Metronidazole induces programmed cell death in the protozoan parasite Blastocystis hominis

A. M. A. Nasirudeen, Yap Eu Hian, Mulkit Singh and Kevin S. W. Tan

Department of Microbiology, Faculty of Medicine, National University of Singapore, 5 Science Drive 2, Singapore 117597

Previous studies by the authors have shown that the protozoan parasite Blastocystis hominis succumbed to a cytotoxic monoclonal antibody with a number of cellular and biochemical features characteristic of apoptosis in higher eukaryotes. The present study reports that apoptosis-like features are also observed in growing cultures of axenic B. hominis upon exposure to metronidazole, a drug commonly used for the treatment of blastocystosis. Upon treatment with the drug, B. hominis cells displayed key morphological and biochemical features of programmed cell death (PCD), viz. nuclear condensation and nicked DNA in nucleus, reduced cytoplasmic volume, externalization of phosphatidylserine and maintenance of plasma membrane integrity with increasing permeability. This present study also supports the authors’ previously postulated novel function for the B. hominis central vacuole in PCD; it acts as a repository where apoptotic bodies are stored before being released into the extracellular space. The implications and possible roles of PCD in B. hominis are discussed.

INTRODUCTION

Programmed cell death (PCD) has been noted in all invertebrate and vertebrate multicellular organisms studied so far, including nematodes, insects, amphibians and mammals (Ellis et al., 1991; Raff, 1992; Vaux, 1993; Steller, 1995). It is thought to have evolved to regulate growth and development in multicellular organisms (Evan, 1994; Vaux et al., 1994) and as a defence mechanism against viral and bacterial infections (Shen & Shenk, 1995; Weinrauch & Zychlinsky, 1999). Unnecessary, damaged, infected and potentially harmful cells are deleted from surrounding healthy ones to ensure structural and functional homeostasis. Apoptosis, a form of PCD, is characterized by a unique pattern of morphological changes in both the cell nucleus and the cytoplasm. Although different cell types do not necessarily display all the hallmarks of apoptosis, characteristic features appear to be conserved in cells undergoing this form of cell death. These include shrinkage of the cell, preservation of membrane integrity with increasing permeability, chromatin condensation and DNA fragmentation, and externalization of plasma membrane phosphatidylserine (PS) residues (Saraste & Pulkki, 2000).

Although it was long assumed that PCD evolved with multicellularity, recent reports have suggested that similar cell-death machinery exists in protozoans such as Leishmania (Leishmania) amazonensis (Moreira et al., 1996), Trypanosoma cruzi (Ameisen et al., 1995), Trypanosoma brucei rhodesiense (Welburn et al., 1996), Dictyostelium discoideum (Cornillon et al., 1994), Plasmodium falciparum (Picot et al., 1997) and Plasmoidium berghei (Al-Olayan et al., 2002).

In our earlier study (Nasirudeen et al., 2001a), we showed that the human intestinal protozoan parasite Blastocystis hominis undergoes PCD when treated with a surface-reactive cytotoxic mAb (1D5), with typical features of apoptotic cells. Ageing agar cultures of B. hominis also displayed ultrastructural features of apoptosis (Tan et al., 2001). The presence of an apoptotic response to undesirable external stimuli led us to investigate if a similar phenotype is observed upon drug exposure. Drugs such as staurosporine have been shown to cause cell death in mammalian cell lines (Nakazono-Kusaba et al., 2002; Koh et al., 1995). In protozoan parasites such as P. falciparum, chloroquine has been reported to induce cell death (Picot et al., 1997). T. brucei rhodesiense undergoes apoptosis when treated with concanavalin A (Welburn et al., 1996). Free-living unicellular organisms have also been reported to undergo apoptosis. Davis et al. (1992) observed apoptotic-like nuclear degeneration in conjugating Tetrahymena cells while Ludovico et al. (2001a, b) reported that PCD can be induced by acetic acid in Saccharomyces cerevisiae. Taken together, these observations suggest that many unicellular organisms have the unusual capacity to activate a complex cell-death programme similar to their multicellular counterparts. This feature has been postulated to aid these microorganisms in regulation of growth and developmental

Abbreviations: PCD, programmed cell death; PI, propidium iodide; PS, phosphatidylserine; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling.
processes, and may also provide us with clues on host–parasite interactions and pathogenesis (Knight, 2002).

