in the presence of 10% FCS. C: Representative images of TUNEL assay from *T. cruzi* epimastigotes maintained for 120 h under different FCS concentrations. a,b: 10%; c,d: 0.5%; e,f: 0%. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

of immunomodulation and promotion of phagocytosis. Diverse protozoa and helmints control their own apoptosis as a way of modulating the activity of the host's immune system, assuring its survival [James and Green, 2004]. *Leishmania amazonensis* amastigotes expose PS that, through binding to receptors on the surface of macrophages, promotes parasites internalization, diminishing the inflammatory response and favoring the intracellular multiplication of the parasite [de Freitas Balanco et al., 2001]. A similar response has been demonstrated during *T. cruzi* trypomastigotes invasion to macrophages, where PS externalization was evidenced in the infective forms but not in epimastigotes or amastigotes [DaMatta et al., 2007]. This phenomenon may be considered as the induction of apoptosis in some parasites assuring the internalization and multiplication of the remainder population. Another possibility is that PS exposure would not necessarily be indicative of apoptosis but that would constitute a parasitic strategy of "imitation," to promote phagocytosis and to modulate the host immune response [Wanderley et al., 2006]. Our evidences point to the first hypothesis, since the stain with annexin V-FITC is accompanied by several apoptotic characteristics, such as oligonucleosomal DNA fragmentation observed by TUNEL and agarose electrophoresis from the beginning of the stationary phase of growth

