

In vivo programmed cell death of *Entamoeba histolytica* trophozoites in a hamster model of amoebic liver abscess

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Entamoeba histolytica trophozoites can induce host cell apoptosis, which correlates with the virulence of the parasite. This phenomenon has been seen during the resolution of an inflammatory response and the survival of the parasites. Other studies have shown that *E. histolytica* trophozoites undergo programmed cell death (PCD) *in vitro*, but how this process occurs within the mammalian host cell remains unclear. Here, we studied the PCD of *E. histolytica* trophozoites as part of an *in vivo* event related to the inflammatory reaction and the host–parasite interaction. Morphological study of amoebic liver abscesses showed only a few *E. histolytica* trophozoites with peroxidase-positive nuclei identified by terminal deoxynucleotidyltransferase enzyme-mediated dUTP nick end labelling (TUNEL). To better understand PCD following the interaction between amoebae and inflammatory cells, we designed a novel *in vivo* model using a dialysis bag containing *E. histolytica* trophozoites, which was surgically placed inside the peritoneal cavity of a hamster and left to interact with the host's exudate components. Amoebae collected from bags were then examined by TUNEL assay, fluorescence-activated cell sorting (FACS) and transmission electron microscopy. Nuclear condensation and DNA fragmentation of *E. histolytica* trophozoites were observed after exposure to peritoneal exudates, which were mainly composed of neutrophils and macrophages. Our results suggest that production of nitric oxide by inflammatory cells could be involved in PCD of trophozoites. In this modified *in vivo* system, PCD appears to play a prominent role in the host–parasite interaction and parasite cell death.

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INTRODUCTION

Entamoeba histolytica is the aetiological agent of amoebic dysentery and its main extra-intestinal complication, amoebic liver abscess (ALA). The infection constitutes an important public health problem worldwide, especially in developing countries. The mechanisms by which this parasite causes intestinal and liver tissue damage have been largely defined using various methods, which have been facilitated by the development of different *in vivo* experimental models of amoebiasis (Tsutsumi & Shibayama, 2006). In the last two decades, multiple studies have been conducted to understand the molecular basis of

host–parasite interactions (Ravdin *et al.*, 1989; Leippe *et al.*, 1991; Que & Reed, 2000). The virulence of *E. histolytica* trophozoites has been considered a multifactorial process governed by specific interactions at the cellular and molecular levels. These host–parasite interactions take place in a series of sequential steps, including adherence (Petri *et al.*, 1987), contact-dependent cytolysis (Huston *et al.*, 2000) and phagocytosis (Orozco *et al.*, 1983). These steps allow the parasite to invade and damage host tissues, as well as to evade detection by the immune system.

Programmed cell death (PCD), or apoptosis, has been reported in all multicellular invertebrate and vertebrate lineages; however, various forms of regulated PCD have now been described in unicellular organisms, the phylogenetic divergence of which is believed to have occurred between two million and one billion years ago (Ameisen, 2002). PCD is an essential part of cell biology and is

Abbreviations: ALA, amoebic liver abscess; FACS, fluorescence-activated cell sorting; NO, nitric oxide; NOS, nitric oxide synthase; PCD, programmed cell death; PI, propidium iodide; TUNEL, terminal deoxynucleotidyltransferase enzyme-mediated dUTP nick end labelling.

thought to be involved in growth regulation and development of multicellular organisms. In unicellular organisms, in addition to eliminating deficient mutant individuals from the community, PCD could also be involved in maintaining the optimal population density to facilitate organization into cell populations and establish patterns of intercellular communication (Palková *et al.*, 1997). During the course of evolution, it seems that PCD has also changed gradually, and throughout this process, caspases and apoptosis-inducing factors appeared before other proteins, such as death receptors (Gordeeva *et al.*, 2004). Some studies have reported the occurrence of PCD in unicellular organisms, such as bacteria (Sat *et al.*, 2001), yeast (Ludovico *et al.*, 2002), trypanosomatids (Nguewa *et al.*, 2004), *Plasmodium berghei* (Al-Olayan *et al.*, 2002), *Peridinium gatunense* (Vardi *et al.*, 1999), *Dictyostelium discoideum* (Cornillon *et al.*, 1994), *Tetrahymena* (Davis *et al.*, 1992), *Blastocystis hominis* (Nasirudeen *et al.*, 2004) and, more recently, *E. histolytica* (Ramos *et al.*, 2007; Villalba *et al.*, 2007). Several notions of how amoebae kill target cells *in vitro* have been proposed, but the *in vivo* cytotoxic mechanisms have not yet been determined.

The ability of *E. histolytica* to kill multiple host cells correlates with parasite virulence. There are certainly many factors involved in these processes. Currently, there is evidence suggesting roles for Gal/GalNac lectin subunits (Petri *et al.*, 2002), amoebapores (Leippe *et al.*, 1991) and cysteine proteinases (Que & Reed, 2000). Huston *et al.* (2000) demonstrated rapid caspase 3-dependent apoptosis of Jurkat leukaemia T cells killed by amoebic trophozoites *in vitro*. More evidence of a role for apoptosis in *E. histolytica* infection was obtained by treating mice with Z-VAD-fmk, a pan-caspase inhibitor, which yielded protection from ALA formation, showing a direct involvement of apoptosis in *E. histolytica* infection (Yan & Stanley, 2001). However, further *in vivo* studies are needed to provide insight into how effector cells induce *E. histolytica* apoptosis and why this is an important event in the damage caused by the amoeba.

Studies reported more than 20 years ago (Tsutsumi *et al.*, 1984; Tsutsumi & Martínez-Palomo, 1988) suggested the importance of inflammatory reactions in *E. histolytica* infection pathophysiology in hamster livers. In the sequential morphological analysis of tissue lesions, host cell damage has been associated with previous leukocyte lysis caused by the parasites. Based on these early reports, several *in vivo* studies have focused on determining the molecules involved in host cell death, which include proteases (Que & Reed, 2000), amoebapores (Bruhn *et al.*, 2003), nitric oxide (Pacheco-Yépez *et al.*, 2001) and cytokines (Sharma *et al.*, 2005). However, during the destruction of host tissue, *E. histolytica* trophozoites also die, although little attention has been paid to the process of parasite death, and whether PCD occurs in the amoebae.