The present study demonstrates characteristic features of PCD in the human intestinal protozoan parasite B. hominis after exposure to 5 × 10⁻⁷ M metronidazole. Metronidazole is a nitroimidazole antibiotic used clinically to treat B. hominis infections as well as for the treatment of anaerobic infections in humans (Garavelli, 1991; Hager & Rapp, 1992). Dunn & Boreham (1991) developed an assay to measure the sensitivity of drugs against B. hominis and arrived at an ID₅₀ value of 3.34 × 10⁻⁷ M for metronidazole.

The pathogenicity of B. hominis is a controversial issue: some authors consider it to be a pathogen (Lee, 1991; Llibre et al., 1997, 1999), whereas others conclude that it is a harmless commensal (Rosenblatt, 1990; Sun et al., 1989). Symptoms reported in B. hominis infection include diarrhoea, abdominal pain, flatulence, nausea and constipation (Ok et al., 1999). Several reports show the parasite to be more pathogenic in immunosuppressed patients such as those with AIDS (Libre et al., 1989). Although metronidazole has been used clinically as a drug to treat B. hominis infections, little effort has been made to evaluate the pattern of death induced by this drug. In the present study, we have employed flow cytometry and electron microscopy to study the effects of metronidazole on B. hominis.

**METHODS**

**Parasite and growth conditions.** B. hominis isolate B was obtained from a local patient and maintained axenically in Iscove’s modified Dulbecco’s medium (IMDM) containing 10% horse serum (Ho et al., 1993). Cells were grown anaerobically (Concept Plus Anaerobic Workstation; Ruskin Technology) at 37°C.

**Metronidazole.** Metronidazole, 2-methyl-5-nitroimidazole-1-ethanol, is a 5-nitroimidazole drug used in the treatment of bacterial infections and other anaerobic infections. Stock solutions of metronidazole M-1547 (Sigma) were prepared in double distilled water and further diluted in IMDM to obtain the desired concentration.

**Treatment with metronidazole to induce cell death.** Metronidazole (5 × 10⁻⁷ M) was used to induce cell death in B. hominis. Cells were inoculated into pre-reduced IMDM to give a final concentration of 2 × 10⁶ cells ml⁻¹. Metronidazole was then added to a final concentration of 5 × 10⁻⁷ M. Two controls were included in the experiments: (i) cells grown in normal culture conditions (IMDM with 10% horse serum); (ii) necrotic cell control of B. hominis was obtained by incubating cells with 0-1% sodium azide and harvesting 24 h post-treatment. Cells were harvested at 3, 6, 9 and 12 h for flow cytometry analysis and phase-contrast microscopy. Similar conditions were followed for ultrastructural studies except that necrosis was induced by heating the cells in an 80°C water bath for 15 min.

**Membrane integrity analysis and detection of PS externalization.** The detection of loss of membrane permeability and exposure of PS was detected using an Annexin V/FITC kit (BenderMed Systems) as described previously (Nasirudeen et al., 2001a). Briefly, 2 × 10⁶ cells incubated in the presence and absence of metronidazole were harvested at 3, 6, 9 and 12 h. Cells were washed twice in 1 ml PBS (pH 7.4). Then, 190 μl calcium-containing binding buffer, 0.21 μg ml⁻¹ FITC–Annexin V and 2.5 μg ml⁻¹ propidium iodide (PI) were added sequentially. The samples were measured by flow cytometry (Coulter Epics Elite ESP) for FITC/PI fluorescence and results were displayed using the WINMDI 2.7 software program. Exclusion of PI from these cells was taken as a quantitative marker for membrane integrity (Cornillon et al., 1994).

**DNA ladder assay.** In the presence and absence of metronidazole, 5 × 10⁶ cells ml⁻¹ were incubated and harvested at 3, 6, 9 and 12 h intervals. An apoptotic DNA ladder kit (Boehringer Mannheim) was used to extract DNA from apoptosis-induced and uninduced cells according to the manufacturer’s instructions. Apoptotic U937 cells (histiocytic lymphoma cells), included in the kit, were used as a positive control. DNA was electrophoresed in 2% agarose gels at 100 V for 2 h and visualized by using an UV transilluminator; the gels were photographed with a Polaroid camera.