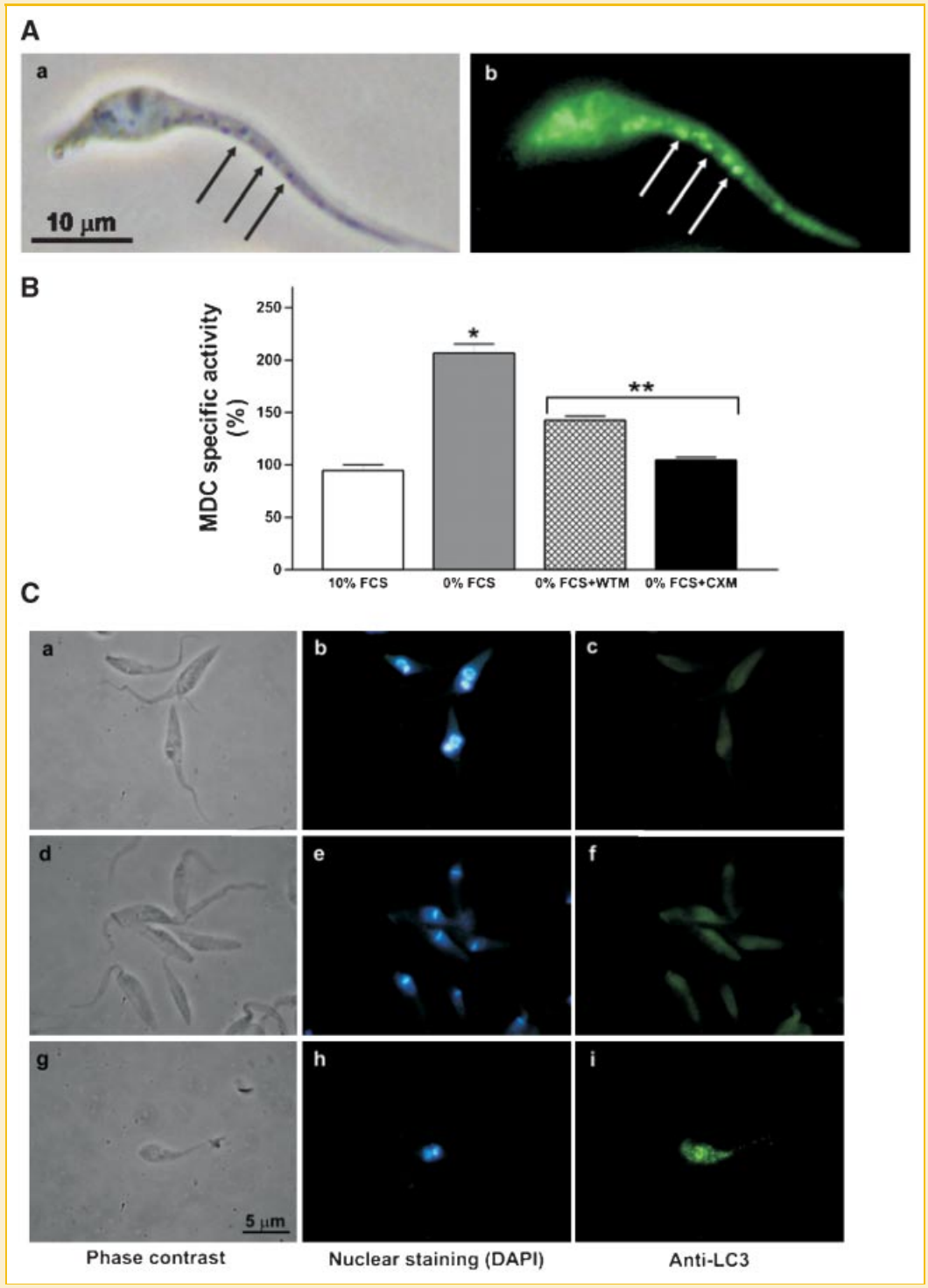

Fig. 5. Autophagic induction by serum deprivation in *T. cruzi* epimastigotes. A: Representative fluorescence image of epimastigotes, maintained 5 days in the absence of fetal calf serum. a: Phase contrast. b: MDC labeling of vacuoles. Arrows indicate the stained autophagic structures. B: MDC-labeling quantification. Parasites cultured in 0% of FCS were treated with 200 nM wortmaninn (WTM) or 2 μg/ml cicloheximide (CXM). MDC stain was measured by fluorescent photometry. Parasites grown with 10% FCS were considered as control. The data represent mean ± SEM of two independent experiments in duplicate. C: Immunocytochemical localization of LC3 in epimastigotes maintained 5 days at the indicate conditions. a–c: parasites in 10% FCS; d–f: epimastigotes grown in the absence of FCS + 200 nM wortmaninn; g–i: parasites in culture medium without FCS. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

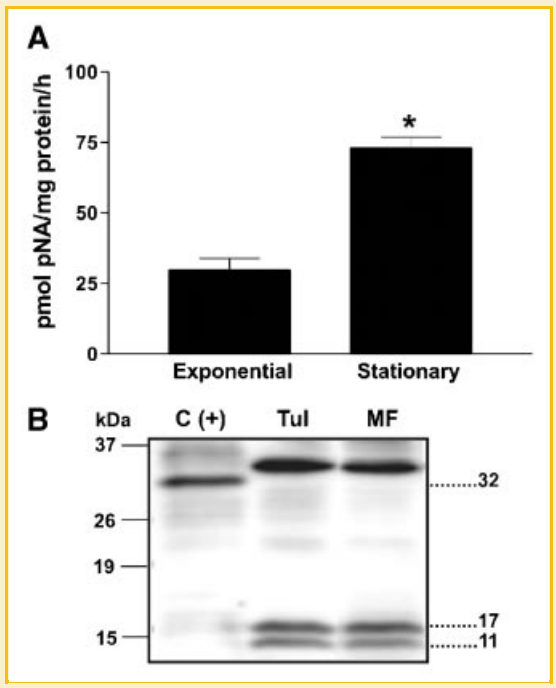

Fig. 6. Caspase 3-like detection. A: Caspase activity in Tulahuen strain epimastigotes at exponential and stationary phases of growth. Bars correspond to caspase-3 like specific activity. * Significant differences between both conditions, $P < 0.001$ ($n = 3$ independent experiments, in duplicate). B: Western blot of caspase 3-like protein in *T. cruzi* epimastigotes at stationary phase of growth. C(+): Total proteins from HeLa cells treated with 2 μg/ml of actinomycin. Tul: total proteins from Tulahuen strain epimastigotes. MF: total proteins from MF strain epimastigotes.

on (Fig. 3). Indeed, the significant lower epimastigote proliferation in 0.5% or in total absence of FCS compared to 10% FCS is accompanied by an increase in DNA fragmentation and in the number of apoptotic cells (Fig. 4).

During cell death induction by serum deprivation, an increase in cytoplasmic vacuolization was observed, a feature that is not present in typical morphological patterns of apoptosis. Indeed, vacuole formation is considered as indicative of other alternative mechanisms of cell death, as necrosis or autophagy [Tsujimoto and Shimizu, 2005]. Autophagy or PCD type II is a well-known physiological process involved in protein turnover, organelle destruction and recycling of materials during starvation [Lockshin and Zakeri, 2004]. Evolutionarily conserved, this mechanism has been documented in ancient organisms such as yeast [Klionsky and Emr, 2000] and unicellular eukaryotes, including Kinetoplastids [Bera et al., 2003; Besteiro et al., 2007]. In protozoa, autophagic pathway seems to be simpler than that described in other cell types, although they have all the essential proteins to carry out this process [Herman et al., 2006]. Recently, Atg4 and Atg8 homologous from *T. cruzi* have been cloned and expressed in yeast, demonstrating the presence and functionality of autophagic pathways in this parasite [Alvarez et al., 2008]. Here we present evidences that serum deprivation produces a significant increase in MDC-labeled vesicles and this effect is partially abrogated by PI3-K inhibitors such as wortmannin. On the other hand, treatment with cycloheximide blocks autophagic vacuole formation indicating that protein synthesis is necessary in at least one of the multiple steps of autophagy (Fig. 5B). At this respect, Klionsky and co-workers have demonstrated that protein synthesis is not necessary to trigger autophagy but for the expansion of autophagosomes [Abeliovich et al., 2000].

