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A Form of Cell Death with Some Features Resembling Apoptosis in the Amitochondrial Unicellular Organism *Trichomonas vaginalis*

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One of hallmarks of apoptosis is the degradation and concomitant compaction of chromatin. It is assumed that caspases and caspase-independent pathways are rate limiting for the development of nuclear apoptosis. The caspase-independent pathway involves apoptosis-inducing factor (AIF) and leads to DNA fragmentation and peripheral chromatin condensation. Both pathways are the result of activation of death signals that the mitochondrion receives, integrates, and responds to with the release of various molecules (e.g., cytochrome c and AIF). In fact, both pathways have in common the final point of the DNA fragmentation and the mitochondrial origin of molecules that initiate the apoptotic events. Here, we examine the question of whether apoptosis or apoptotic-like processes exist in a unicellular organism that lacks mitochondria. We herein show that a form of cell death with some features resembling apoptosis is indeed present in *Trichomonas vaginalis*. Characterization of morphological aspects implicated in this event together with the preliminary biochemical data provided may lead to new insight about the evolutionary relationships between the different forms of programmed cell death identified so far.

Key Words: **programmed cell death; apoptosis; paraptosis; hydrogenosomes; protists; mitochondrion; *Trichomonas vaginalis*; AIF; unicellular organisms; amitochondrial.**

INTRODUCTION

The main universal known function of mitochondria is the energy production (ATP) through a process in which electrons are transferred along a series of respiratory enzyme complexes. It is accepted that another fundamental function of mitochondria is to regulate and promote apoptosis, releasing proteins such as cytochrome c and apoptosis-inducing factor (AIF)[2] from mitochondrial intermembrane space into the cytosol [1–3]. Through at least these two proteins, the mitochondrion appears thus largely implicated in caspase-dependent and -independent apoptotic pathways [4, 5]. However, if the mitochondrion is one if not the sole integrator of pathways cited above, what are the mechanisms used to promote cell death in amitochondrial organisms? Indeed, a variety of phylogenetically distinct eukaryotes, including ciliates, fungi, amoeba, and flagellates, lack typical eukaryotic organelles such as mitochondrion and peroxisome [6, 7]. These organisms, called amitochondriate protists, can be subdivided into two distinct types. Type II comprizes, e.g., *Trichomonas vaginalis* and is characterized by the presence of a hydrogenosome, a structure not found in multicellular organism cytoplasm. Type I, to which *G. lamblia* belongs, lacks both hydrogenosome and mitochondrion [8].

The paraxostylar and paracostal granules of *T. vaginalis* are typical hydrogenosomes. They are DNA-free double membrane-bound organelles producing H_2, a metabolic end product highly unusual among eukaryotes [9–11]. The presence of enzymes not found in mitochondria (e.g., pyruvate:ferrodoxin oxidoreductase and hydrogenase) [12] and the absence of a number of mitochondrial functions (e.g., oxidative phosphorylation) are the main features of this unique organelle [9, 11, 13]. It has been suggested that mitochondrion and hydrogenosome share a common ancestral origin. This idea is supported by the recent identification of Hmp31, a homologue of ADP/ATP carrier (ANT), identified in the *Trichomonas* hydrogenosomal membrane [13, 14].

Apoptosis is described as one form of morphologically

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[2] Abbreviations used: AIF, apoptosis inducing factor; ANT, ADP/ATP carrier; BA, bongkrekic acid-3NH3; DOXO, doxorubicin; ETO, etoposide; PBS, phosphate-buffered saline; PCD, programmed cell death; PS, phosphatidylserine; STS, staurosporine; TUNEL, terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling; TYM, trypticase–yeast extract–maltose medium.

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multifaceted programmed cell death (PCD) in multicellular organisms [15]. The term "apoptosis" was subsequently extended to unicellular mitochondrial organisms, where PCD exhibiting apoptotic features was also demonstrated to occur [16–19]. More recently, paraptosis, a distinct non-apoptotic variant of PCD in multicellular organisms, was also reported [20]. All descriptions made so far rely on mitochondrial organisms and bacteria [21–24].

We have studied the response of *T. vaginalis*, an amitochondrial unicellular organism, to pro-apoptotic drugs. We describe here a form of cell death that fails to fulfill completly the criteria for apoptosis or paraptosis previously described for multicellular organisms.

MATERIALS AND METHODS

Culture of organisms. *T. vaginalis* strain C1:NIH (ATCC 30001) was grown axenically at 37°C in liquid trypticase–yeast extract–maltose (TYM) medium supplemented with 10% (v/v) heat-inactivated horse serum (GibcoBRL, Life Technologies, Cergy Pontoise, France), 100 U/ml of penicillin G, and 50 mg/ml of streptomycin sulfate (Sigma, Saint Quentin Fallavier, France).

Chemicals. Stock solutions of etoposide (ETO) and staurosporine (STS) were purchased from Sigma (St. Louis, MO) and were prepared in DMSO (20 mM and 800 μM, respectively). Stock solution of doxorubicin (DOXO) was prepared in water (400 μg/ml). Stock solution of bongkrekic acid-3NH3 (BA, BIOMOL Research Laboratories, Inc., Butler Pike, U.S.A.) was prepared in water (1 mg/ml). z-VAD-.fmk was prepared in DMSO (10 mM). All stock solutions were stored at −20°C for further use.