**Nick-labelling of internucleosomal DNA fragments.** Terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick-end labelling (TUNEL) was performed using the ApoAlert™ DNA Fragmentation Assay kit (Clontech). Metronidazole-treated and untreated cells (2 × 10⁶ cells ml⁻¹) were washed twice with 1 ml PBS and fixed with 4% formaldehyde/PBS for 25 min at 4°C. After two washes with PBS, the pellet was resuspended in 5 ml permeabilization solution (0.2% Triton X-100 in PBS) and incubated on ice for 5 min. Eighty microlitres of equilibration buffer were added and the cells were incubated at room temperature for 5 min. The cells were labelled by adding 50 μl TUNEL mix followed by incubation for 60 min at 37°C in a dark, humidified incubator. One millilitre of 20 mM EDTA was then added to terminate the tailing reaction. The samples were washed with PBS and the pellet was resuspended in 250 μl PBS prior to flow cytometry using an argon-ion laser tuned to 488 nm. Green fluorescence was detected using a 525 nm band-pass filter. A region (M1) was defined to exclude background fluorescence by unstained cells. Stained parasites that fell within the M1 region were represented as a percentage of total cells analysed.

**Transmission electron microscopy analysis.** In the presence and absence of metronidazole, 1 × 10⁶ cells were incubated and harvested at 3, 6, 9 and 12 h intervals. Necrosis was induced by heating the cells in an 80°C water bath for 15 min. Cells were washed twice in 0-1 M sodium cacodylate buffer with 5% sucrose and fixed sequentially in each of the following fixatives: 1% glutaraldehyde in 0-5 M sodium cacodylate buffer. DNA was nicked by using a 1% triton X-100 and visualized by using an UV transilluminator; the gels were photographed with a Polaroid camera.

**Reproducibility of results.** All experiments were repeated at least twice, except for transmission electron microscopy analysis, which was performed once.

**RESULTS**

**Cell shrinkage in metronidazole-treated cells**

Phase-contrast microscopy revealed that B. hominis cells grown in normal culture conditions displayed healthy morphology, as indicated by a spherical shape, clear central vacuole and peripheral cytoplasm (Fig. 1a). Metronidazole-treated B. hominis cells, however, appeared smaller and darker (Fig. 1b). Cell shrinkage was confirmed using flow
Fig. 1. Effect of metronidazole on B. hominis cell size as determined by light microscopy and flow cytometry. (a) Cells grown in normal culture conditions; (b) cells exposed to $5 \times 10^{-7}$ M metronidazole. Note cell shrinkage, condensed cytoplasm and darkening of cells after exposure to metronidazole. Bars, 20 μm. (c) Flow cytometry analysis of cell size and granularity. Dot plots show two distinct populations of cells. The population on the left (R2; green) is due to dead cells and debris while the population on the right (R1; red) could be a mixture of healthy cells and cells undergoing PCD.
cytometry where metronidazole-treated cells showed progressive reduction in cell size (Fig. 1c). Dot plot displayed two distinct populations of cells. The population on the right (R1-gated) represents larger cells (healthy and early apoptotic cells) while the population on the left (R2-gated) represents late apoptotic and necrotic cells, and cellular debris.

Preservation of membrane integrity and externalization of PS in cells undergoing PCD

Loss of cell viability is most often measured as loss of membrane integrity. A short incubation with PI was used to investigate membrane integrity in B. hominis cells undergoing PCD. PI can only enter cells with an altered plasma membrane and then stain the nucleic acid, giving red fluorescence. Exclusion of PI by membrane-intact cells was scored as preservation of membrane integrity. This dye was used together with Annexin V, which binds PS exposed during apoptosis. The results were analysed by flow cytometry (Fig. 2): 2·87% of the metronidazole-treated cells were PI-positive at 3 h and 4·60% were PI-positive at 12 h. Cells grown in normal culture media showed 1·45% PI-positive cells at 3 h and 1·13% PI-positive cells at 12 h. Flow cytometry data revealed a gradual increase in dying cells (increase in cell permeability) due to metronidazole treatment. Approximately 30% of the B. hominis cells were PI-positive when treated with 0·1% sodium azide for 24 h.

