Programmed cell death in *Entamoeba histolytica* induced by the aminoglycoside G418

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This study presents morphological and biochemical evidence of programmed cell death (PCD) in *Entamoeba histolytica* induced by exposure of trophozoites to the aminoglycoside antibiotic G418. Morphological characteristics of PCD, including cell shrinkage, reduced cellular volume, nuclear condensation, DNA fragmentation and vacuolization were observed, with preservation of trophozoite membrane integrity. PCD is orchestrated biochemically by alterations in intracellular ion fluxes. In G418-treated trophozoites, overproduction of reactive oxygen species (ROS), decreased intracellular K+ increased cytosolic calcium, and decreased intracellular pH levels were observed. However, externalization of phosphatidylserine was not detected. These results suggest that amoebae can undergo PCD under stress conditions, and that this PCD shares several properties with PCD reported in mammals and in a variety of unicellular organisms.

**INTRODUCTION**

*Entamoeba histolytica*, the causal agent of amoebiasis, is a protozoan parasite that resides in the colon of infected humans. The invasive trophozoites adhere to mucus and epithelial cells, proliferate by binary fusion, and release proteolytic factors that destroy the intestinal mucosa, resulting in amoebic dysentery. In one in ten patients with intestinal *E. histolytica* infection, the trophozoites migrate through the portal vein to the liver and give rise to amoebic abscesses, the main cause of death by this parasite (Espinosa-Cantellano & Martínez-Palomo, 2000). The apoptosis of host cells such as macrophages induced by contact with *E. histolytica* trophozoites has been widely studied, and it is considered an important feature of the host–parasite relationship (Ragland et al., 1994; Berninghausen & Leippe, 1997).

Programmed cell death (PCD) has been considered a critical mechanism of development, differentiation and control of cellular proliferation in metazoans. However, increasing evidence indicates that PCD is also present in unicellular organisms. Forms of PCD such as apoptosis, apoptosis-like processes and necrosis-like processes have been identified in several bacteria (Lewis, 2000), yeast (Madeo et al., 1999), the slime mould *Dictyostelium discoideum* (Cornillon et al., 1994), the dinoflagellate *Peridinium gatunense* (Vardi et al., 1999), the euglenoid *Euglena gracilis* (Scheuerlein et al., 1994), the dinoflagellate *Trypanosoma brucei* (Nguewa et al., 2004) and *Plasmodium* (Al-Olayan et al., 2002). Recently, results reported by Ramos et al. (2007) have suggested the induction of an apoptotic-like process by nitric oxide species in *E. histolytica*. Apoptosis is the result of a genetic program that induces cellular and biochemical changes, including caspase activation, externalization of phosphatidylserine (PS), an increase in intracellular Ca2+ and mitochondrial dysfunction, as well as physical changes such as cell shrinkage, alteration in cell volume, cytoplasmic blebbing and vacuolization, chromatin condensation, and nucleosomal fragmentation. Apoptosis is energy dependent, requiring ATP for signalling from the cytoplasm to the nucleus of the cell. The physiological role of apoptosis in protozoa is unknown. Although there is no obvious advantage at the individual level for unicellular organisms to carry the complex machinery required for PCD, the phenomenon has been related to altruistic behaviour, with clear benefits for the entire population, or as a mechanism to avoid host death (Wanderley et al., 2007).
2005). Under conditions of limited nutrients or excessive expansion of the parasite population in the host, a subset of the population might ‘commit suicide’ by PCD.

Apoptosis, however, is not limited to physiological processes; it can also be induced by cellular damage such as treatment with antibiotics (Chen et al., 1995). G418 has been described as an apoptotic inducer in kidney cells, ear sensory hair cells and the Trypanosoma cruzi parasite. This drug is an aminoglycoside antibiotic used extensively for the treatment of human Gram-negative bacterial infections and in molecular biology research for the selection of prokaryotic and eukaryotic cells that have accepted neomycin-resistance genes. In kidney cells, G418-induced apoptosis is dependent on the release of cytochrome c from mitochondria and the endoplasmic reticulum (Jin et al., 2004). Similarly, in ear sensory hair cells, G418-induced apoptosis is dependent on caspasas activated by the phosphorylation of c-jun, the translocation of cytochrome c, and increased cytoplasmic calcium (Matsui et al., 2004). However, apoptosis in the protozoan parasite T. cruzi has been poorly studied. T. cruzi undergoes apoptosis in old cultures as well as in the presence of G418. Under both of these conditions, apoptosis is associated with the translocation from the cytosol to the nucleus of elongation factor 1, a protein involved in eukaryotic protein biosynthesis (Billaut-Mulot et al., 1996). The role of elongation factor 1 in apoptosis is unknown, but it has been suggested to be involved in transcriptional processes.