We have previously demonstrated that PCD of *E. histolytica* trophozoites can be induced *in vitro* (Villalba *et al.*, 2007). In an attempt to determine if PCD also occurs

in vivo, we designed a novel model of cellulose membrane dialysis bags containing *E. histolytica* trophozoites. The bags were placed in the peritoneal cavity of hamsters, expecting that an interchange of soluble substances between host and parasite would take place. The model was developed to determine if the cellular immune response of the host contributes to the damage caused by *E. histolytica* trophozoites by releasing products that induce trophozoite PCD.

METHODS

Amoeba culture and maintenance. Trophozoites of *E. histolytica* strain HM1:IMSS, grown axenically in TYI-S-33 medium (Diamond *et al.*, 1978), were passed several times through hamster livers to increase their virulence, which was determined by the size of liver abscesses produced. Virulent trophozoites were harvested at the end of the exponential growth phase (72 h) by chilling at 4 °C and were used in all experiments.

Animals. Male golden hamsters (*Mesocricetus auratus*), aged 10–12 weeks with a mean weight of 100 g, were intraperitoneally injected with 1 ml commercial mineral oil 1 week before placing the dialysis bag into the peritoneal cavity. The mineral oil stimulated and increased the peritoneal inflammatory exudates. All animals used in this study were handled in accordance with the guidelines of the Institutional Animal Care and Use Committee, with a previous approval of the protocol by the institutional committee (IACUC; ID 423-08). Our institution fulfils all the technical specifications for the production, care and use of laboratory animals and is certified by national law (NOM-062-ZOO-1999). All hamsters were killed by an overdose of sodium pentobarbital at the end of the experiments and were handled according to the guidelines of the 2000 AVMA Panel of Euthanasia.

Amoebic liver abscess formation. For ALA production, 10⁶ *E. histolytica* trophozoites in the exponential phase of growth were directly inoculated into the left lobe of the hamster liver in a volume of 200 µl culture medium as previously described (Tsutsumi *et al.*, 1984). Fragments of liver from the lesion areas were taken at different times (30 min, 1, 3, 6, 9, 12, 24, 48 and 72 h, and 7 days). Tissues were fixed in 4% (w/v) paraformaldehyde in PBS. After embedding in paraffin, 4–6 µm sections were mounted on silanized glass slides and processed for terminal deoxynucleotidyltransferase enzyme-mediated dUTP nick end labelling (TUNEL) staining.

Preparation and implantation of dialysis bags. Symmetrical, cylindrical fragments of dialysis cellulose membrane (50 mm long and 7.5 mm in diameter), which were permeable for the passage of molecules up to 25 kDa (Spectrum Laboratories), were prepared under sterile conditions. After closing one end of the membrane with silk thread no. 001, 4 × 10⁶ trophozoites were added to the bag in a total volume of 1 ml medium. The opposite side of the bag was similarly closed. These bags were maintained in sterile Petri dishes with culture medium until use (Fig. 1). To test in an *in vitro* system whether the trophozoites would remain viable inside the dialysis bags, we inoculated amoebae into 120 ml glass bottles filled with Diamond culture medium supplemented with serum, which were incubated at 37 °C. Amoebae were recovered at different times for determination of cell viability and TUNEL assays. Cells remained viable from 3 to 6 h; after this time (12 and 24 h), parasite viability decreased progressively (85%).

Surgical procedure. Animals were anaesthetized intraperitoneally with sodium pentobarbital (Anestesia; Smith Kline) at a dose of

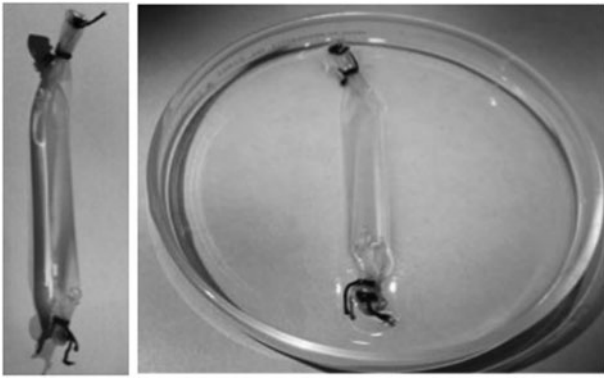


Fig. 1. The dialysis bag model. A cylindrical dialysis bag prepared with cellulose membrane containing trophozoites (4×10^6 cells ml^{-1}) is placed into the hamster peritoneal cavity, where it remains for 3 or 6 h.

4.72 mg per 100 g body weight. After a mid-longitudinal incision of the abdominal wall, the dialysis bag containing amoebae was carefully introduced into the peritoneal cavity, and the abdominal wall was closed under aseptic conditions for 3 or 6 h. For negative control animals, the dialysis bag was filled only with culture medium. To recover the inflammatory cells prior to killing the animal and to obtain the dialysis bag, the hamster was injected intraperitoneally with 10 ml RPMI medium, and the abdomen was massaged for 30 s to stimulate the production of peritoneal exudate. Through a small incision in the abdominal wall, the tip of a Pasteur pipette was used to collect most of the peritoneal liquid, which was washed twice in PBS. Suspensions of inflammatory cells were maintained in RPMI at 37 °C until use. The animals were killed 3 or 6 h after exposing the dialysis bag to the peritoneal liquid environment. The recovered bag was immediately placed in PBS, and *E. histolytica* trophozoite viability was determined by trypan blue exclusion before performing morphological analyses and other tests, including TUNEL and transmission electron microscopy.

Detection of DNA fragmentation. TUNEL to detect DNA fragmentation was carried out using an assay kit according to the manufacturer's instructions and as described previously (Villalba *et al.*, 2007). Briefly, cells were fixed in 4% (w/v) paraformaldehyde, permeabilized with 0.2% (v/v) Tween 20 for 10 min at room temperature and incubated in buffer containing a nucleotide mixture (50 μM fluorescein-12-dUTP, 100 μM dATP, 10 mM Tris/HCl, pH 8.0, 1 mM EDTA, pH 7.6) for 1 h at 37 °C. Finally, cells were counter-stained with propidium iodide (PI) and observed with a confocal microscope (Olympus FV500) with a dual-pass FITC/PI filter set. In a second attempt to detect PCD in *E. histolytica* trophozoites present in ALA, we used a TUNEL-mediated peroxidase nick end labelling technique, which was performed according to the recommended protocol for the ApopTag Peroxidase *In situ* Apoptosis Detection kit (Chemicon).