Incubation of T. vaginalis with pro-apoptotics drugs and caspase activity inhibitors. Cells were counted using a Malassez device and subsequentlty distributed in 24-well culture dishes (Dutscher, Trumath, France) with an initial density of 1.10^6 cells per well (1-ml culture volume). After 1-h-preincubation at 37°C, cells were treated for 15 h at 37°C using a range of concentrations of ETO (400, 200, and 100 μM), STS (15, 10, and 4 μM), or DOXO (16, 10, and 2 μg/ml). Equivalent amounts of DMSO were added to control samples, the highest final DMSO concentration never exceding 2%. When BA and z-VAD.fmk were used, the cells were preincubated for 1 h at 37°C with inhibitors before the incubation with pro-apoptotics drugs.

Nuclear staining. Drug-treated and control cells were treated with 0.5 μg/ml of vital Hoechst dye 33342 (DAPI, Sigma) for at least 15 min, harvested by centrifugation (5 min at 900 rpm), washed with phosphate-buffered saline (pH 7.4; PBS), and subsequently mounted on slides. Nuclear morphology of the cells was visualized using an Olympus fluorescence microscope.

The TUNEL (Terminal deoxynucleotidyltransferase-mediated dUTP nick-end labeling) method. TUNEL reaction was carried out by using the In Situ Cell Death Detection Kit (Roche Diagnostics, Meylan, France) on cytospin slides as follows: a cell suspension was diluted to $1.5 \cdot 10^5$ cells/ml from treated and control cultures; 100 μl of the suspension was cytocentrifuged 6 min at 1200*g* using a cytospin cup (SHANDON). Slides were air-dried for 5 min, fixed at room temperature for 1 h in PBS supplemented with 4% paraformaldehyde, and then washed thrice with PBS. Endogenous peroxidases were blocked with a 3% H_2O_2 solution in methanol and cells were permeabilized for 2 min on ice in a 0.1% Triton X-100, 0.1% sodium citrate solution. The labeling and signal conversion were carried out according to the manufacturer's instructions. Finally, samples were counterstained with eosin prior to analysis by light microscope. Cell controls correspond either to samples without drug traitement or to spontaneously dead cells (collected from culture tube bottoms).

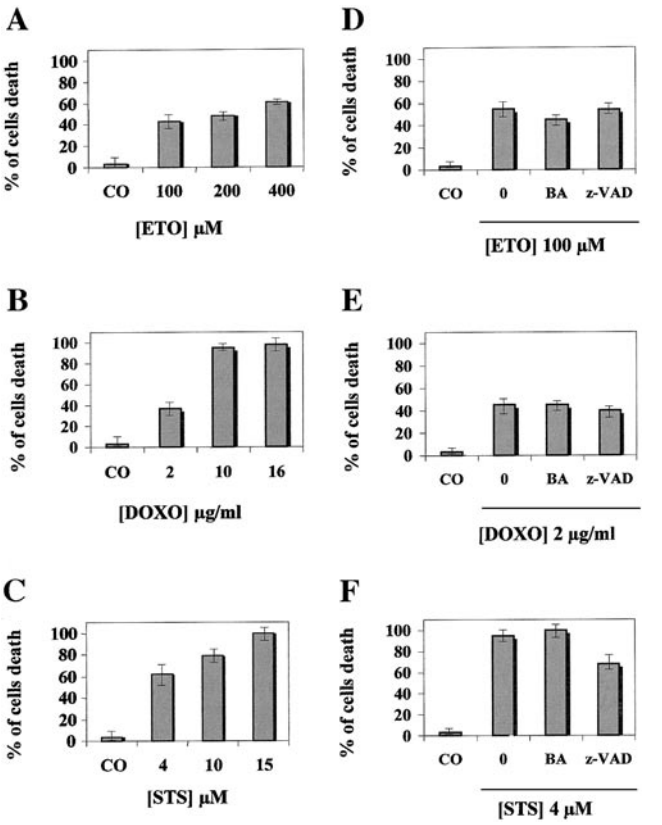

FIG. 1. *T. vaginalis* treated with pro-apoptotics drugs (A, ETO; B, DOXO; C, STS) and caspase activity inhibitors (D, ETO; E, DOXO; F, STS). Histograms reflect percentages of living cells as determined from morphology and flagella mobility (standard deviation of four independent hemocytometer counts). *T. vaginalis* cells were treated as described under Materials and Methods. z-VAD.fmk (a pan-caspase inhibitor) and BA (a mitochondrial membrane permeability transition inhibitor) were both used at a final concentration of 25 μM, 0 corresponding to inhibitor untreated samples. CO refers to control cells without any treatment at all. This experiment was reproduced four times.