The present study is believed to be the first to report PCD in trophozoites of the human intestinal parasite E. histolytica exposed to the antibiotic G418. PCD features that were determined in G418-treated trophozoites include nuclear staining by terminal deoxynucleotidyl transferase-mediated biotin–dUTP nick end labelling (TUNEL), DNA fragmentation and compaction, production of reactive oxygen species (ROS), potassium release, increased cytoplasmic calcium, acidification of intracellular pH (pHi), and decreased cellular volume. Our results suggest that amoebae can undergo PCD under stress conditions such as treatment with G418. The possible significance of this phenomenon in the host–amoeba relationship is discussed.

METHODS
Parasite and growth conditions. Trophozoites of clone A (strain HM1:1IMSS) were cultured axenically in TYI-S-33 medium (Diamond et al., 1978). PCD was induced in trophozoites by incubation with 10 μg G418 ml⁻¹ for different periods of time, as indicated.

Kinetics of growth. Growth curves of trophozoites were determined in the absence (not treated; NT) or presence of 10 μg G418 ml⁻¹. Trophozoite viability was measured every 12 h by using Trypan Blue exclusion.

Flow-cytometry assays and microscopic analysis. Changes in size and in the light-scattering properties of trophozoites were determined by flow cytometry, as described by Hawley et al. (2004), using a Becton Dickinson FACSCalibur equipped with CellQuest software (Becton Dickinson). Trophozoites (1 x 10⁶), non-treated or treated with 10 μg G418 ml⁻¹, were analysed using a 488 nm argon laser. A specific gate based on the properties of control trophozoites was selected to determine their positions on a forward scatter vs side scatter dot plot. Light scattered in the forward direction is roughly proportional to cell size, whereas light scattered at a 90° angle (side scatter) is proportional to cell density. For microscopic analysis, G418-treated or NT trophozoites were washed twice with PBS and placed on glass slides. Trophozoites were fixed in 2 % formaldehyde and observed using an Olympus BX41 inverted microscope coupled to a Media Cybernetics CoolSNAP-Pro digital video camera with Image-Pro Plus software.

Effect of cysteine protease inhibitor E-64. Ten-thousand trophozoites were cultured in TYI-S-33 medium in the presence of 20 or 50 μM E-64 [trans-epoxysuccinyl-L-leucylamido (4-guanidino) butane; Sigma Aldrich] or without the drug. One hour later, trophozoites were incubated with G418 by co-incubation with 10 μg G418 ml⁻¹ for 6 h. Finally, DNA electrophoresis, TUNEL and transmission electron microscopy assays (described below) were conducted.

Nuclear extracts and DNA isolation. Nuclei from G418-treated, G418-treated co-incubated with E-64, or NT trophozoites were obtained as reported by Gómez et al. (1998), with some modifications. Briefly, 1 x 10⁶ trophozoites were washed twice with PBS, pH 6.8, resuspended in four volumes of buffer A (0.01 M HEPES, pH 7.9, 0.0015 M MgCl₂, 0.01 M KCl, 0.01 M DTT, 0.0005 M PMSF), and incubated on ice for 35 min. The trophozoites were homogenized with 25 strokes in an all-glass Dounce homogenizer and centrifuged at 6000 r.p.m. at 4°C for 10 min. Integrity of the nuclei was monitored by phase-contrast microscopy. To isolate DNA from nuclei, the nuclear pellet was mixed with 750 μl extraction buffer (0.02 M EDTA, 0.01M Tris, 0.5% SDS) containing 50 μg proteinase K ml⁻¹ and incubated at 65°C for 20 min. Then, DNA was extracted with phenol/chloroform/isomyl alcohol (25:24:1). Nucleic acids from G418-treated, NT and co-incubated with E-64 trophozoites were precipitated at −20°C by addition of 0.2 M NaCl and one volume of isopropyl alcohol. DNA was analysed by 2% agarose gel electrophoresis at 100 V for 45 min and stained with 0.5 μg ethidium bromide ml⁻¹.

Nick-labelling of internucleosomal DNA fragments. Ten-thousand G418-treated, G418-treated co-incubated with E-64, or NT trophozoites were fixed in 4 % formaldehyde in a humidified atmosphere for 10 min. After washing twice with PBS, 50 μl TUNEL reaction mixture (Roche) was added and incubated for 60 min at 37°C in a humidified atmosphere in the dark. Trophozoites were rinsed three times with PBS, loaded on slides, and observed with a Zeiss LSM Pascal confocal microscope. As a positive control, trophozoites were treated with 20 μg ml⁻¹ DNase I endonuclease for 10 min.