Flow cytometry analysis. Cells (1×10^6) were analysed on a flow cytometer (FACSCalibur; Becton Dickinson) equipped with a 15 mV, 488 nm air-cooled argon ion laser. Trophozoites were detected and analysed according to their relative fluorescence intensities compared with unstained cells. Analyses were performed on 10 000 gate events, and numerical data were processed using WinMDI shareware. Data for TUNEL and PI staining data are depicted as histograms. All results are representative of three experiments.

Ultrastructure. For electron microscopy, trophozoites from dialysis bags and peritoneal exudates from 3 and 6 h were harvested and washed twice with 0.1 M sodium cacodylate buffer and fixed for 1 h with 2.5% (w/v) glutaraldehyde solution prepared in the same buffer. Samples were then post-fixed with 1% (w/v) osmium tetroxide, dehydrated with ethanol and propylene oxide and embedded in epoxy resin. Semi-thin sections (0.5 μm) were stained with toluidine blue for light microscopy analysis. Thin sections (60–90 nm) were contrasted with uranyl acetate and lead citrate and observed under a Zeiss EM-910 transmission electron microscope.

Determination of protein patterns. Protein patterns were determined by SDS-PAGE. Briefly, crude extracts from each condition were prepared in lysis buffer (0.05 M Tris, 0.15 M NaCl) containing 20 mM *p*-hydroxymercuribenzoate, 10 mM *N*-ethylmaleimide, 3 mM PMSF, 3 mM EDTA, 3 mM tosyl-L-phenylalanine chloromethyl ketone and 3 mM iodoacetamide (Sigma Aldrich). Cells were disrupted by five freeze-thaw cycles, and protein quantification of each condition was done by the Bradford method (Bradford, 1976). Finally, 50 μg protein from each condition was loaded per well. SDS-PAGE was performed with 10% (w/v) SDS at constant voltage (100 V) for 1 h. Gels were stained with 0.5% (w/v) Coomassie brilliant blue R-250 for 30 min. The relative density of the bands was determined using ImageJ software.

Nitrite and nitrate determination. To prepare Griess reagent, equal volumes of *N*-(1-naphthyl)ethylenediamine and sulphanilic acid were mixed. By using a microplate (300 μl per well), 50 μl Griess reagent, 150 μl of the nitrite-containing sample and 100 μl deionized water were mixed and incubated for 10 min at room temperature. A photometric reference sample was prepared by mixing 20 μl Griess reagent and 280 μl deionized water. The absorbance of nitrite-containing samples was measured in a spectrophotometric microplate reader at a wavelength of 570 nm. A standard curve generated using an aqueous sodium nitrite solution from 2.5 to 100 μM was used as a reference.

RESULTS

Examination of ALA development

As a first attempt to find *E. histolytica* trophozoites with PCD characteristics, we reproduced the different stages of ALA development, as reported by Tsutsumi *et al.* (1984). Samples of the infected liver were obtained from animals killed at different times post-infection ranging from 30 min to 7 days. The livers were then processed for histology. Light microscopy results were similar to those reported previously, but in the present study we intentionally focused our search on apoptotic features at all analysed times. We first observed parasite changes possibly related to PCD, such as cell shape changes and shrunken nuclei, at 1 h, when one or several layers of polymorphonuclear leukocytes and mononuclear cells were seen surrounding the trophozoites (Fig. 2a, b). To determine whether *E. histolytica* trophozoites died by PCD in ALA, we performed a TUNEL assay, as has previously been carried out in a mouse model of disease (Seydel & Stanley, 1998). A significant number of hepatocytes and inflammatory cells stained TUNEL-positive in areas associated with ALA, and these were more pronounced when in close proximity to amoebic trophozoites (Fig. 2c, d). Some parenchymal

areas farther away from ALA showed TUNEL-positive labelling as well (Fig. 2e). When TUNEL with a FITC label was used, we were unable to detect positive trophozoites, although granular and rounded cells were regarded as dead cells. To determine clearly whether *E. histolytica* trophozoites were dying by PCD in ALA, we performed a peroxidase-labelled TUNEL technique on the same sections. Light microscopy analysis showed positive parasites at 3 days post-infection (Fig. 2f). Collectively, the above data suggest that apoptotic death can occur *in vivo* in *E. histolytica* trophozoites.

Viability of *E. histolytica* trophozoites from dialysis bags and inflammatory cells from the peritoneal cavity

After establishing that PCD can occur in the parasite during ALA development, we sought an alternative way to

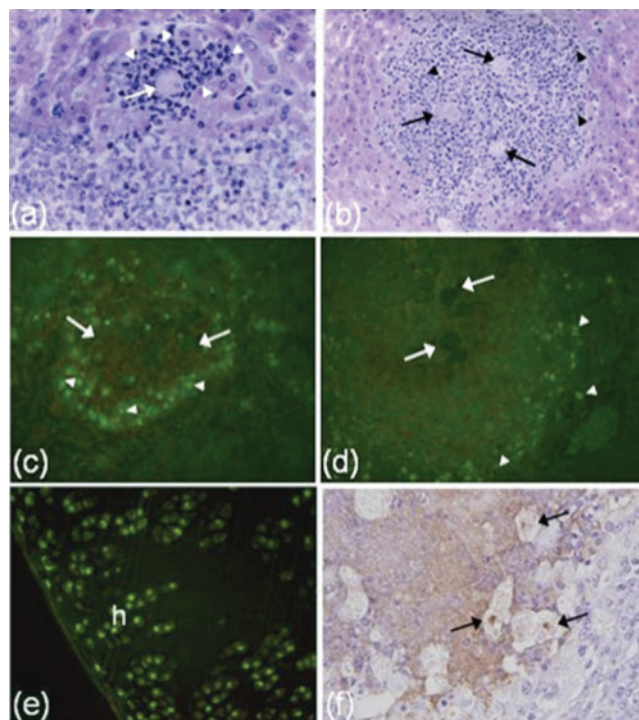


Fig. 2. Microscopic analysis of ALA production. (a) At 6 h after amoebic inoculation, a trophozoite (arrow) is surrounded by several layers of inflammatory cells (arrowheads). Haematoxylin and eosin stain. (b) At 12 h, liver parenchyma shows a major inflammatory infiltrate. Trophozoites (arrows) are localized within the area of inflammatory reaction (arrowheads). Haematoxylin and eosin stain. (c, d) TUNEL-FITC staining of a liver section including an ALA (12 h). Note the intense positive labelling of inflammatory cells (arrowheads) surrounding amoebae (arrows). (e) Hepatocytes (h) near an ALA also show TUNEL-FITC staining. (f) TUNEL-peroxidase assay shows positive *E. histolytica* trophozoite nuclei (arrows) 3 days after intrahepatic inoculation of amoebae. Images acquired at magnification of $\times 40$ (a, c–f) or $\times 20$ (b).