In normal cells, under physiological conditions, PS is segregated to the inner leaflet of the plasma membrane. During the early stages of apoptosis/PCD, this asymmetry collapses and PS is exposed onto the outer surface of cells (Gatti et al., 1998) while maintaining membrane integrity. Annexin V (a PS-binding protein) preferentially binds PS in a calcium-dependent manner. An Annexin V assay was used to detect the presence of PS on the plasma membrane, while the simultaneous presence and absence of PI accumulation indicates apoptotic or necrotic states, respectively. Increase in PS translocation was detected by flow cytometry in metronidazole-treated cells over a period of 9 h. As shown in Fig. 3, before treatment with metronidazole, only 2·57% of the cells were Annexin V-positive. However, within 3 h of metronidazole treatment, a population of cells with dramatically increased Annexin V binding began to emerge in these cultures and increased rapidly thereafter. Annexin V binding in these cells increased from 32·39% at 3 h to 51·09% at 9 h as compared to 2·72% of control cells at 9 h. At 12 h, the Annexin V binding of metronidazole-treated cells dropped to 30·78%. Necrotic cells showed only 9·90% Annexin V binding.

Absence of DNA laddering in metronidazole-treated B. hominis cells

DNA fragmentation and the display of DNA ladders, often of 200 bp and multiples thereof, in agarose gel electrophoresis are common features of apoptosis. Agarose gel electrophoresis of B. hominis DNA in the presence and absence of metronidazole and in the presence of 0·1% sodium azide is shown in Fig. 4. No DNA ladder was observed in any of the experiments. A positive control consisting of apoptotic U937 cells (Boehringer Mannheim) showed a DNA-laddering profile typical of apoptosis (Fig. 4).

Presence of in situ DNA fragmentation in the nucleus of metronidazole-treated cells

Endonuclease activity was evaluated with the TUNEL assay. TUNEL relies on the specific binding of terminal deoxyribonucleotidyl transferase to exposed 3'-OH ends of DNA followed by the synthesis of a labelled polydeoxyribonucleotide molecule. Nuclear DNA is first exposed by proteolytic treatment; this is followed by the incorporation of biotinylated deoxyuridine triphosphate by terminal deoxyribonucleotidyl transferase into the sites of DNA breaks. The signal is amplified by avidin–peroxidase, enabling conventional histochemical identification of PCD by flow cytometry. Approximately 12·4% of metronidazole-treated cells were TUNEL labelled by 3 h and 58·8% were maximally labelled by 9 h. Control cells in normal growth media showed minimal DNA fragmentation. Formation of DNA strand breaks as a consequence of endonuclease activity can be detected by the TUNEL assay and expressed as a percentage of TUNEL-positive cells. Such activity in parasites cultured in normal growth media was approximately 2·0 and 3·1% at 3 and 9 h, respectively. At 12 h, TUNEL of metronidazole-treated B. hominis dipped to 34·8%. B. hominis cells that were exposed to 0·1% sodium azide (necrotic control) showed 11% TUNEL (Fig. 5).
Ultrastructural features of apoptosis in metronidazole-treated cells

Transmission electron microscopy micrographs showed that cultures in normal growth media had classical *B. hominis* morphology (Fig. 6a). Normal DNA chromatin, which is usually seen as a crescentic mass at the nuclear periphery, was visible within the nucleus (Fig. 6a). In contrast, *B. hominis* cells treated with metronidazole showed morphological changes suggestive of apoptosis. In the presence of metronidazole, the nuclei appear distinctively smaller and the nuclear chromatin had segregated as distinct clumps along the nuclear periphery (Fig. 6b). The presence of large cytoplasmic vacuoles, appearing empty or containing some inclusions, the invagination of the cytoplasm into the central vacuole (Fig. 6c) and the presence of membrane-bound electron-dense particles (apoptotic bodies) in the central vacuole (Fig. 6d) were noted. The apoptotic bodies in the central vacuole appeared to be released into the extracellular space. At 12 h, a number of cells showed a break in the central vacuole, suggesting that most or all of the apoptotic bodies had been released (Fig. 6e). Cells that underwent necrosis presented a morphology distinctively different from that of apoptotic *B. hominis*. Necrotic cells were electron-lucent and showed swelling of organelles such as mitochondria and nucleus, and the central vacuole seemed devoid of any electron-dense particles (Fig. 6f).