Transmission electron microscopy analysis. Trophozoites grown in the absence or presence of 10 μg G418 ml⁻¹ were harvested after 3, 6, 9 and 12 h of incubation. Trophozoites co-incubated with E-64 were harvested after 9 h of incubation. Trophozoites were washed twice with 0.1 M sodium cacodylate buffer and fixed for 1 h with 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4. Fixed trophozoites were washed twice with 0.1 M sodium cacodylate buffer, post-fixed with 2.0 % osmium tetroxide, dehydrated with ethanol at increasing concentrations, and treated with propylene oxide. The trophozoites were then embedded in epoxy resins. Semithin sections were stained with toluidine blue for light-microscopic examination. Thin sections were stained with uranyl acetate followed
by lead citrate, and examined with a Zeiss EM-10 electron microscope.

Detection of PS. PS externalization was assessed by monitoring annexin V–FITC binding in viable cells. Briefly, 1 × 10⁶ G418-treated or NT trophozoites were resuspended in 500 μl 1 × binding buffer (Aptoptosis Detection kit, BioVision) containing annexin V–FITC and propidium iodide (PI). After 10 min of incubation in the dark, trophozoites were washed twice with fresh binding buffer. Annexin V–FITC-stained trophozoites were detected by flow cytometry (excitation wavelength, 488 nm; emission wavelength, 530 nm) using an FITC signal detector (FL1), and PI staining was detected by the phycoerythrin emission signal detector (FL2). Alternatively, staining of trophozoite membranes was visualized by confocal microscopy using a Zeiss LSM Pascal confocal microscope.

Measurement of ROS. To determine the levels of ROS, the cell-permeant probe dichlorodihydrofluorescein (DCFDA; Sigma Aldrich) was used. In the presence of a suitable oxidant, DCFDA is oxidized to the highly fluorescent 2,7-dichlorofluorescein. NT or G418-treated trophozoites (1 × 10⁶) were resuspended in 500 μl phosphate buffer, pH 7.4, containing 0.02 M DCFDA, incubated in the dark for 15 min, and analysed by flow cytometry (excitation wavelength, 485 nm; emission wavelength, 525 nm) using the CellQuest software.

Measurement of intracellular potassium (K⁺) levels. K⁺ levels were determined by using 5 μM potassium-binding benzofuran isophthalate (PBFI-AM; Sigma Aldrich) as a cell-permeant probe and a FACSCalibur flow cytometer. Briefly, 1 × 10⁶ trophozoites were grown in the presence or absence of 10 μg G418 ml⁻¹ for 6 h, harvested, and washed twice with a buffer containing 0.116 M NaCl, 0.0054 M KCl, 0.0008 M MgCl₂, 0.0055 M glucose and 0.05 M MOPS, pH 7.4. Trophozoites were resuspended in the same buffer and incubated with PBFI-AM for 1 h at 37 °C. Then, the trophozoites were pelleted at 1500 r.p.m. for 2 min. After two washing steps, trophozoites were resuspended in fresh buffer. Prior to flow-cytometric analysis, PI was added to each sample to a final concentration of 10 μg ml⁻¹. Ten-thousand trophozoites were loaded with 10 μg PBFI-AM for 1 h with occasional agitation. Then, trophozoites were pelleted at 1500 r.p.m. for 2 min. After two washing steps, trophozoites were resuspended in fresh buffer and incubated with PBFI-AM for 1 h at 37 °C. Then, the trophozoites were pelleted at 1500 r.p.m. for 2 min. After two washing steps, trophozoites were resuspended in fresh buffer. Prior to flow-cytometric analysis, PI was added to each sample to a final concentration of 10 μg ml⁻¹. Ten-thousand trophozoites were loaded with 10 μg PBFI-AM and PI, respectively, and emission was registered at 540 nm.

Cytosolic Ca²⁺ concentrations. Changes in intracellular Ca²⁺ concentration ([Ca²⁺]) were monitored with the fluorescent probe Fura-2/AM. After harvesting, trophozoites were washed twice at 1500 r.p.m. for 2 min at 4 °C in buffer I, which contained 0.116 M NaCl, 0.0054 M KCl, 0.0008 M MgSO₄, 0.0055 M D-glucose and 0.05 M HEPES, pH 7.4. Amoebae were resuspended in loading buffer (1 × 10⁶ trophozoites ml⁻¹) that contained 0.116 M NaCl, 0.0054 M KCl, 0.0008 M MgSO₄, 0.0055 M D-glucose, 1.5 % sucrose, 0.05 M HEPES, pH 7.4, and 6 μM Fura-2/AM. The trophozoite suspension was incubated for 1 h at 37 °C with occasional agitation. Then, trophozoites were washed four times with ice-cold buffer I to remove extracellular dye. For fluorescence measurements, 125 μl of the trophozoite suspension was diluted to 2.4 ml buffer I. Fura-2/AM was excited at 340 nm, and emission at 510 nm was registered by a Perkin Elmer MPF44A fluorimeter. The [Ca²⁺], in nM, was determined at 30 °C using the formula:

\[ [Ca^{2+}] = K_d \times \frac{(F_2 - F_3)}{(F_1 - F_3)} \]

where \( F_1 \) is the fluorescence signal obtained from the entire cell, \( F_2 \) represents the fluorescence signal after addition of 0.001 M EGTA, \( F_3 \) is the fluorescence following cell lysis with 0.04 % Triton X-100 in 0.03 M Trizma base, and \( F_3 \) is the fluorescence after adding 0.004 M CaCl₂. \( K_d \) represents the dissociation constant value of 224 nM, as reported by Grynkiewicz et al. (1985).

pH, measurements. Trophozoites (1 × 10⁶) were resuspended in TYI-S-33 medium and washed twice with buffer A (0.14 M KCl, 0.004 M CaCl₂, 0.025 M HEPES-Tris, pH 7.4). Then, trophozoites were loaded with 10 μM 2,7-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein (BCECF; Sigma Aldrich) for 45 min in 1 ml buffer A. Nigericin (1 μg ml⁻¹) was added to the loading incubation. After loading, trophozoites were washed twice with buffer and resuspended in fresh buffer. Fluorescence was registered at 535 nm in a Perkin Elmer MPF44A fluorimeter. At the end of each experiment, an in situ pH calibration procedure with nigericin was used to relate the fluorescence intensities at 485 nm to the pH value. When cells are exposed to depolarizing high-K⁺ buffers of different pH values (pH 5.7–7.7), nigericin, an H⁺-K⁺ exchanger ionophore, sets \([K^+]_i = [K^+]_o\) and \(pH_o = pH_i\), where \([K^+]_o\) is extracellular potassium concentration, \([K^+]_i\) is intracellular potassium concentration, and \(pH_o\) is extracellular pH.

RESULTS

Cytotoxic effect of G418 on E. histolytica trophozoites

The effect of G418 on trophozoite viability was assessed by Trypan Blue exclusion staining. Compared with control NT trophozoites, 70 % of trophozoites had died after 48 h of incubation with 10 μg G418 ml⁻¹ (Fig. 1a). Morphological changes in G418-treated trophozoites were observed by light microscopy. Whereas NT parasites had typical amoebic forms, trophozoites exposed to G418 showed rounded forms and cell shrinkage (Fig. 1b). Moreover, the mean volume of G418-treated trophozoites was significantly lower than that of NT trophozoites.

Changes induced by G418 in cell size and granularity

To determine whether G418 induces trophozoite shrinkage, cell size was measured by the decrease in forward scatter in flow-cytometry analysis. As shown in Fig. 1(c), the trophozoite population treated with G418 showed a marked reduction in cell size. Whereas the sizes of 51.3 % of NT trophozoites were greater than the mean value, G418-treated trophozoites had obviously diminished sizes, with only 16.9 % of the total population having sizes greater than the mean y axis value taken from the R1 population (Fig. 1c). Interestingly, changes in granularity were also observed by the increase in side scatter, from 13 % in NT trophozoites to 50.1 % in G418-treated parasites. These morphological changes resemble those observed during PCD. Accordingly, the biochemical changes induced by G418 were studied to explore a putative PCD process in this parasite.

G418 induces DNA fragmentation in E. histolytica

In mammalian cells, internucleosomal DNA fragmentation is one of the most important and typical nuclear features that define the PCD phenomenon (Collins et al., 1992). When electrophoresed on an agarose gel, nuclear DNA...
from trophozoites treated with 10 μg G418 ml⁻¹ appeared to be degraded (Fig. 2a, lane 3, b, lane 2), whereas NT trophozoite DNA did not (Fig. 2a, lane 2). No obvious ladder pattern was detected for G418-treated trophozoite DNA; instead, five or six smeared DNA bands were observed. To confirm nuclear DNA fragmentation, TUNEL assays were conducted. Less than 10% of nuclei of NT trophozoites were stained (Fig. 2c). In contrast, 70% of trophozoites showed positive nuclear staining after 6 h of G418 incubation. As a positive control, trophozoites were incubated with DNase I endonuclease, and a negative staining control is also shown.

**Effect of E-64 on PCD induced by G418**

Incubation of parasites with the E-64 inhibitor abolished DNA fragmentation induced by G418. As shown in Fig. 2(b), lanes 3 and 4, the co-incubation of trophozoites with 50 and 20 μM, respectively, of E-64 inhibited DNA fragmentation, and DNA degradation almost disappeared, especially with the higher E-64 concentration used. Instead, a high-molecular-mass DNA band was observed, similar to that observed with the NT trophozoites (Fig. 2a, lane 2). TUNEL assays of trophozoites treated with the E-64 inhibitor also showed a remarkable reduction in nuclear staining, so that less than 15% of trophozoite nuclei were stained (Fig. 2c).