DNA purification. DNA purification of treated or untreated *T. vaginalis* was performed using two distinct methods. The first one was initially described by Gong *et al.* [25], namely, 2.10^6 cells in 1 ml of Hanks' buffered salt solution were transferred into 10 ml of 70% ethanol solution on ice. Cells were fixed for 24 h at −20°C and subsequently centrifuged (5 min, 800*g*). The cell pellets were resuspended in 40 μl of 0.1 M phosphate–citrate buffer (pH 7.8) and incubated for 30 min at room temperature. Lysats were centrifuged again (5 min, 1000*g*) and supernatants were vacuum-concentrated for 15 min. Samples were supplemented with 3 μl of 0.25% Nonidet P-40 (NP-40) solution and 3 μl of 1 mg/ml RNase solution (Eurogentec, Seraing, Belgium) previous to new incubation (30 min, 37°C). This was followed by proteinase K (Gibco BRL, Life Technologies) treatment (3 μl of 1 mg/ml solution) for 30 min at 37°C; 12 μl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol) was then added to raw DNA extracts, which were directly loaded on 2% agarose (Eurogentec) gels for analysis. The DNA markers was purchased from Eurogentec (Smart Ladder, Seraing, Belgium). The second procedure was carried out using the Apoptotic DNA Ladder Kit (Roche Diagnostics, Meylan, France)

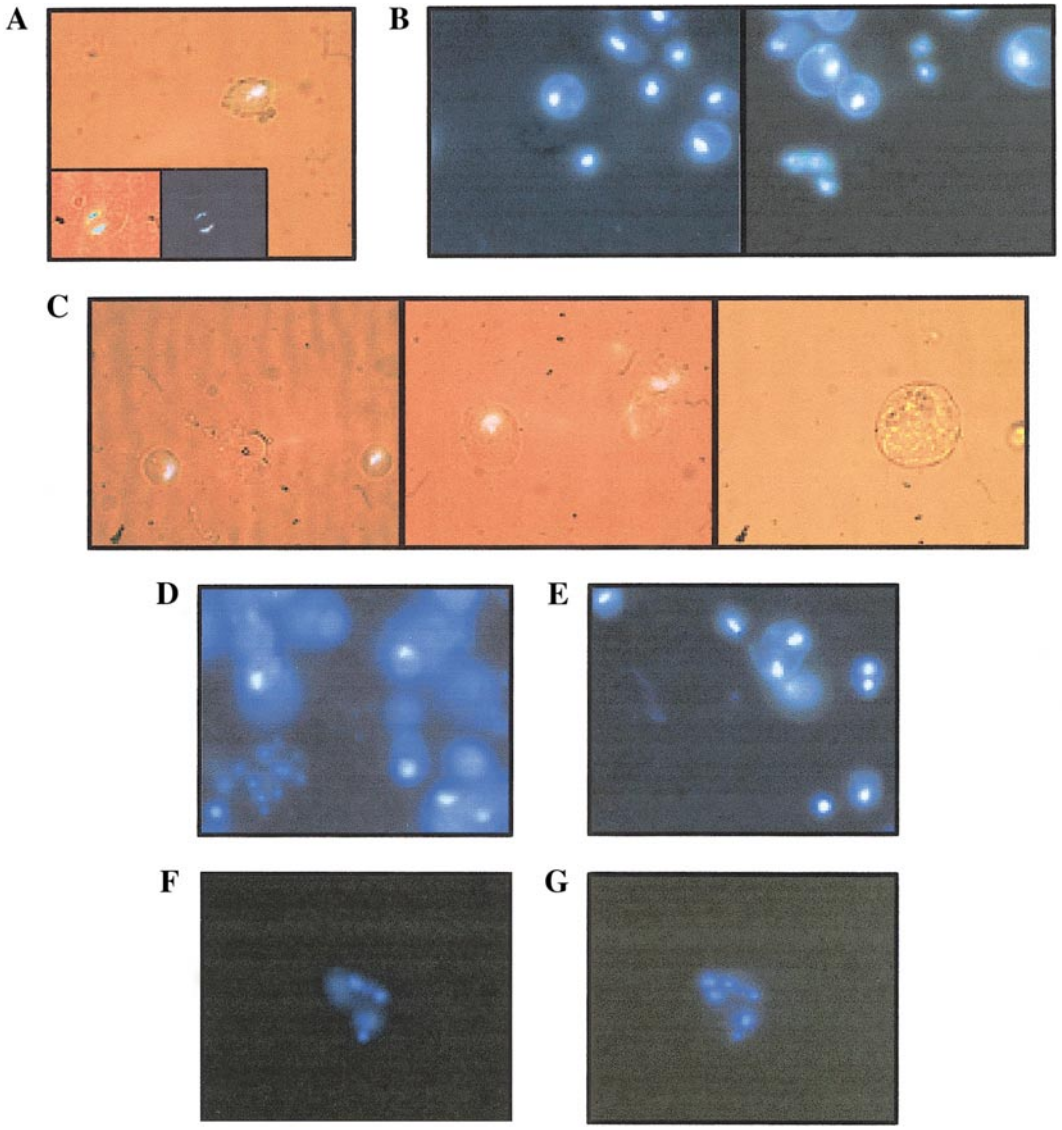

FIG. 2. Morphological aspects of *T. vaginalis* treated with STS. Unfixed samples were stained with Hoechst dye and observed under fluorescent or visible light. (A) Untreated *T. vaginalis* cells. The single or dual nuclei coloration reflects differences in mitotic state. The insert compares visible and fluorescent images for the same cell; treated *T.vaginalis* cells; typical morphology includes round cytoplasm and condensation with reduction of nuclei size as shown from fluorescent (B) and visible (C) light. In fluorescence, different forms of condensation and deformation of nuclei can be observed together with already formed corpuscular bodies (D, E). The formation process of corpuscular bodies can be followed and increases with time (F, G). The same profiles were obtained with other pro-apoptotic drugs (not shown).

according to the manufacturer's instructions and visualized as described above.