In agreement with previously published data [Alvarez et al., 2008], we were able to detect immunoreactivity with polyclonal antibodies against LC3, confirming the presence of orthologues proteins related to Atg8 in *T. cruzi* (Fig. 5C). These data reinforce the idea that PCD has a central role during the life cycle of *T. cruzi*, not only by induction of apoptosis but also through other cell death mechanism that can contribute to the surveillance of the total parasite population. More studies are necessary to elucidate whether autophagy is a protective mechanism that allows trypanosomatids to respond to environmental changes recycling its own proteins or, in opposition, it is a form of self-destruction in response to nutrient scarcity, or both.

Despite morphological and biochemical data about the presence of PCD in unicellular organisms, there is no conclusive evidence respecting to the presence and activity of caspases, necessary to define the mechanism of death. Using synthetic caspase substrates we have detected an increase in caspase-like activity in epimastigotes at the stationary phase of growth, indicating that these enzymes may be involved in some of the apoptotic features described here (Fig. 6A). Recent works report the presence and functionality of metacaspases in *L. major*, *T. brucei*, and *T. cruzi* [Szallies et al., 2002; Helms et al., 2006; Kosec et al., 2006; Gonzalez et al., 2007; Ambit et al., 2008].

At this point, there is enough evidence to conclude that *T. cruzi* epimastigotes activates a PCD process during the transition from the proliferative to the stationary phase of growth in axenic cultures.

Our present efforts are focused on the possibility of stimulating with drugs the natural apoptotic process in *T. cruzi* cells in order to pursue new and specific therapies against Chagas' disease.

ACKNOWLEDGMENTS

We thank Dr. Roberto Docampo and Dr. Arturo Ferreira for helpful discussion and corrections of the manuscript.

REFERENCES

Abeliovich H, Dunn WA, Jr., Kim J, Klionsky DJ. 2000. Dissection of autophagosome biogenesis into distinct nucleation and expansion steps. J Cell Biol 151:1025–1034.

Al-Olayan EM, Williams GT, Hurd H. 2002. Apoptosis in the malaria protozoan, Plasmodium berghei: A possible mechanism for limiting intensity of infection in the mosquito. Int J Parasitol 32:1133–1143.

Alvarez VE, Kosec G, Sant'anna C, Turk V, Cazzulo JJ, Turk B. 2008. Autophagy is involved in nutritional stress response and differentiation in Trypanosoma cruzi. J Biol Chem 283:3454–3464.

Ambit A, Fasel N, Coombs GH, Mottram JC. 2008. An essential role for the Leishmania major metacaspase in cell cycle progression. Cell Death Differ 15:113–122.

Ameisen JC. 1996. The origin of programmed cell death. Science 272:1278–1279.

Ameisen JC, Idziorek T, Billaut-Mulot O, Loyens M, Tissier JP, Potentier A, Ouaissi A. 1995. Apoptosis in a unicellular eukaryote (Trypanosoma cruzi): Implications for the evolutionary origin and role of programmed cell death in the control of cell proliferation, differentiation and survival. Cell Death Differ 2:285–300.

Arnoult D, Tatischeff I, Estaquier J, Girard M, Sureau F, Tissier JP, Grodet A, Dellinger M, Traincard F, Kahn A, Ameisen JC, Petit PX. 2001. On the evolutionary conservation of the cell death pathway: Mitochondrial release of an apoptosis-inducing factor during Dictyostelium discoideum cell death. Mol Biol Cell 12:3016–3030.

Arnoult D, Akarid K, Grodet A, Petit PX, Estaquier J, Ameisen JC. 2002. On the evolution of programmed cell death: Apoptosis of the unicellular eukaryote Leishmania major involves cysteine proteinase activation and mitochondrion permeabilization. Cell Death Differ 9:65–81.

Assuncao GC, Linden R. 2004. Programmed cell deaths. Apoptosis and alternative deathstyles. Eur J Biochem 271:1638–1650.