**Transmission electron microscopy analysis**

By transmission electron microscopy, it was observed that G418 altered the typical morphology of *E. histolytica* nuclei. After 3 h of incubation with G418, trophozoites did not show any morphological differences from the control. Cell size was normal, with abundant vacuoles and glycogen deposits in the cytoplasm. The nucleus had dense peripheral chromatin with a central ‘endosome’. The nuclear and plasma membranes appeared intact (Fig. 2d). After 9 h of incubation with G418, a different distribution of fragmented chromatin was observed, with the chromatin displaced to one side of the amoeba nucleus. The cytoplasm contained large vacuoles, and the amount of glycogen was increased. The nucleus was smaller than in NT trophozoites. After 12 h of G418 incubation, a smaller nucleus containing fragmented, dense chromatin was observed, and the round nuclear bodies were more
Fig. 2. DNA fragmentation and ultrastructural changes in trophozoites after G418 and E-64 treatments. (a) Agarose gel electrophoresis analysis of DNA. Lanes: 1, M, 1000 DNA marker; 2, DNA from NT trophozoites; 3, DNA from G418-treated trophozoites. (b) Agarose gel electrophoresis analysis of DNA from trophozoites treated with E-64. Lanes: 1, M, DNA marker; 2, DNA from G418-treated trophozoites; 3 and 4, DNA from trophozoites co-incubated with G418 and 50 and 20 μM E-64, respectively. (c) Confocal microscopy analysis showing nuclear TUNEL staining of trophozoites after 6 h of incubation with G418, G418/E-64, and in NT trophozoites. As a positive control, trophozoites were treated with 20 μg DNase I ml⁻¹ and negative staining is also shown. Bars, 20 μm. (d) Ultrastructure of trophozoites after G418 and G418/E-64 treatments. A healthy NT trophozoite displaying a round nucleus (N) and dense peripheral chromatin (arrows) is shown in the upper-left panel; a central endosome is also seen. The upper-right panel shows the ultrastructure after 9 h of G418 treatment. Clumps of chromatin have gathered mainly at one side of the nuclear envelope (arrows). The cytoplasm contains large clear vacuoles (V) and areas of glycogen (G). The lower-left panel shows the ultrastructure after 12 h of incubation with G418. A condensed nucleus is occupied by dense chromatin, with loss of the nuclear membrane (Nm). Round, dense nuclear bodies are conspicuous (arrows). Irregular, clear areas that correspond to glycogen are abundant (G). The plasma membranes in all trophozoites appeared intact (arrowheads). Bars, 1 μm. The lower-right panel shows the ultrastructure after 9 h of coincubation with G418 and 50 μM E-64. A central endosome is seen with a round nucleus (N) and dense peripheral chromatin. No DNA lesions were observed.
conspicuous. The outer limits of the nuclear envelope were not clearly defined. Cytoplasmic glycogen was increased significantly, and the number and size of vacuoles decreased substantially. For all times of incubation studied, cytoplasmic membranes appeared normal. In trophozoites co-incubated with G418 and 50 μM E-64, the cell size and nucleus appeared normal. As in the control trophozoites, the nucleus had dense peripheral chromatin, and no obvious DNA lesion was observed.

G418 does not produce detectable changes in PS externalization

During the early stages of typical PCD, translocation of PS from the inner to the outer layer of the plasma membrane occurs. To look for this phenomenon in G418-treated amoebae, we used flow cytomtery and annexin V–FITC, which binds with high affinity to PS. No positive fluorescence was detected after incubation of trophozoites with G418 (Fig. 3). Aley and co-workers (Aley et al., 1980) reported that PS forms less than 10% of E. histolytica trophozoite membrane lipids. Thus, we searched for PS by confocal microscopy in permeabilized and unpermeabilized trophozoites. As shown in Fig. 3, no fluorescence signal was observed in the outer or inner plasma membrane. This result suggests that either annexin V is unable to recognize E. histolytica PS, or PS is not a component of the E. histolytica plasma membrane. As an internal control, PS was detected in apoptotic lymphocytes (data not shown).