PCR. Specific human AIF primers used were forward primer, 5′-TTGAGAATGGTGGTGTGGC-3′, and reverse primer, 5′-TGCTGACTCCAACGGATTG. Specific *Dictyostelium discoideum* AIF primers were forward primer, 5′-GGTGGAACAGCAGCTTACC-3′, and reverse primer, 5′-TTGGAATGATACCGGCG-3′. PCR reactions were set essentially as follows: 40 ng of genomic DNA, 0.5 μM each primer, 200 μM dNTPs (Sigma) 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.1 mM $MgCl_2$, and 0.01% gelatin were mixed in distilled water to a final volume of 50 μl; 2.5 units of REDTAQ DNA polymerase (Sigma) was then added and PCR was performed for 30 cycles (1 min 93°C, 1 min 30°C, 1 min 72°C) in a Mastercycler Personal (Eppendorf AG, Hamburg, Germany). Amplification products were subsequently visualized on 1.5% agarose gels (Eurogentec).

Flow cytoflurometry. Flow cytoflurometry immunostainings for of *T. vaginalis* and hybridoma control cells were prepared as previously described [26]. Cells were labeled either for 10 min at 37°C with 40 nM 3-3′-dihexyloxacarbocyanineiodide $DiOC_6(3)$. (Molecular Probes, Eugene, OR) or with the annexin V-FITC kit (Beckman-Coulter, Brea, CA) followed by propidium iodide staining according to manufacturer's specifications. Aliquots corresponding to 1.10^6 cells were analyzed for each suspension by Coulter EPICS XL flow cytometer (Beckman Coulter)

Caspase immunohistochemical staining. Cells were treated essentially as for TUNEL (see above) excepted that permeabilization was omitted. After preincubation with 20% normal horse serum slides were then incubated with anti-caspase-3 monoclonal antibody CPP32 (Beckman Coulter) according to the manufacturer's specifications. Immunobinding was detected using biotinylated horse anti-