determine if the interaction of *E. histolytica* trophozoites with cells of the immune system or soluble mediators of immunity triggered apoptosis. Parasites were placed into dialysis bags (prepared as described in Methods), which prevented their spread into the peritoneal cavity (Fig. 1), and incubated for 3 or 6 h. In the negative control using dialysis bags filled with culture medium, no changes were observed in the peritoneal cells (data not shown). A differential leukocyte count of peritoneal exudates was performed by light microscopy analysis of multiple semithin sections stained with toluidine blue. The majority ($\geq 75\%$) comprised polymorphonuclear neutrophils with typical multilobular nuclei. The proportion of mononuclear macrophages showing distinctive horseshoe-shaped nuclei was about 25–30%. These results were confirmed by Wright's staining. Likewise, under light microscopy, some amoebae had the typical pleomorphic appearance seen in axenically cultured trophozoites. Trophozoites obtained from dialysis bags had a distinctive round appearance with large cytoplasmic vesicles with microfibrillar content (Fig. 3). To distinguish between necrosis and apoptosis, we determined cell membrane integrity. The plasma membrane changes that were induced in parasites after contact with the peritoneal cells were monitored by time-course experiments. Trypan blue exclusion after incubation with parasites showed 90% viability for 6 h, and similar results were obtained with freshly isolated inflammatory cells. In a separate experiment aiming to determine whether the reduced space inside the dialysis bag was itself a factor that induced death, we introduced a dialysis bag with amoebae into glass bottles filled with culture medium and determined the viability of cells recovered at 3 and 6 h by trypan blue exclusion. The limited space was not involved in the induction of cell death (data not shown). All data were also confirmed by flow cytometry using PI.

Cleavage of DNA

TUNEL staining was used to determine if DNA degradation occurred in the parasites. By this procedure, we observed that many *E. histolytica* trophozoites contained in the dialysis bag after 3 and 6 h of incubation showed positive labelling, which was confirmed by confocal microscopy in combination with fluorescence-activated cell sorting (FACS) analysis. Confocal microscopy revealed that TUNEL-positive cells indicative of massive DNA breaks constituted 90% of the inflammatory cells and 70% of the parasites at 6 h (Fig. 4). These results were verified by FACS analysis, in which the fluorescent signals were quantified in both amoebae and immune cells that were subjected to the TUNEL assay. For these studies 10 000 events were acquired from the positive labelled region. The peritoneal cells were 75 and 98% positive for TUNEL staining at 3 and 6 h, respectively, compared with 16% of control cells. TUNEL-positive amoebae represented 52% and 86% of the cell population at 3 and 6 h, respectively, compared with 8% of amoebae from axenic culture (Fig. 5). This phenomenon

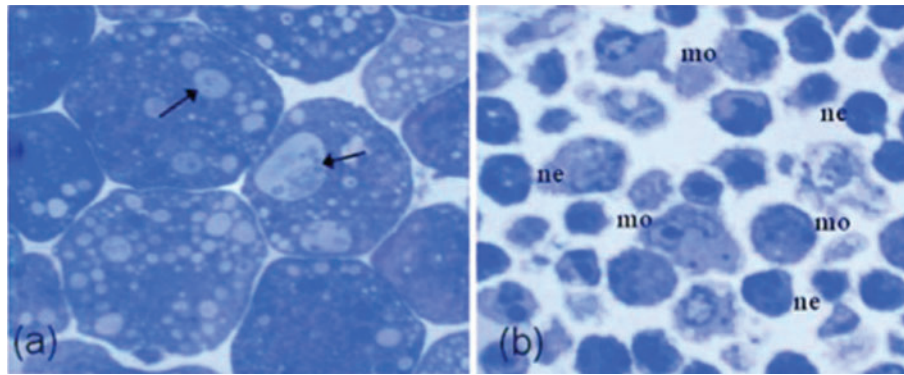


Fig. 3. Analysis of *E. histolytica* trophozoites from inside the dialysis bag and inflammatory cells from peritoneal exudates obtained after 6 h in the peritoneum. (a) Amoebae showing several large vacuoles with microfibrillar content (arrows). (b) Peritoneal infiltrate with abundant neutrophils (ne) and mononuclear cells (mo). Images acquired at $\times 40$ magnification. Toluidine blue stain.

was observed in ALA development, exclusively in hepatocytes and inflammatory cells directly contacting amoebic trophozoites, as well as staining in more distantly located hepatocytes, but not directly in the amoebae. The last result is in agreement with the *in vivo* system data, corroborating the presence of cell death in the amoebae at 72 h.

Ultrastructural analysis

Most of the ultrastructural features observed on *E. histolytica* trophozoites from dialysis bags exposed to

inflammatory cells of the hamster peritoneal cavity showed indications of PCD. The irregular single nucleus was the most obvious, contrasting with the round or spherical nuclei of control cells with their central endosome and regularly distributed peripheral chromatin, as reported by Martínez-Palomo (1982) (Fig. 6a). During PCD, the phase of chromatin condensation showed the formation of electron-dense nuclear inclusions characteristically distributed peripherally under the nuclear membrane (Fig. 6b–d). Numerous vacuoles were irregularly scattered throughout the endoplasm in the early stages of PCD. No significant