Bera A, Singh S, Nagaraj R, Vaidya T. 2003. Induction of autophagic cell death in Leishmania donovani by antimicrobial peptides. Mol Biochem Parasitol 127:23–35.

Besteiro S, Williams RA, Coombs GH, Mottram JC. 2007. Protein turnover and differentiation in Leishmania. Int J Parasitol. 37:1063–1075.

Biederbick A, Kern HF, Elsasser HP. 1995. Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. Eur J Cell Biol 66:3–14.

Billaut-Mulot O, Fernandez-Gomez R, Loyens M, Ouaissi A. 1996. Trypanosoma cruzi elongation factor 1-alpha: Nuclear localization in parasites undergoing apoptosis. Gene 174:19–26.

Blommaart EF, Krause U, Schellens JP, Vreeling-Sindelarova H, Meijer AJ. 1997. The phosphatidylinositol 3-kinase inhibitors wortmannin and LY294002 inhibit autophagy in isolated rat hepatocytes. Eur J Biochem 243:240–246.

Bortner CD, Cidlowski JA. 2004. The role of apoptotic volume decrease and ionic homeostasis in the activation and repression of apoptosis. Pflugers Arch 448:313–318.

Chou CC, Yung BY. 2001. Increased stability of nucleophosmin/B23 in anti-apoptotic effect of ras during serum deprivation. Mol Pharmacol 59:38–45.

DaMatta RA, Seabra SH, Deolindo P, Arnholdt AC, Manhaes L, Goldenberg S, de SW. 2007. Trypanosoma cruzi exposes phosphatidylserine as an evasion mechanism. FEMS Microbiol Lett 266:29–33.

Danial NN, Korsmeyer SJ. 2004. Cell death: Critical control points. Cell 116: 205–219.

Das M, Mukherjee SB, Shaha C. 2001. Hydrogen peroxide induces apoptosis-like death in Leishmania donovani promastigotes. J Cell Sci 114:2461–2469.

de Freitas Balanco JM, Moreira ME, Bonomo A, Bozza PT, marante-Mendes G, Pirmez C, Barcinski MA. 2001. Apoptotic mimicry by an obligate intracellular parasite downregulates macrophage microbicidal activity. Curr Biol 11:1870–1873.

Debrabant A, Lee N, Bertholet S, Duncan R, Nakhasi HL. 2003. Programmed cell death in trypanosomatids and other unicellular organisms. Int J Parasitol 33:257–267.

Diamond LS. 1968. Improved method for the monoxenic cultivation of Entamoeba histolytica Schaudinn, 1903 and E. histolytica-like amebae with trypanosomatids. J Parasitol 54:715–719.

Dirsch VM, Stuppner H, Vollmar AM. 2001. Helenalin triggers a CD95 death receptor-independent apoptosis that is not affected by overexpression of Bcl-x(L) or Bcl-2. Cancer Res 61:5817–5823.

Figarella K, Rawer M, Uzcategui NL, Kubata BK, Lauber K, Madeo F, Wesselborg S, Duszenko M. 2005. Prostaglandin D2 induces programmed cell death in Trypanosoma brucei bloodstream form. Cell Death Differ 12:335–346.

Freire-de-Lima CG, Nascimento DO, Soares MB, Bozza PT, Castro-Faria-Neto HC, de Mello FG, DosReis GA, Lopes MF. 2000. Uptake of apoptotic cells drives the growth of a pathogenic trypanosome in macrophages. Nature 403: 199–203.

Frohlich KU, Madeo F. 2000. Apoptosis in yeast—A monocellular organism exhibits altruistic behaviour. FEBS Lett 473:6–9.

Gomez-Angelats M, Bortner CD, Cidlowski JA. 2000. Protein kinase C (PKC) inhibits fas receptor-induced apoptosis through modulation of the loss of K+ and cell shrinkage. A. role for PKC upstream of caspases. J Biol Chem 275: 19609–19619.

Gonzalez IJ, Desponds C, Schaff C, Mottram JC, Fasel N. 2007. Leishmania major metacaspase can replace yeast metacaspase in programmed cell death and has arginine-specific cysteine peptidase activity. Int J Parasitol 37:161–172.

Helms MJ, Ambit A, Appleton P, Tetley L, Coombs GH, Mottram JC. 2006. Bloodstream form Trypanosoma brucei depend upon multiple metacaspases associated with RAB11-positive endosomes. J Cell Sci 119:1105–1117.

Hengartner MO. 2000. The biochemistry of apoptosis. Nature 407:770–776.