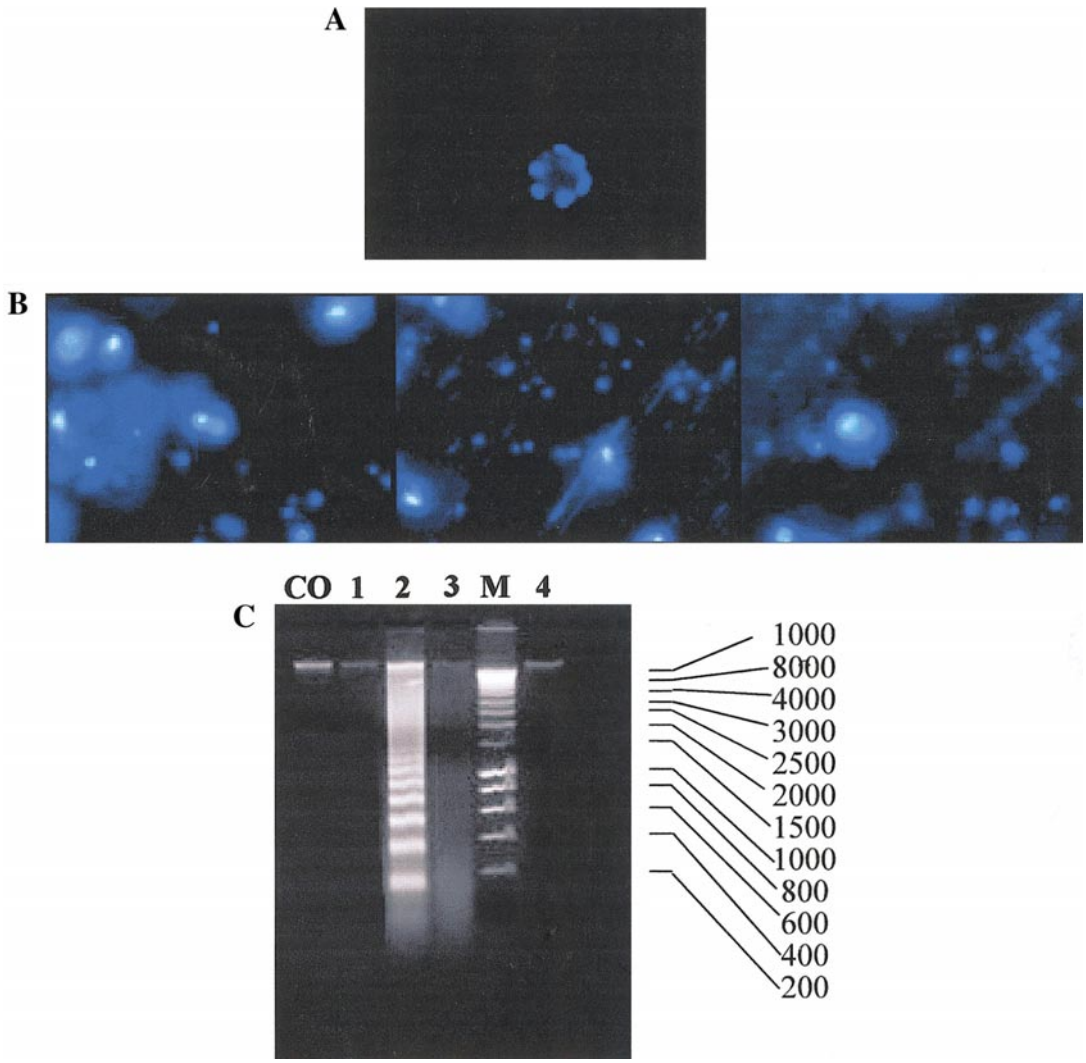

FIG. 3. Nuclear fluorescence of *T. vaginalis* dead cells (A, B). Chromatin segmentation during cell death after treatment with apoptotic drugs (A) is shown together with the naturally occurring death of untreated cells (called "starry sky") (B). Electrophoresis of total *T. vaginalis* genomic DNA (C): CO, untreated *T. vaginalis* control DNA; 1, ETO (100 μM)-treated cell DNA; 2, fragmented DNA control (supplied with kit); 3, natural *T. vaginalis* death cell DNA; M, molecular weight marker; 4, STS (4 μM)-treated cells DNA.

mouse Ig (Vectastain Elite ABC kit, Vector Laboratories, Inc., Burlinegame, CA) and DAB (Roche Diagnostics) substrate. Peroxidase activity was visualized under a light microscope.

RESULTS

Induction of apoptosis or other form of programmed cell death in T. vaginalis. Wild-type *T. vaginalis* was treated with different concentrations of pro-apoptotic drugs (namely, ETO, DOXO, and STS). All of them were able to induce cell death (Figs. 1A–1C). Hoescht staining of nuclear chromatin reflecting level of condensation was used as one morphological criterium for cell death quantification. Other morphological aspects, such as lack of flagelle movement, were also followed in light microscopy on 24-well culture dishes. Mortality rates were estimated to 42, 48, and 61% when cells were treated with 100, 200, or 400 μM ETO, respectively, to 36.8, 94.7, and 98% when cells were treated with 2, 10, and 16 μg/ml of DOXO, respectively, and to 62, 79, and 100% when cells were treated with 4, 10, and 15 μM STS, respectively. When cells were exposed to z-VAD.fmk previous to incubation with pro-apoptotic drugs (100 μM ETO, 2 μg/ml DOXO, and 4 μM STS), a moderate inhibition of cell death was only observed with STS (Fig. 1F). Indeed, the mortality percentage observed with STS treatment was 94.5%, falling to 68% when a z-VAD.fmk preincubation was performed. No significative inhibition could be observed when cells where exposed to ETO and DOXO (Figs. 1D and 1E). Under same conditions, the mitochondrial membrane permeability transition inhibitor BA did not show any significant inhibition whatever

pro-apoptotic drug was used (Figs. 1D–1F). Equivalent amounts of DMSO were added to control samples using identical conditions and result was equal to that of nonsupplemented cells (result not shown).

Morphological aspects of T. vaginalis treated with apoptosis inducers and stressed by nutriment depletion. As noticed above, all three pro-apoptotics drugs tested were able to induce cell death at a significant rate. Various morphological forms closely resembling those widely described for programmed cell death could be observed. These included nuclear fragmentation and apoptotic-like (optically refringent and DAPI fluorescent) bodies emergence, a typical feature described in apoptosis as apoptotic bodies. On the other hand, a cytoplasmic vacuolation could be observed with all tested drugs, a phenomenon that has not been described in typical apoptosis [27], but is present in paraptosis, another recently described form of programmed cell death [20]. The morphology of treated cells can be described in the following way: first, for almost all cells induced with pro-apoptotic drugs, round-shaped cells with emerging bubbles were observed after 15 h of treatment (Figs. 2A–2C). Second, Hoeschst staining revealed that the same cells evolved in a sequence in which chromatin condensation and reduction of nuclei size occurred together with cytoplasmic vacuolation and cytoplasm widening, both events resulting in volume/surface of nuclei/cytoplasmic ratio modifications (Figs. 2B and 2C). Third, condensation and reduction of nuclei size was followed by chromatin segmentation, visible through the emergence of about six to eight mininuclei (Figs. 2D, 2F, and 2G). It is important to note that when *T. vaginalis* was treated with metronidazol (the specific therapeutic agent used for human treatment against this pathogen protist) or with griseofulvine, the same morphological pattern could be observed (result not shown). Fourth, the cells showed various condensed nuclei forms mixed with apoptic-like bodies (Fig. 2D). In both intact dead cells and apoptotic-like bodies, destruction of the plasmic membrane was never observed. To explore the possibility that untreated cells submitted to a stress could enter the same sequence of events, we also observed morphological aspects of dead cells that naturally accumulated at the bottom of culture tube after at least 72 h (refered here as "naturally" spontaneously dead cells). Almost all the visual field was occupied by apoptic-like bodies, a figure that unambiguously evoked a "starry sky" when seen with Hoescht staining against a microscope dark field (Fig. 3A).