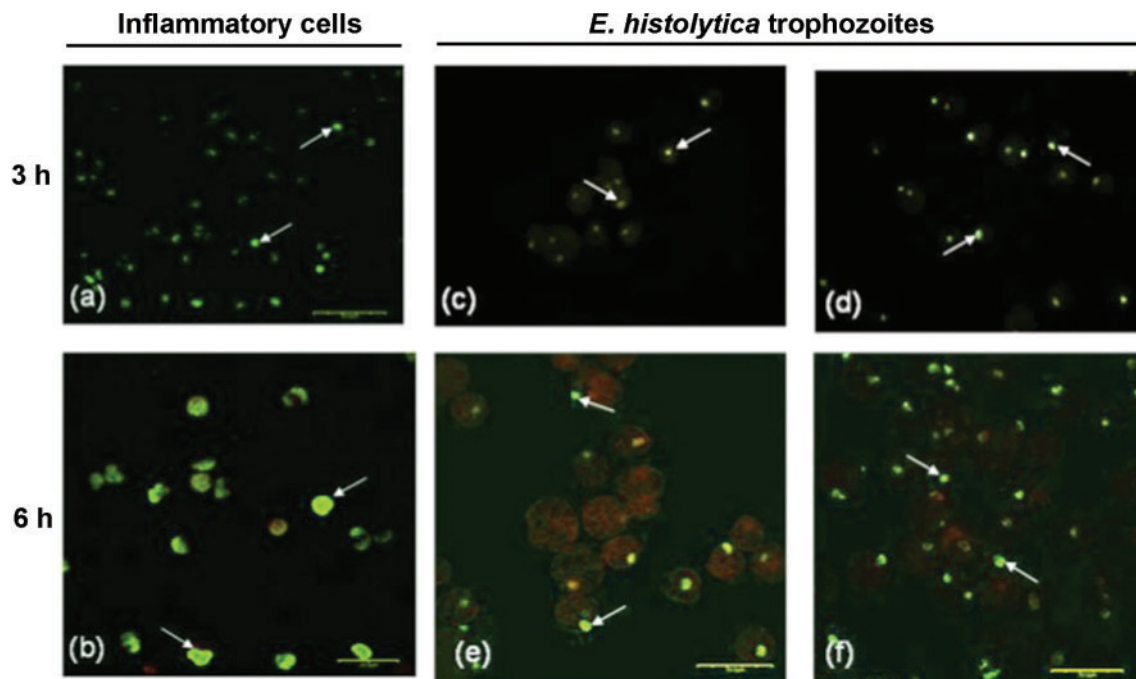


Fig. 4. Confocal microscopy of TUNEL assays at 3 and 6 h post-interaction in the peritoneum. (a, b) Inflammatory cells obtained from the peritoneal cavity. TUNEL-FITC-positive cells (arrows) are seen. Nuclei were counterstained with PI. (c–f) *E. histolytica* trophozoites obtained from the dialysis bag after interaction with the peritoneal exudates are positive for TUNEL (arrows).

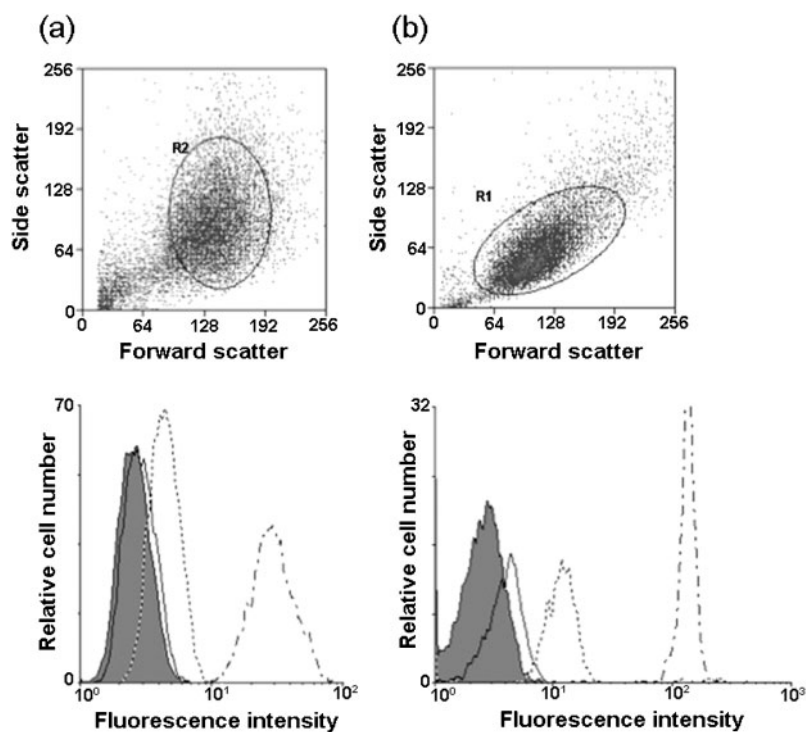


Fig. 5. Flow cytometric analysis of the TUNEL assay: FACS profiles of (a) *E. histolytica* trophozoites and (b) inflammatory cells after *in vivo* interaction for 3 h (dotted line) and 6 h (dot-dash line). The profile of control cells is shaded.

ultrastructural differences were seen between 3 and 6 h post-interaction.

At the light microscopic level, the trophozoites were filled with many vacuoles, some with a small amount of cell debris, suggesting that they were autophagic vacuoles (Fig. 3). During this process, the plasma membrane remained intact (Fig. 6). Transmission electron microscopy revealed evidence of neutrophils and macrophages undergoing apoptosis when exposed to *E. histolytica* trophozoites inside the dialysis bag. Normal neutrophils showed irregular cell surfaces, multi-lobulated nuclei and heterogeneous nuclear chromatin (Fig. 6e). Following incubation with *E. histolytica* for 3 h, the neutrophils became round, with condensation of chromatin and cytoplasm (Fig. 6f). Macrophages showed morphological features characteristic of apoptosis, including cytoplasmic vacuolation (Fig. 6g, h). After 6 h of incubation, neutrophils in close contact with trophozoites displayed an apoptotic appearance, which was evidenced by a marked condensation of nuclear chromatin. The macrophages also showed chromatin condensation with peripheral margination but intact organelles, including mitochondria, although the presence of blebs was evident (Fig. 6g, h).

Protein profile analysis by SDS-PAGE

The changes in the pattern of *in vivo* protein synthesis obtained in *E. histolytica* trophozoites from the dialysis bag after 3 or 6 h of incubation inside the hamster peritoneal cavity were assessed. For separation, polyacrylamide slab gel electrophoresis with SDS was used. Comparison of

protein profiles derived from amoeba dialysis bags and the control (amoeba culture) produced patterns containing approximately nine discrete bands with molecular masses of 20–180 kDa (Fig. 7a), although the PAGE pattern showed differences when densitometric analysis of the gels was performed (Fig. 7b). Qualitative differences were evident principally in the protein bands with molecular masses of 175, 133, 99, 45 and 35 kDa, which were increased in the parasites isolated from the peritoneal cavity, while protein bands corresponding to molecular masses of 31, 23, 21 and 17 kDa were diminished. These changes were more evident at 6 h post-interaction.