G418 induces oxidative stress in E. histolytica trophozoites

Because the generation of intracellular ROS is associated with PCD, we analysed the production of ROS in G418-treated trophozoites by flow cytometry, determining the conversion of DCFDA to the highly fluorescent 2,7-dichlorofluorescein in the presence of a suitable oxidant. As shown in Fig. 4, NT trophozoites displayed ROS signals near to the control histogram peak, whereas G418-treated trophozoites exhibited substantial enhancement of ROS production: 62% of the trophozoite population showed 10-fold increased fluorescence compared with the NT trophozoites.
G418 induces changes in trophozoite K\textsuperscript{+} levels

Overproduction of ROS inactivates the Na\textsuperscript{+}–K\textsuperscript{+} ATPase pump, decreasing the K\textsubscript{z}i level (Sen et al., 2004a). Thus, extrusion of K\textsuperscript{+} ions and the subsequent loss of cell volume are among the most notable events of typical PCD. By using PBFI-AM fluorescent dye, the trophozoite K\textsubscript{z}i concentration was analysed after G418 treatment. Without G418 treatment, 100% of trophozoites had a strong fluorescence signal, reflecting intracellular pools of K\textsuperscript{+}. After G418 treatment, the intensity of fluorescence decreased by two orders of magnitude, evidencing a substantial loss of potassium (Fig. 5). Based on data published elsewhere (Sen et al., 2004b), it appears that impairment of the Na\textsuperscript{+}–K\textsuperscript{+} ATPase pump is a consequence of high ROS levels inside the cell and of lipid peroxidation.

G418 increases trophozoite cytosolic Ca\textsuperscript{2+} levels

Many studies have shown that calcium flux is required for the activation of several apoptotic mechanisms (Tandogan & Ulusu, 2005). The increase in intracellular Ca\textsuperscript{2+} after G418 treatment was measured during a period of 120 min by spectrofluorometric analysis. The [Ca\textsuperscript{2+}]i of NT trophozoites remained stable (20 nM Ca\textsuperscript{2+}) over the time period. However, in parasites treated with G418, the Ca\textsuperscript{2+} concentration increased from 20 nM at the beginning to 44 nM at 80 min, with a maximum of 48 nM Ca\textsuperscript{2+} at 120 min of incubation with G418 (Fig. 6). The chelator EGTA was used as a control. As expected, EGTA greatly diminished the intracellular Ca\textsuperscript{2+} in both G418-treated and NT parasites.

Acidification of trophozoite pH\textsubscript{i}

In other systems, increased endogenous ROS and intracellular Ca\textsuperscript{2+} are responsible for the loss of mitochondrial and endoplasmic reticulum membrane potentials, which subsequently decreases pH\textsubscript{i} levels (Demaurex et al., 2003). To evaluate the pH\textsubscript{i} as a consequence of G418-induced
PCD, a fluorescence method was utilized. Trophozoites were loaded with an acetomethyl ester derivative of BCECF, a dye whose fluorescence emission is sensitive to pH variations. The excitation wavelengths were 440 and 490 nm, and the emission was recorded at 535 nm. The pH of treated and NT parasites was recorded at pH 6.8. The pH of NT trophozoites remained constant at 7.8 over the incubation period. In contrast, a significant decrease in pH, from 7.8 to 6.0, was observed after 3 h of G418 incubation (Fig. 7). These results indicate that the pH of trophozoites undergoing PCD is more acidic than that of NT trophozoites.

DISCUSSION

It has been assumed that apoptosis, a form of PCD, was developed by multicellular organisms to regulate growth and development (Jacobson et al., 1997). However, recent reports have indicated that PCD also occurs in some species of unicellular organisms, including bacteria (Sat et al., 2001), yeast (Madeo et al., 1999), D. discoideum (Cornillon et al., 1994), and several protozoa such as P. gatunense (Vardi et al., 1999), Eu. gracilis (Scheuerlein et al., 1995), Tet. thermophila (Christensen et al., 1995), trypanosomatids (Nguewa et al., 2004), Plasmodium (Al-Olayan et al., 2002) and Blastocystis hominis (Nasirudeen et al., 2004). Evidence for a cell-suicide pathway in unicellular organisms that is analogous to metazoan apoptosis strongly suggests that PCD confers evolutionary advantages upon micro-organisms, including (i) selection of the best-adapted individuals in response to environmental changes (Lee et al., 2002; Verma & Dey, 2004), (ii) regulation of the competition of parasites for limited resources in the gut or within the host (Dale et al., 1995), (iii) regulation of the cell cycle and cell differentiation (Hesse et al., 1995), and (iv) selection of specific parasitic forms, as non-infectious forms do not contribute to perpetuation of the parasite and might compete with the infectious parasites for available nutrients (Welburn et al., 1997). Some pathogens that infect mammalian hosts have developed mechanisms to repress programmed death in the cells required for pathogen replication or persistence, as well as mechanisms to induce programmed death in immune cells that may target the infected cell for destruction (Williams, 1994). These mechanisms not only favour immune evasion (Ameisen et al., 1994) but also might allow the growth of pathogens in host cells through uptake of apoptotic cells (Freire-de-Lima et al., 2000).