Lack of detection of hAIF or aAIF and internucleosomal DNA fragmentation in programmed cell death in the amitochondrial protist T. vaginalis. In order to investigate the presence of AIF-related DNA in *T. vaginalis,* two alternative sets of AIF-specific primers were used in a PCR approach. The primers were designed according to human (AF100928) and *D. discoideum* amoeba (AJ272500) AIF sequences and temptatively used to amplify an AIF counterpart in *T. vaginalis*. Both failed to amplify any specific band when *T. vaginalis* genomic DNA was used as a matrix under low-stingency conditions (data not shown). We also studied apoptotic-like bodies of induced (Fig. 3A) or "naturally" dead cells (stress-occurring cell death) (Fig. 3B) through purification of total genomic DNA. Subsequent electrophoretic profiles did not reveal any of the patterns previously reported for PCD (Fig. 3C).

Assessment of PS exposure. The exposure of phosphatidylserine (PS) in the outer leaflet has been previously described as one of the hallmarks of apoptosis [26]. Using annexin V labeling followed by flow cytofluorometry analysis, we studied PS exposure in *T. vaginalis* untreated control cells (Fig. 4A) or STS-treated cells without (Fig. 4B) or with (Fig. 4C) preincubation of z-VAD.fmk, a caspase inhibitor. Assessment of membrane integrity was performed through propidium iodide staining. STS treatment provokes the exposure of PS in the outer leaflet of *T. vaginalis*. However, the presence of z-VAD.fmk does not prevent this effect.

DNA fragmentation with the TUNEL technique. We wanted then to investigate DNA fragmentation, another biochemical feature of programmed cell death. TUNEL showed a positive staining in treated cells (Fig. 5A) and a negative one in untreated cells (Fig. 5B).

Assessment and activity of Hmp31 protein. The Hmp31 protein is phylogenetically related to the mitochondrial ANT [14]. To explore the possibility that an induced form of PCD could promote the activity of Hmp31 in *T. vaginalis* hydrogenosomes, we used a $DiOC_6(3)$ marker in a flow cytometry experiment to measure the hydrogenosomal membrane potential dissipation. (Figs. 6A–6C). Interestingly, a weak but significative membrane potential "$\Delta\psi_m$ dissipation" was only observed when *T. vaginalis* cells were treated with pro-apoptotics drugs followed by 15-min exposure with $DiOC_6(3)$ (Fig. 6C).

DISCUSSION

T. vaginalis morphological aspects after treatment with ETO, DOXO, and STS showed some features resembling apoptosis [15–28]. Indeed, we observed "apoptotic-like" conformations including nuclear fragmentation, chromatin condensation, and apoptotic-like body formation (the latest closely resembling apoptotic bodies) [29, 30]. This lead us to verify if any caspase inhibitor-responsive protein could be present in *T. vaginalis* cells. For this purpose the classical pan-caspase pathway inhibitor z-VAD.fmk was used in combination with the pro-apoptotic drugs ETO, DOXO, and STS. This inhibitor did not abolish cell death when

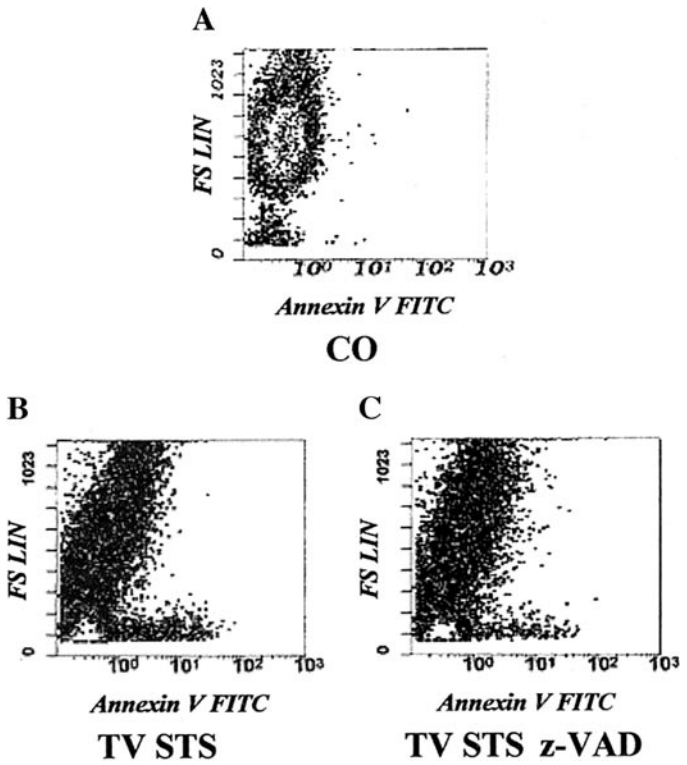

FIG. 4. Annexin V FITC flow cytometric analysis of *T. vaginalis* membrane out leaflet phosphatidylserine (PS) exposure. (A) Untreated control cells; (B, C) STS (4 μM) cells treated without (B) or with z-VAD.fmk inhibitor preincubation (C). Membrane integrity was controlled using propidium iodide staining.