Cytotoxicity against *E. histolytica*

Previous results have demonstrated that nitric oxide (NO) is one of the major cytotoxic molecules released by activated phagocytes during interaction with *E. histolytica* trophozoites *in vitro* (Lin & Chadee, 1992; Lin *et al.*, 1994). To confirm the role of NO as a part of the self-defence system, we measured the generation of nitrites and nitrates, which are the stable end products of NO, in the serum, the peritoneal fluid and the supernatant of the dialysis bag. The concentration of nitrates and nitrites in serum was higher in the group of stimulated hamsters (1.9 and 3.3 μM at 3 and 6 h, respectively) than in controls (0.2 μM). In the peritoneal cavity, the concentration of nitrates and nitrites was 0.6 and 0.7 μM at 3 and 6 h, respectively, whereas the concentration in the control group was 0.1 μM at both time points. To determine whether NO was capable of crossing the dialysis bag, the concentration of nitrates and nitrites was also determined in the supernatant contained

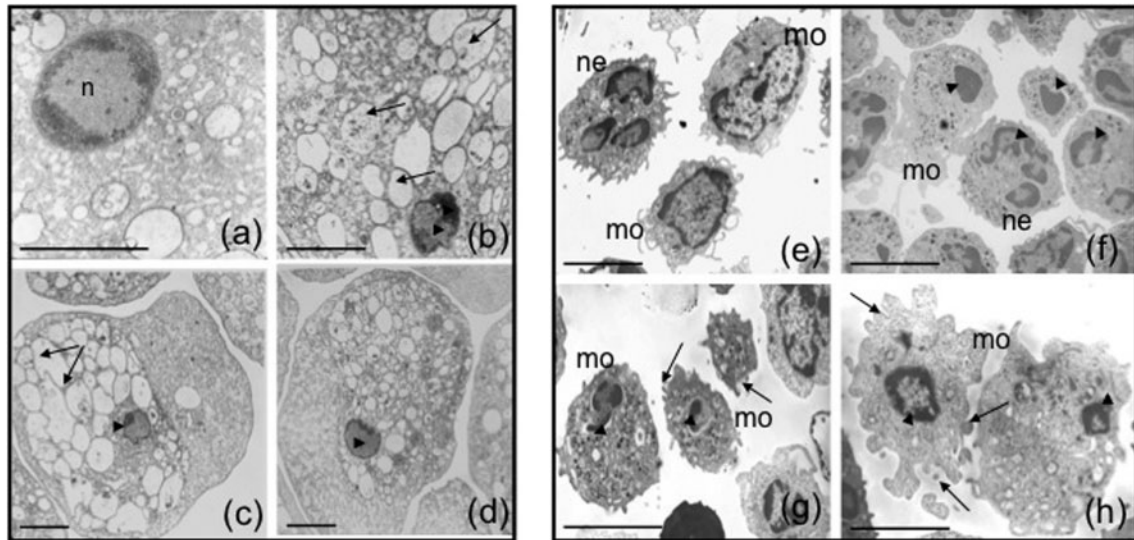


Fig. 6. (a–d) Ultrastructure of *E. histolytica* trophozoites and inflammatory cells. (a) Control trophozoite: typical amoeba nucleus (n) showing peripherally localized chromatin. (b–d) Trophozoites at 3 h post-interaction in the hamster peritoneum, showing irregular nuclei with dense chromatin (arrowheads) attached to the nuclear envelope. The cytoplasm appears condensed with many vacuoles and vesicles (arrows), suggestive of apoptosis. (e–h) Ultrastructure of freshly isolated inflammatory cells from the peritoneum. (e) Control: neutrophil with typical morphology (ne), showing dense multilobular nucleus; two monocytes (mo) with normal morphology showing cytoplasmic projections with peripheral chromatin. (f) 3 h post-interaction. Changes suggestive of apoptosis, such as nuclear condensation (arrowheads) are seen. (g, h) 6 h post-interaction. Cell shrinkage, rounding and the presence of blebs (arrows) are seen. Bars, 5 μ m.

within the bag. Stimulated hamsters had peak concentrations of 1.6 and 1.7 μ M at 3 and 6 h, respectively, compared with a constant concentration of 0.4 μ M in the control group (Fig. 8).

DISCUSSION

Our first aim in the present work was to determine if morphological features of PCD in experimental acute ALA

can be detected, either in the parasites or in any of the associated host cells. As described more than 20 years ago (Tsutsumi *et al.*, 1984), a sequential histological study of *E. histolytica*-infected hamster livers revealed that multiple foci of acute inflammation with irregularly shaped foci were formed in the hepatic parenchyma by a few centrally located trophozoites surrounded by several layers of polymorphonuclear leukocytes. The number of phagocytes increased proportionately with time, and areas of focal necrosis surrounded by rings of inflammatory cells limited

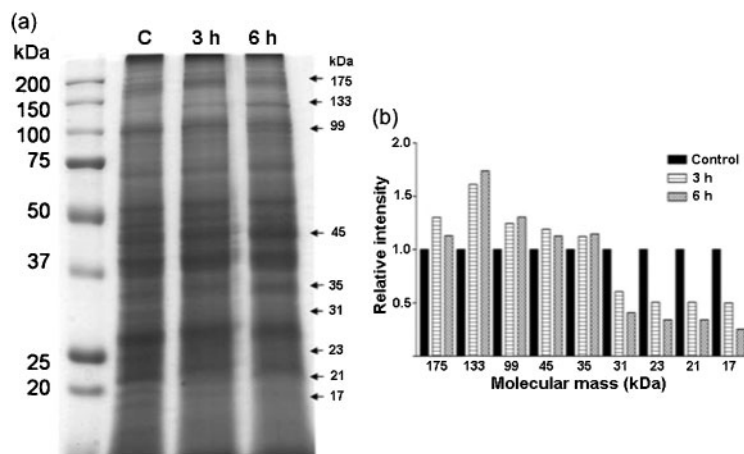


Fig. 7. (a) SDS-PAGE of proteins from *E. histolytica* trophozoites grown in axenic culture (control, C) and recovered from dialysis bags at 3 and 6 h post-interaction in the hamster peritoneum. (b) Densitometric analysis. Proteins of 175, 133, 99, 45, 35, 31, 23, 21 and 17 kDa were the most evident as compared with the control. ImageJ software was used.