DISCUSSION

Metronidazole is an antibiotic used for the treatment of anaerobic microbes including *B. hominis*. Dunn & Boreham (1991) showed that metronidazole treatment of *B. hominis* impaired its growth at an ID$_{50}$ value of $3.34 \times 10^{-7}$ M. We therefore reasoned that the concentration of $5 \times 10^{-7}$ M used in this study should be sufficient to induce cell death in a majority of the parasites in culture.

Metronidazole enters cells and mitochondria by simple diffusion. In mitochondria, metronidazole competes efficiently with the natural electron acceptor for electrons.
Organisms susceptible to 5-nitroimidazoles transfer electrons generated by their electron-transport systems to the nitro group of the drugs and not to their natural electron acceptor (Kulda, 1999). The reduction of the metronidazole nitro group results in the synthesis of cytotoxic radicals (R-NO$_2$) (Kulda, 1999). Hence, the antimicrobial effect of metronidazole depends on its metabolic reduction within the target cell resulting in the release of cytotoxic radicals (Kulda, 1999).

The present study shows that metronidazole causes apoptotic-like death in _B. hominis_. In an earlier study, we described similar apoptotic-like features in this organism in response to a cytotoxic antibody exposure (Nasirudeen _et al._, 2001a). When treated with 5 x 10$^{-7}$ M metronidazole, light microscopic data showed cell shrinkage and darkening of the cytoplasm in _B. hominis_. Cell shrinkage due to compaction of organelles in the cytoplasm represents important morphological evidence of apoptosis. This could be due to loss of cytoplasmic fluids and denaturation of proteins in apoptotic cells (Huppertz _et al._, 1999). Flow cytometry data further supported cell shrinkage observed under light microscopy.

In apoptosis research, electron microscopy is often employed to observe key ultrastructural features. Transmission electron micrographs of metronidazole-treated _B. hominis_ clearly showed ultrastructural characteristics of apoptosis. Nuclear condensation, cell shrinkage, deposition of membrane-bound apoptotic bodies, maintenance of cytosolic organelle structure and size (unlike necrosis where the organelles swell and appear disoriented) and heavy vacuolization clearly provide ultrastructural evidence of apoptosis in metronidazole-treated _B. hominis_.

Flow cytometry analysis revealed that metronidazole-treated _B. hominis_ preserved its membrane integrity with PI-exclusion levels comparable to those of normal _B. hominis_ cells (<10% of cells PI-positive). These were much lower than those observed in control necrotic cells, which gave PI levels of approximately 30%. Upon exposure to metronidazole, only a small percentage of cells became permeable to PI while most of the cells maintained membrane integrity. In cells grown in normal media, there was no significant increase in the uptake of PI, indicating normal growth and an intact plasma membrane.

**Fig. 5.** Representative histograms and graph showing _in situ_ DNA fragmentation analysis (TUNEL) of _B. hominis_ cells by flow cytometry. Note significant TUNEL intensity increase in metronidazole-treated cells. ■, Metronidazole-treated cells; ◆, untreated cells. In the lower graph, results are given based on three replicates and are shown ±SD.