Seydel & Stanley (1998) and Huston et al. (2003) demonstrated that E. histolytica trophozoites kill host cells by inducing apoptosis followed by phagocytic cell clearance, suggesting that this mechanism may limit inflammation and enable amoebae to evade the host immune response. Recently, Ramos et al. (2007) reported the in vitro induction of apoptosis in trophozoites after treatment with nitric oxide species. Taking into consideration the putative role of programmed death in the host–amoeba relationship, the present study investigated the induction of PCD in E. histolytica in response to an undesirable external stimulus, the exposure of trophozoites to the aminoglycoside antibiotic G418. G418 induces apoptosis in kidney cells (Jin et al., 2004) and in ear sensory hair cells (Matsui et al., 2004) by a caspase-3-dependent mechanism, and in T. cruzi (Billaut-Mulot et al., 1996) by an unknown mechanism.

In E. histolytica trophozoites, G418 caused a reduction in cell size and shrinkage of the cytoplasm, two of the most reliable morphological criteria for defining PCD (Huppertz et al., 1999). Flow cytometry and electron microscopy were employed to examine key ultrastructural features. Flow cytometry analysis showed that the decrease in cell size is...
accompanied by an increase in cell granularity, suggesting that vacuolization may also be related to cell death. Transmission electron micrographs of PCD-induced trophozoites confirmed the characteristics of the PCD process: cell shrinkage with an increased number and size of vacuoles, nuclear condensation, chromatin fragmentation, and, importantly, preservation of trophozoite cell-membrane integrity. Vacuolization has been reported in PCD of Caenorhabditis elegans (Robertson & Thomson, 1982), D. discoideum (Cornillon et al., 1994), and some types of higher eukaryote cells (Wyllie et al., 1980; Clarke, 1990). During apoptosis, early ultrastructural nuclear lesions at a high level of chromatin organization lead to the appearance of large DNA fragments (300 and/or 50 kb) revealed by PFGE (Walker et al., 1991; Tomei et al., 1993). This is often followed by lower-level DNA fragmentation (Wyllie, 1980), resulting in a gel electrophoresis ladder pattern of DNA fragments of 180–200 bp and multiples thereof. In the present study, an obvious DNA fragmentation ladder could not be detected by gel electrophoresis analysis. Instead, a smear of degraded DNA and faint ladder bands were observed. However, DNA condensation and cleavage without disintegration of the cellular membrane were observed by transmission electron microscopy.

The highly sensitive TUNEL technique confirmed that an intracellular suicide program, rather than a necrotic process, is triggered in trophozoites during incubation with the antibiotic G418. TUNEL detects 3’ OH groups at the ends of single- and double-stranded DNA breaks, whereas DNA cleavage in early necrosis is characterized by selective generation of 5’ overhangs but no 3’ overhangs (Didenko et al., 2003). Similar positive results have been obtained in E. histolytica by TUNEL and YOPRO-1 after induction with nitric oxide species (Ramos et al., 2007). However, some differences were observed in DNA fragmentation patterns: while the above authors reported four bands smaller than 500 bp, our results showed a more heterogeneous digestion pattern. The irregular nucleosomal organization of chromatin in E. histolytica reported by Torres-Guerrero et al. (1991) accords with our findings. Similarly, D. discoideum PCD is not characterized by DNA laddering (Cornillon et al., 1994). Alternatively, there are some reports that indicate that DNA fragmentation cannot always be regarded as a hallmark of apoptosis, as certain cells display morphological and biochemical features of apoptosis without a typical ladder-like DNA fragmentation (Collins et al., 1992; Howell & Martz, 1987; Barbieri et al., 1992; Mesner et al., 1992; Falcieri et al., 1993; Vaux et al., 1994; Hirata et al., 1998).

We searched by in silico analysis for the presence of a putative caspase-like protein in the E. histolytica genome (TIGR 9712) (data not shown). The results did not show any matches that suggested the presence of a caspase-like protein, although the parasite contains 50 cysteine protease genes (Bruchhaus et al., 2003; Tillack et al., 2007). Ramos et al. (2007) have clearly demonstrated that E-64, a specific cysteine protease inhibitor, efficiently blocks E. histolytica cysteine protease activity. Thus, we decided to investigate the effect of E-64 on one of the most important features of PCD, DNA alteration. We showed that E-64 abolishes DNA degradation, as demonstrated by gel electrophoresis, TUNEL and electron microscopy ultrastructure, strongly suggesting that at least one of the cysteine proteases reported participates in G418-induced PCD. Our results contrast with those published by Ramos et al. (2007), in which the authors speculate that nitric oxide species induce a cysteine protease-independent apoptosis. This affirmation was based on the fact that E-64 treatment failed to abolish the death of trophozoites; however, no experiments were carried out to determine the effects with respect to the morphological and molecular characteristics of PCD.