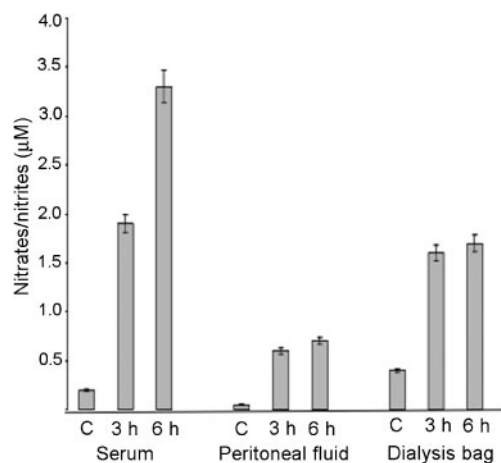


Fig. 8. Nitric oxide (NO) quantification. Colorimetric analysis at 570 nm by the Griess method. NO (based on nitrate/nitrite levels) was determined in serum, peritoneal fluid and liquid from inside the dialysis bag recovered from the hamsters at 3 and 6 h. Data are expressed as mean \pm SD ($n=5$); $P<0.05$ for all experimental treatments compared with controls (C).

the necrotic tissue encroaching in the normal-appearing liver parenchyma. Detailed analysis of the necrotic areas showed that relatively few trophozoites had clear signs of cell death, such as cell rounding with shrinkage (Fig. 2).

Because only a few amoebae morphologically suggested PCD in liver tissue, it was necessary to employ other methodologies to examine cell death, such as TUNEL staining. First, we performed TUNEL with FITC labelling on sections from ALAs in hamsters and observed them by fluorescence microscopy. As shown in Fig. 2, we found significant TUNEL staining of hepatocytes and inflammatory cells in areas of ALA, and the staining was most pronounced in areas immediately adjacent to amoebic trophozoites. However, this sensitive procedure did not allow us to detect positive TUNEL staining in the trophozoites, although the observation that the morphology of parasites correlated with rounded forms suggests that most of the trophozoites arriving in the liver die by PCD, while the surviving trophozoites multiply and cause significant tissue damage. To confirm the death of parasites, a TUNEL–peroxidase labelling assay was performed. The results presented in Fig. 2 show parasite death, as evidenced by TUNEL-positive staining of nuclei at 3 days post-inoculation, indicating that a PCD process was occurring.

Based on our previous experience in working with experimental animals, especially with the ALA model in hamster, and to better understand the mechanisms involved in the elimination of intraperitoneally inoculated *E. histolytica* in the pathogenesis of ALA (Shibayama *et al.*, 1998, 2008), we first designed a small dialysis cellulose membrane bag containing trophozoites of *E. histolytica*.

These bags were introduced into the peritoneal cavity of the hamster and left for specific periods of time, after which the viability of the amoebae remained at over 90% (3 and 6 h). We used mineral oil injected intraperitoneally as a pro-inflammatory agent to increase the peritoneal cell exudate that interacts with the components inside the bags before their introduction into the peritoneal cavity. This exudate was composed of neutrophils (70%) and macrophages (30%) (Fig. 3).

The use of the intraperitoneal model of ALA production (Shibayama *et al.*, 1998) to analyse PCD of *E. histolytica* trophozoites was also considered. However, as reported in that study, intraperitoneally inoculated parasites interact rapidly with inflammatory cells from the peritoneal exudates, producing multiple foci, which in turn invade the capsule and liver parenchyma and produce tissue damage. Therefore, the possibility of separately obtaining clean and abundant samples of trophozoites and inflammatory cells to properly analyse PCD is significantly reduced. Moreover, the factors operating in this peritoneal milieu are multiple and difficult to relate directly to PCD. By contrast, the use of intraperitoneal dialysis bags allows us to analyse the role of diffusible molecules that can be related to PCD, with the additional advantage of obtaining large numbers of trophozoites for multiple studies related to apoptosis.

A relatively simple and rapid procedure to detect PCD in cell populations is to identify various morphological changes typical of cells undergoing apoptosis. However, the determination of DNA fragmentation is one of the most convincing indications of PCD, and this procedure permits the distinction of *E. histolytica* trophozoites in the process of death by labelling the DNA strand breaks, as occurs with other micro-organisms, such as *Candida albicans* (Phillips *et al.*, 2003). Fig. 4 shows data from TUNEL assays observed by confocal microscopy. Cells undergoing PCD were characterized by bright green condensation of the nuclei. Flow cytometry was also useful in determining the percentage of cells undergoing PCD during the course of experiments. Peritoneal neutrophils and macrophages underwent apoptosis beginning at 3 h and apoptotic cells were more noticeable 6 h after the interaction with amoebae (75% and 98% apoptosis, respectively, in the peritoneal cell cavity versus 16% in non-stimulated cells). While 52% and 86% of the amoebae were TUNEL-positive at 3 and 6 h, respectively, only 8% of trophozoites from controls were TUNEL-positive (Fig. 5). These data suggest that PCD plays a prominent role in the pathogenicity of *E. histolytica* in ALA in this hamster model and that leukocytes can induce *E. histolytica* trophozoites to undergo cell death. We also observed that amoebae can induce apoptosis in inflammatory cells, as reported by Sim *et al.* (2004).

Much of the characterization of apoptosis has been derived from morphological data, including ultrastructural observations. Intracellular and plasma membrane modifications

have been widely recognized as crucial factors involved in cell death (Kerr *et al.*, 1972). Fig. 6 shows ultrastructural features observed in typical *E. histolytica* trophozoites from axenic culture. The amoeba possesses a single nucleus, an endosome suggesting DNA condensation in the centre of the nucleus and peripheral chromatin close to the nuclear membrane. Vesicles were irregularly scattered throughout the cytoplasm. On the other hand, as evidenced by light microscopy, the ultrastructural changes in the parasites from dialysis bags included distorted nuclear shape (Fig. 6), in contrast to the rounded nuclei of control cells, accompanied by condensation of the nuclear chromatin to form dense granular caps or toroidal structures underlying the nuclear membrane, an increase in the number of small vesicles, a decrease in the number of vacuoles with a clear content and preservation of an intact plasma membrane. The inflammatory cells also revealed features of cells undergoing apoptosis, including chromatin condensation, shrinkage and fragmentation of nuclei, formation of micronuclei and apoptotic bodies, condensation of cytoplasm, and cell membrane blebs (Fig. 6). Therefore, *E. histolytica*-induced neutrophil and macrophage apoptosis may help prevent unwanted tissue inflammation and damage in the amoeba-invaded lesions *in vivo*. In addition, our data suggest that *E. histolytica* trophozoites show apoptotic characteristics, probably due to molecules secreted by inflammatory cells as a means to protect the host against infection.