T. vaginalis was exposed to ETO and DOXO, but a weak response was observed after exposure to STS. This observation is a quite surprising feature since extensive genetic screening among a large number of mitochondriated unicellular eukaryotes failed to detect typical caspase proteins [31]. However, this result does not exclude the presence of more ancestral caspase-like proteins, such as metacaspases and paracaspases [32]. In order to check this hypothesis, the presence of an equivalent of caspase 3, a protein that is activated by caspase 9 in apoptotic eukaryotic cells, was investigated with anti-caspase-3 monoclonal antibody. A positive reactivity could be observed (data not shown), which may indicate at least the presence of a caspase-like protein in *T. vaginalis*. However, since this antibody shows mostly reactivity against mouse and human caspase 3, this histochemical observation has to be confirmed with molecular studies as we cannot rule out a cross-reaction with some unrelated proteins sharing the same epitope. On the other hand, we observed positive TUNEL staining in *T. vaginalis* treated with ETO and STS. This unambiguously shows that *T. vaginalis* DNA undergoes fragmentation, a feature that is considered a point of no return in cell death. When analyzing electrophoretic fragmented DNA profiles, none of previously described patterns such as, e.g., laddering or high-molecular-weight banding, could be detected.

Taking this altogether, the results presented here imply that a caspase-like pathway (or more ancient equivalent proteases such as those described in *C. elegans*) may exist in amitochondrial protists. In addition, the exposure of PS on the outer leaflet of the plasma membrane, another event that is one of the hallmarks of apoptosis, could be induced in *T. vaginalis* with the pro-apoptotic drug STS [26]. Furthermore, the behavior revealed by $DIOC_6(3)$ treatment (Fig. 6) may perhaps be explained by the presence of a molecule that complies with a function similar to mitochondrial ANT. The weak dissipation of the hydrogenosomal membrane potential may be thus a counterpart of mitochondrial membrane potential. In this opinion, a good potential candidate may be Hmp31, a protein that is phylogenetically related to mitochondrial ANT [14]. Since this molecule is found in the hydrogenosomal membrane, it would be worthwhile to imagine that the hydrogenosome can alter its membrane permeability in order to release harmful molecules that mimick, e.g., AIF or the cytochrome c effect [33].

Interestingly, when we examined *T. vaginalis* "naturally" dead cells (issuing from stress-induced naturally occurring death after at least 72 h of culture), an enormous number of apoptic-like bodies could be detected (presenting a "starry sky" aspect when stained with Hoescht dye). This is in good agreement with the results reported for conditional stress-induced PCD in the dinoflagellate *P. gatunense* [19]. In this organism, DNA fragmentation was demonstrated by the TUNEL technique. The absence of an electrophoretic laddering pattern is explained by the differences in chromatin organization (e.g., lack in nucleosomal arrangement). *T. vaginalis* naturally dead cells also undergo DNA fragmentation (TUNEL positive) without any specific pattern, a fact that contrasts with classical nucleosomal organization. However, a deep rearrangement in chromatin organization (chromatin segmentation) is visible directly during STS-induced cell death through the emergence of about six to eight mininuclei (see Fig. 3A), a feature also seen when cells were treated with griseofulvine (E. Viscogliosi, personal communication). This striking observation rises new questions about the real morphological aspect of chromosomal organization and its role during cell death in amitochondriate protists. PCD, the main frequent form of which is named apoptosis, is found throughout the animal kingdom and culminates in the execution, packaging, and disposal of dying cells [4, 5, 15, 23, 28–31]. Such cell suicide also occurs in nonanimal cells, e.g., in plants, during development as well as in the senescence of flowers and leaves or in response to injury and infection [15–23]. A well-documented example of nonanimal cell PCD has been described in some strains of *Esche-*