In the early stages of eukaryote apoptosis, cells externalize PS, while maintaining membrane integrity (Gatti et al., 1998). As evidenced by electron microscopy, E. histolytica trophozoites induced to undergo PCD maintain membrane integrity, although annexin V–FITC failed to detect PS in the outer leaflet of the plasma membrane of NT or G418-treated trophozoites. Aley et al. (1980) reported that PS makes up less than 10% of total membrane lipids in the plasma membrane of E. histolytica trophozoites. Martin et al. (1993) did not detect PS as a constituent of the E. histolytica plasma membrane by using 31P-NMR spectroscopy. They reported that the major phospholipids in whole amoebic extracts were phosphatidylycholine and two phosphatidylethanolamine species. Taking these findings into consideration, the results obtained here suggest three possibilities: (i) the abundance of PS is insufficient for detection by the method used here, (ii) the E. histolytica plasma membrane does not contain PS, or (iii) PS interacts with other membrane components that block its interaction with annexin V.

In a typical apoptotic process, cell shrinkage is due to loss of cytoplasmic fluids and to the denaturation of proteins (Huppertz et al., 1999), producing characteristic biochemical features. It has been proposed that the generation of ROS inside cells causes an increase in the level of lipid peroxidation (Sen et al., 2004b). Lipid peroxidation decreases membrane fluidity and increases the leakage of the membrane, leading to complete loss of cytoplasmic fluids and membrane integrity (Halliwell & Gutteridge, 1989). This, in turn, causes a decrease in K+ and an increase in intracellular Ca2+ levels. The present study showed that E. histolytica PCD induced by G418 was accompanied by twofold increased intracellular ROS levels. Several studies (Kroemer & Reed, 2000) support the hypothesis that disruption of membrane potential is an irreversible commitment to cell death. Most cells achieve and maintain balance of osmotic pressure through continuous activity of the Na+-K+ ATPase pump, which creates and maintains an intracellular environment high in K+ and low in Na+. It has been proposed that ROS inactivate the ATPase pump with the subsequent movement of ions (specifically K+) out of the cell, resulting in
the loss of cell volume during apoptosis (Bortner et al., 1997). Our flow-cytometry results revealed that K\textsuperscript{i} levels decreased by more than 90% in the G418-treated trophozoites compared with NT trophozoites. Additionally, oxidative stress causes increased cytosolic Ca\textsuperscript{2+} levels, another common feature of apoptosis (Jiang et al., 1994). Our results showed a significant increase in the Ca\textsuperscript{2+} level in PCD-induced trophozoites, suggesting that Ca\textsuperscript{2+} has a pivotal role in this process in *E. histolytica*. Ca\textsuperscript{2+} is necessary for the activation of different enzymes, including cysteine proteases, that participate in PCD (Tagliarino et al., 2001). Finally, it has been suggested that immediately after the loss of the membrane potential, protons are released into the cytosol, thus contributing to intracellular acidification (Facompre et al., 2001). pH changes modulate the apoptotic responsiveness of the cell, and also amplify the apoptotic program by regulating enzymic activities (Matsuyama et al., 2000). As a consequence of the overproduction of ROS and the loss of K\textsuperscript{i}, a diminished pH\textsuperscript{i} was observed for PCD-induced trophozoites.

In conclusion, the present study demonstrates, for what is believed to be the first time, PCD in *E. histolytica* induced by an external drug stimulus. This process is orchestrated by coordinated alterations in intracellular ion fluxes and subsequent morphological changes and ultrastructural alterations in DNA that are analogous to the events observed during PCD in other organisms (Table 1). Work currently in progress will allow us to determine the molecular components and steps involved in this intricate process, and also how this mechanism of cell death can be induced by other drugs. This knowledge will provide new insights into the host–parasite relationship and potential molecular targets for drug design.

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**REFERENCES**


**Table 1. Characteristics of PCD in unicellular organisms**

Abbreviations: –, absence; +, presence; ND, not determined.

<table>
<thead>
<tr>
<th>Organism</th>
<th>Cellular morphology</th>
<th>Nuclear alteration</th>
<th>Biochemical change</th>
<th>Caspase</th>
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<tr>
<td></td>
<td>Cell shrinkage</td>
<td>Chromatin condensation</td>
<td>DNA fragmentation</td>
<td>TUNEL</td>
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<tr>
<td><em>D. discoideum</em></td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>ND</td>
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<tr>
<td><em>B. hominis</em></td>
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<td>−</td>
<td>+</td>
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<tr>
<td><em>Saccharomyces cerevisiae</em></td>
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<td><em>Leishmania</em></td>
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<tr>
<td><em>E. histolytica</em></td>
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*Increase.
†Decrease.
$Paracaspase.
§Caspase-like.
||Metacaspase.
‡Cysteine protease.


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