E. histolytica is known to kill multiple cell types; *in vitro* studies have demonstrated that after adherence, amoebic trophozoites are capable of damaging a variety of host cells. Hence, adherence is required for cytolysis, but this is apparently insufficient, as we demonstrated that apoptotic cell death of neutrophils and macrophages induced by *E. histolytica* trophozoites can occur without target cell contact. There are undoubtedly many factors involved in these processes, such as amoebapores (8 kDa; Leippe & Müller-Eberhard, 1994) and cysteine proteinases (16–96 kDa; Irmer *et al.*, 2009). PCD processes involve the coordinated activity of a plethora of proteins that can be separated into activators, effectors, and negative regulators, such as kinases (Franklin & McCubrey, 2000). The electrophoretic method proved to be useful in identifying amoebic proteins from trophozoites (Fig. 7). Visual changes in the protein profiles of these parasites were observed; however, PAGE profiles showed these differences more clearly. Some proteins were downregulated in the parasites isolated from the peritoneal cavity, such as the 31, 23, 21 and 17 kDa proteins. However, the 35, 45, 99 and 133 kDa proteins increased at 6 h. Several cysteine proteinases with molecular masses in the range of 16 to >100 kDa have been detected in trophozoite extracts of pathogenic *E. histolytica* (Singh *et al.*, 2004; Bruchhaus *et al.*, 2003). Among these, some of the molecules of low molecular mass could cross the membrane of the dialysis bag and might be important virulence factors contributing to the death of host immune cells. To determine more

specifically the possible molecules involved, we are presently carrying out experiments such as Western blotting and PCRs for amoebapore and proteases, including the transcriptomic analysis of PCD.

Production of NO by the host plays an important role in the mammalian immune system by killing or inhibiting the growth of many pathogens, including parasites, viruses and bacteria (Chakravorty & Hensel, 2003). The biochemical basis of its effects on the parasite targets appears primarily to involve the inactivation of enzymes that are crucial to energy metabolism and growth, although other biological activities also exist, such as mediating the S-nitrosylation of cysteine-containing proteins (e.g. cysteine proteases) (Colasanti *et al.*, 2002). For this reason, NO produced and released by macrophages and neutrophils was quantified in serum, peritoneal fluid and the supernatant of dialysis bags containing amoebae. Of the liquids tested, the concentration of nitrates and nitrites was highest in serum. The mean concentration at 3 h was 1.9 μM , which increased to 3.3 μM at 6 h, maintaining a relationship with the induction of death in *E. histolytica* trophozoites. The concentration of reactive nitrogen compounds in the peritoneal cavity was lower than in all other samples analysed: the NO concentration was 0.6 and 0.7 μM at 3 and 6 h, respectively (Fig. 8). To demonstrate that NO could pass through the dialysis bag, the supernatant contained in the bag was also analysed. We found a peak concentration greater than 1.7 μM at 6 h. Therefore, under our conditions, it appears that NO participates significantly in the elimination of amoebae by inducing PCD. Ramos *et al.* (2007) demonstrated that diverse NO donors were capable of inducing apoptosis in *E. histolytica* trophozoites. Lin & Chadee (1992) demonstrated that NO is the major cytotoxic molecule released by activated macrophages, and NO-releasing molecules are effective in treating amoebiasis (Sannella *et al.*, 2003). Other studies indicate that NO is involved in host defences *in vivo*, and this molecule is required for the control of ALA in a murine model (Seydel *et al.*, 2000). NO concentration is increased in patients with acute intestinal amoebiasis (Pérez-Fuentes *et al.*, 2000). Moreover, Pacheco-Yépez *et al.* (2001) suggested that NO does not have any effect on *E. histolytica* trophozoites and is unable to provide protection; several studies have begun to clarify, at the cellular level, the specific effects of *E. histolytica* on immune cells and effector cell functions (Campbell & Chadee, 1997). Our future *in vivo* experiments may employ specific nitric oxide synthase (NOS) inhibitors, such as N^G -nitro-L-arginimethyl ester (L-NAME), aminoguanidine and dexamethasone, or possibly NOS knockout mice.

While *in vitro* studies have described PCD in several eukaryotic micro-organisms, such as *Trichomonas* (Mariane *et al.*, 2006), *Giardia* (Chose *et al.*, 2003) and *Entamoeba* species (Ramos *et al.*, 2007; Villalba-Magdaleno *et al.*, 2007; Nandi *et al.*, 2010; Ghosh *et al.*, 2010), the data that can be obtained *in vivo* are also important. In this *in vivo* study we have shown the occurrence of PCD in

amoebae in the liver. To our knowledge, this is the first *in vivo* demonstration of PCD in *E. histolytica*. The main indicator of PCD in protozoa is nuclear DNA fragmentation, which can be revealed using either electron microscopy, by which the earliest definitive changes in PCD have been reported (White & Cinti, 2004), or TUNEL staining followed by fluorescence microscopy, cytochemistry or flow cytometry (Didenko, 2002). On the other hand, our group has been using molecular techniques to analyse the progression of cell death. The first genes identified during the early stage of the process that could be implicated in cellular death in *E. histolytica* are *glutaminyl-tRNA synthetase*, *sir-2* and *grainins*. All of these are anti-apoptotic signals that could be implicated in the regulation of apoptotic pathways (Sánchez-Monroy *et al.*, 2010).

Although previous studies have suggested the induction of host cell death by *E. histolytica*, our *in vivo* system provided additional information regarding the death signalling pathway. These results are useful for better understanding the molecular mechanisms of amoebic PCD and may contribute to the development of new therapeutic agents against amoebiasis.

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