Herman M, Gillies S, Michels PA, Rigden DJ. 2006. Autophagy and related processes in trypanosomatids: Insights from genomic and bioinformatic analyses. Autophagy 2:107–118.

James ER, Green DR. 2004. Manipulation of apoptosis in the host-parasite interaction. Trends Parasitol 20:280–287.

Kerr JF, Wyllie AH, Currie AR. 1972. Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. Br J Cancer 26:239–257.

Klionsky DJ, Emr SD. 2000. Autophagy as a regulated pathway of cellular degradation. Science 290:1717–1721.

Koonin EV, Aravind L. 2002. Origin and evolution of eukaryotic apoptosis: The bacterial connection. Cell Death Differ 9:394–404.

Kosec G, Alvarez VE, Aguero F, Sanchez D, Dolinar M, Turk B, Turk V, Cazzulo JJ. 2006. Metacaspases of Trypanosoma cruzi: Possible candidates for programmed cell death mediators. Mol Biochem Parasitol 145:18–28.

Kroemer G, Martin SJ. 2005. Caspase-independent cell death. Nat Med 11: 725–730.

Lee N, Bertholet S, Debrabant A, Muller J, Duncan R, Nakhasi HL. 2002. Programmed cell death in the unicellular protozoan parasite Leishmania. Cell Death Differ 9:53–64.

Lettre G, Hengartner MO. 2006. Developmental apoptosis in C. elegans: A complex CEDnario. Nat Rev Mol Cell Biol 7:97–108.

Lockshin RA, Zakeri Z. 2004. Apoptosis, autophagy, and more. Int J Biochem Cell Biol 36:2405–2419.

Maudlin I, Welburn SC. 1994. Maturation of trypanosome infections in tsetse. Exp Parasitol 79:202–205.

Mousavi SA, Robson GD. 2003. Entry into the stationary phase is associated with a rapid loss of viability and an apoptotic-like phenotype in the opportunistic pathogen Aspergillus fumigatus. Fungal Genet Biol 39:221–229.

Munafo DB, Colombo MI. 2001. A novel assay to study autophagy: Regulation of autophagosome vacuole size by amino acid deprivation. J Cell Sci 114:3619–3629.

Razik MA, Cidlowski JA. 2002. Molecular interplay between ion channels and the regulation of apoptosis. Biol Res 35:203–207.

Sen N, Das BB, Ganguly A, Mukherjee T, Bandyopadhyay S, Majumder HK. 2004. Camptothecin-induced imbalance in intracellular cation homeostasis regulates programmed cell death in unicellular hemoflagellate Leishmania donovani. J Biol Chem 279:52366–52375.

Sperandio S, de BI, Bredesen DE. 2000. An alternative, nonapoptotic form of programmed cell death. Proc Natl Acad Sci USA 97:14376–14381.

Sperandio S, Poksay K, de BI, Lafuente MJ, Liu B, Nasir J, Bredesen DE. 2004. Paraptosis: Mediation by MAP kinases and inhibition by AIP-1/Alix. Cell Death Differ 11:1066–1075.

Sudhandiran G, Shaha C. 2003. Antimonial-induced increase in intracellular Ca2+ through non-selective cation channels in the host and the parasite is responsible for apoptosis of intracellular Leishmania donovani amastigotes. J Biol Chem 278:25120–25132.

Szallies A, Kubata BK, Duszenko M. 2002. A metacaspase of Trypanosoma brucei causes loss of respiration competence and clonal death in the yeast Saccharomyces cerevisiae. FEBS Lett 517:144–150.

Tsujimoto Y, Shimizu S. 2005. Another way to die: Autophagic programmed cell death. Cell Death Differ 12(Suppl 2):1528–1534.

Wanderley JL, Moreira ME, Benjamin A, Bonomo AC, Barcinski MA. 2006. Mimicry of apoptotic cells by exposing phosphatidylserine participates in the establishment of amastigotes of Leishmania (L) amazonensis in mammalian hosts. J Immunol 176:1834–1839.

Welburn SC, Maudlin I, Ellis DS. 1989. Rate of trypanosome killing by lectins in midguts of different species and strains of Glossina. Med Vet Entomol 3:77–82.

Welburn SC, Barcinski MA, Williams GT. 1997. Programmed cell death in trypanosomatids. Parasitol Today 13:22–26.

WHO Expert Committee. 2002. Control of Chagas disease. World Health Organ Tech. Rep. Ser. 905:i-vi, 1-109, back cover.:i-109, back.

Zangger H, Mottram JC, Fasel N. 2002. Cell death in Leishmania induced by stress and differentiation: Programmed cell death or necrosis? Cell Death Differ 9:1126–1139.