**Fig. 6.** Transmission electron micrographs of _B. hominis_ cells exposed to metronidazole for 3–12 h. (a) Healthy _B. hominis_ cell displaying normal morphology and DNA chromatin seen as a crescentic mass (arrow). (b) Segregation of condensed chromatin to the nuclear periphery and condensation of nucleus (arrow), resulting in a large intramembranous space. (c) Large cytoplasmic vacuoles (V), cytoplasm pinching inwards into central vacuole (arrow). (d) Membrane-bound organelle-containing vesicles within the central vacuole. (e) At 12 h, the cells have apparently released most or all of their apoptotic bodies. (f) Necrotic cells with swollen mitochondria and nucleus and chromatin seen in large clumps in necrotic cells. Note clear central vacuole in necrotic cells. N, nucleus; CV, central vacuole; M, mitochondria; V, vacuole. Bars, 1 μm.
Metronidazole-induced apoptosis in *Blastocystis*
In higher eukaryotes, apoptotic cells exhibit externalization of PS in the early stages of cell death while maintaining membrane integrity (Gatti et al., 1998). This can easily be detected by Annexin V staining. At 9 h exposure to metronidazole, Annexin V-positive B. hominis cells had increased 10-fold from ~5 to 50 %, while membrane integrity was largely intact. At 12 h of metronidazole treatment, the number of B. hominis cells stained with Annexin V decreased. This decrease in Annexin V staining could be largely due to the release of apoptotic bodies where part of the cell membrane might be lost in the process (Nasirudeen et al., 2001a). Necrotic B. hominis showed low Annexin V staining. In this study, cells were treated with 0-1 % sodium azide for 24 h to induce necrosis. As such, PS residues may have already been degraded by cytosolic lipases, which may explain the relatively low Annexin V staining in necrotic cells. These results suggest that PS exposure precedes the loss of membrane integrity by several hours. The preservation of membrane integrity and the simultaneous exposure of PS to the outer leaflet of the plasma membrane (in cells treated with metronidazole) clearly demonstrate the apoptotic effect of the drug on B. hominis.

In mammalian apoptotic machinery, externalization of PS is necessary to signal neighbouring immune cells (Adayev et al., 1998). The immune cells then phagocytose the apoptotic cells and/or apoptotic bodies to minimize damage to the neighbouring healthy cells. The role of PS externalization in Blastocystis has yet to be determined. Perhaps, in vivo, it could be used for attracting neighbouring phagocytic cells. Penfold & Provis (1986) reported that most of the cellular debris resulting from cell death of the retina is taken up by adjacent cells rather than by macrophages. Without proper disposal of dying cells, the cells or the apoptotic bodies could undergo secondary necrosis. Hence, the externalization of PS residues during apoptotic death of B. hominis cells could have a similar function in the clearance of apoptotic bodies.

The appearance of the nucleosomal DNA ladder on an agarose gel was regarded as a hallmark of apoptosis (Collins et al., 1992). DNA extracts of metronidazole-treated B. hominis did not result in any apparent ladder-like fragmentation such as that seen in many apoptotic cells. But it has also been shown that internucleosomal DNA fragmentation cannot always be regarded as a hallmark of apoptosis since certain cells display morphological and biochemical features of apoptosis without ladder-like DNA fragmentation (Howell & Martz, 1987; Barbieri et al., 1992; Collins et al., 1992; Mesner et al., 1992; Falcieri et al., 1993; Cornillon et al., 1994; Vaux et al., 1994; Hirata et al., 1998). Furthermore, Darzynkiewicz et al. (1992) showed that DNA degradation, in many cell types, does not proceed to nucleosomal-sized fragments but rather results in 50–300 kb range DNA fragments which do not readily generate a characteristic ‘ladder’ pattern during agarose gel electrophoresis. In our previous studies, on the exposure of a cytotoxic antibody to B. hominis (Nasirudeen et al., 2001a), no DNA ladder was noted in apoptotic B. hominis except for two distinct fragments. However, when cells were treated with 10 μg ml⁻¹ Rnase A for 3 h at 30 °C, these two fragments were absent after electrophoresis (data not shown). Hence, the two bands from the earlier run correspond to contaminating rRNA fragments.

Though agarose gel electrophoresis of B. hominis DNA did not show typical ladder-like DNA fragmentation pattern, TUNEL indicated that metronidazole-treated cells do indeed undergo in situ DNA fragmentation. Flow cytometry data showed increasing TUNEL staining of metronidazole-treated B. hominis up to 9 h. At 12 h post-treatment, TUNEL intensity dipped. This could be due to secondary necrosis where much of the nuclear material may have been lost in apoptotic bodies. Normal B. hominis cells and those exposed to 0-1 % sodium azide (necrosis control) showed only minimal TUNEL fluorescence. TUNEL detects 3′-OH groups at the end of single-strand and double-strand DNA cuts. Didenko et al. (2003) reported that DNA cleavage in early necrosis is characterized by selective generation of 5’ overhangs, but not 3’ overhangs. This may be the reason why necrotic B. hominis cells showed low TUNEL. Another reason could be that much of the nuclear material may have degraded during the 24 h necrosis induction and therefore have been lost during the washing of cells.