richia coli. Indeed, these strains produce an inactive form of protease, which can be bound and activated by a phage protein. The activated protease degrades another bacterial protein required for general protein synthesis, leading to bacterial death. Although this bacterial program shares a number of factors with caspase-dependent apoptosis in animal cells, the proteases involved in the two programs are unrelated [22]. Besides *P. gutanense* [19], characteristic PCD processes have also been described as apoptotic in mitochondriated unicellular protists such as *Trypanosoma cruzi* [17], *Trypanosoma brucei rhodensiense* [34], *Leishmania amazonensis* [18], and *Tetrahymena thermophila* [34]. Recently, a form of non-apoptotic programmed cell death (paraptosis) has been reported in Apaf-1 null mouse embryonic fibroblasts. This form is distinct from apoptosis by morphology, biochemistry, and response inhibitor criteria [20, 21]. Interestingly, the form of cell death we describe here in *T. vaginalis* presents some morphological aspects similar to both apoptosis and paraptosis, among which are cytoplasmic vacuolization, chromatin condensation, and the presence of apoptic-like bodies. On some aspects, cell death features presented here can be related to those described, e.g., in Bax-transfected *Schizosaccharomyces pombe* (cytoplasmic vacuolation) [36] and *D. discoideum* (cytoplasmic vacuolation without nuclear fragmentation) [16, 37]. We are aware that actual data are not sufficiently relevant to ensure that *T. vaginalis* cell death is indeed a form of PCD. Additional molecular studies are obviously necessary to determine what form of cell death occurs here and particularly if it may be related to an already known form of PCD. However, to our knowledge, this study constitutes the first report of a form of cell death with some features resembling apoptosis in an amitochondrial unicellular organism. In conclusion, we show for first time that, in an amitochondrial organism such as *T. vaginalis*, cell death with some features resembling apoptosis can either be induced with drugs classically used for chemotherapeutic apoptosis induction of both caspase-dependent and -independent pathways or may occur in natural stress-induced death. Since *T. vaginalis* can be considered a kind of intermediate between amitochondriated (type I) and mitochondriated eukaryotes, data presented here may thus represent a particularly interesting example in the evolution of cell death, of which the molecular mechanism remains to be investigated.

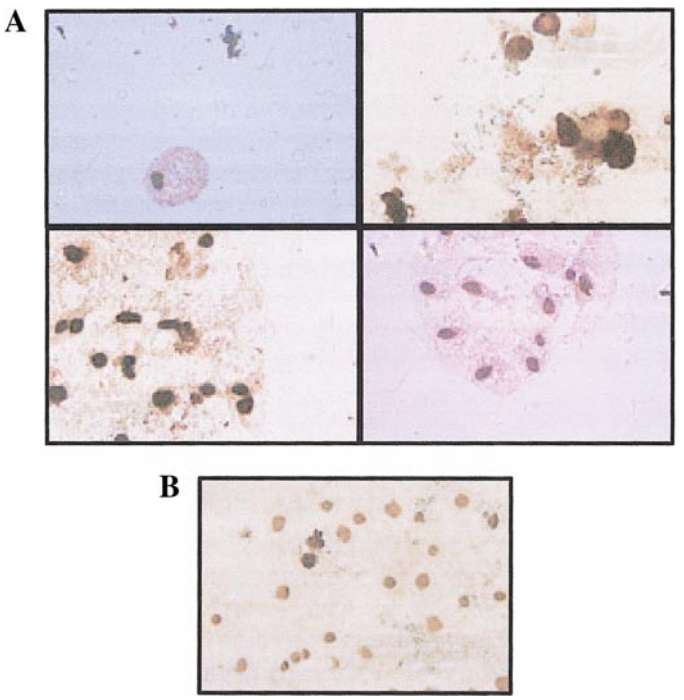

FIG. 5. Morphological aspects of cell death in *T. vaginalis* stained with a TUNEL kit. The strong brown nuclei coloration of STS (4 μM)-treated cells (A) contrasts with the weak background-like picture of untreated controls (B).

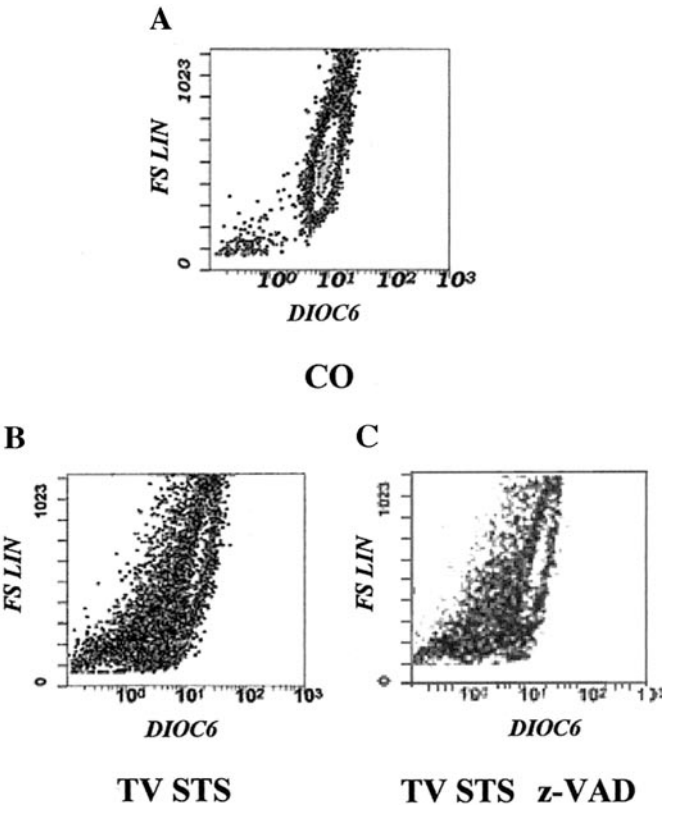

FIG. 6. $DiOC_6(3)$ flow cytometric analysis of *T. vaginalis* transmembrane potential dissipation ($\Delta\psi_m$) after STS treatment. (CO) Untreated control; (B, C) STS (4 μM)-treated cells without (B) or with (C) z-VAD.fmk inhibitor pretreatment.

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