What accounts for the inconsistency between the TUNEL and agarose gel electrophoresis methods? Apoptosis may have occurred in an asynchronous fashion so that distinct accumulation of DNA fragments was not observed. Another possible reason for the lack of DNA laddering in dying B. hominis cells could be the low sensitivity in the method of detection. In apoptotic chloroquine-sensitive P. falciparum, DNA laddering was reported using autoradiography methods when no visible DNA ladder pattern was seen using conventional agarose gel electrophoresis (Picot et al., 1997). Another reason for the inconsistency could be that the fragments could have been of larger sizes and hence not resolved in a 2 % agarose gel. Furthermore, Linfert et al. (1997) demonstrated that of the 21 DNA samples extracted from ischaemic tissue and subsequently electrophoresed, only three showed DNA laddering – despite the immunohistochemical in situ DNA fragmentation detected in all tissue samples.

In our earlier study, we postulated that the central vacuole acts as a repository for the storage of apoptotic bodies during apoptosis (Nasirudeen et al., 2001a). The current research provides more evidence for this role. Membrane-bound apoptotic bodies containing portions of the fragmented nucleus and an array of intact organelles such as mitochondria were noted in the central vacuole of metronidazole-treated B. hominis cells. The central vacuole of B. hominis grown in normal culture media under normal physiological conditions may contain lipid granules or electron-dense particles. But the central vacuole...
In all the assays performed in this study, the metronidazole host immune response (e.g. inflammation) and favour been suggested that effective apoptosis would reduce the resistance of strains could be a result of an efficient and effective apoptotic machinery. The characteristics of mammalian apoptosis are clearly reflected in metronidazole-treated B. hominis, namely preservation of plasma membrane integrity, in situ fragmentation of nuclear DNA, externalization of PS and formation of apoptotic bodies. In our earlier studies on apoptosis, we described the presence and activity of caspase 3-like proteins in B. hominis undergoing apoptosis (Nasirudeen et al., 2001b). The potential to undergo apoptosis appears to be present in many protozoans and more work is warranted before we can better understand this process at the molecular level. Interestingly, earlier reports have suggested that the genes involved in apoptosis in protozoa are not homologous to those in higher eukaryotes (Welburn & Murphy, 1998; Welburn et al., 1999).

Vaux & Strasser (1996) remarked that cells might detect early changes caused by drugs or other agents and respond by activating physiological death mechanisms. In the case of B. hominis, it appears that the parasite activates its apoptotic machinery when exposed to undesirable stimuli such as cytotoxic mAb 1D5 (Nasirudeen et al., 2001a) and metronidazole. We believe that B. hominis undergoes apoptosis for its own survival as a population of cells. We postulate that, as in the case of T. cruzi, some cells undergo apoptosis in vivo, significantly decreasing their population size, as to evade the immune response. When the immune response is minimal, the surviving parasites re-emerge to infect the host (DosReis & Barcinski, 2001). Some authors have reported resistance of B. hominis cells to metronidazole treatment (Haresh et al., 1999; Zaman & Zaki, 1996). These resistant strains could be a result of an efficient and effective apoptotic response to, and (or) an insufficient dose of, metronidazole. The existence of apoptosis in protozoa may also be relevant to host–parasite interactions, as it has been suggested that effective apoptosis would reduce the host immune response (e.g. inflammation) and favour overall parasite survival (Knight, 2002).

In all the assays performed in this study, the metronidazole concentration was 5 × 10⁻⁷ M. Further research needs to be done to investigate the effects of higher concentrations of the drug so that dose-dependent effects can be studied. For example, it has been shown in other systems that a higher drug concentration could induce necrosis (Anselmi et al., 2002). In summary, the observations described here strongly suggest the apoptotic effect of metronidazole on B. hominis. The results of this research lead one to speculate that cell-death mechanisms in B. hominis and higher eukaryotes may be conserved at the morphological and biochemical levels. Further detailed research is required to identify molecules involved in apoptotic mechanisms and regulation of this cell-death pathway. Currently, we are employing subtractive hybridization methods to identify cell-death-associated genes in B. hominis.

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