Cell Death and Differentiation (2002) 9, 53–64

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Programmed cell death in the unicellular protozoan parasite *Leishmania*

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Received 2.5.01; revised 23.7.01; accepted 31.8.01
Edited by G Melino

Abstract

In the present study we have demonstrated some features characterizing programmed cell death (PCD) in the unicellular protozoan parasite *Leishmania donovani*, the causative agent of visceral *Leishmania*sis. We report that PCD is initiated in stationary phase cultures of promastigotes and both in actively growing cultures of axenic amastigotes and promastigotes upon treatment with anti *Leishmanial* drugs (Pentostam and amphotericin B). However, the two cell types respond to *antileishmanial* drugs differently. The features of PCD in *L. donovani* promastigotes are nuclear condensation, nicked DNA in the nucleus, DNA ladder formation, increase in plasma membrane permeability, decrease in the mitochondrial membrane potential ($\Delta\Psi_m$) and induction of a PhiPhiLux (PPL)-cleavage activity. PCD in both stationary phase culture and upon induction by amphotericin B resulted first in the decrease of mitochondrial membrane potential followed by simultaneous change in plasma membrane permeability and induction of PPL-cleavage activity. Of the total PPL-cleavage activity, several caspase inhibitors inhibited a significant amount (21–34%). Inhibitors of cathepsin or calpain did not inhibit PPL-cleavage activity. Taken together this study demonstrates that the characteristic features of PCD exist in unicellular protozoan *Leishmania donovani*. The implication of PCD on the *Leishmania* pathogenesis is discussed. *Cell Death and Differentiation* (2002) **9,** 53–64. DOI: 10.1038/sj/cdd/4400952

Keywords: programmed cell death; *Leishmania*; promastigote; amastigote; Pentostam; amphotericin B

Abbreviations: PCD, programmed cell death; TMRE, tetramethylrhodamine ethyl ester; CCCP, carbonyl cyanide p-trifluoromethoxyphenylhydrazone; TUNEL, terminal deoxy uridine triphosphate nick end labeling; PPL, PhiPhiLux; $\Delta\Psi_m$, mitochondrial membrane potential

Introduction

Leishmania, a unicellular *Trypanosomatid protozoan* parasite causes a wide range of human diseases ranging from the localized self-healing cutaneous lesions to fatal visceral infections. *Leishmania* have a digenic life cycle, which is characterized by the presence of a flagellated promastigote form in the sand fly vector and a nonmotile amastigote stage within phagolysomes of mammalian macrophages.[1–3] The ability of this parasite to undergo differentiation from the promastigote to the amastigote form is crucial for its pathogenesis.[4] Further, during its life cycle, *Leishmania* grows in diverse and hostile environmental conditions[5] and has to control growth as well as preserve individuals that are fit to continue infectivity.

Programmed cell death (PCD) is an essential part of cell biology and is thought to have evolved not only to regulate growth and development in multicellular organisms[6,7] but also to guard against viral infections[8–10] and the emergence of cancer.[11,12] However, recent studies, which showed the existence of PCD in unicellular organisms, have postulated a functional role of PCD in the biology of unicellular organisms.[13–18] Further, in light of a recently reported nonapoptotic programmed cell death, parapoptosis,[15,19] it is important to understand the type of PCD that exists in unicellular organisms. It has been postulated that in order to promote and maintain clonality within the population, the *Trypanosomatids* must have developed an altruistic mechanism to control growth.[14] Recently, PCD has been shown to be involved in the control of cell proliferation of *T. cruzi in vitro*[16] and in the insect vector mid gut.[20] Features of PCD also have been observed in procyclic insect form of *T. brucei rhodesiense* upon treatment *in vitro* with lectin.[17] Ca^{2+} modulation by heat shock during differentiation in *L. amazonnesis* also induced PCD.[18] A cell death process akin to PCD was also demonstrated to exist in *Dictyostelium discoideum*, an organism that exists both as a unicellular and multicellular form,[15] thus reinforcing the idea that the pathway for PCD must have evolved before the evolution of multicellularity.[15] All these observations point towards the existence of a PCD pathway in *Trypanosomatids*. Whether the type of PCD that exists in *Trypanosomatids* is the same as in multicellular organism remains to be seen.

In the present study we explored the existence of PCD in *Leishmania donovani*. We showed for the first time that the parasite death during stationary phase in culture and by various anti*leishmanial* drugs involves PCD. In both of these cases, we demonstrated that PCD began with depolarization of mitochondrial membrane potential followed by induction of a caspase-like activity. Further, the induction of caspase-like activity is also observed in freshly isolated *L. donovani* from infected animals and in an another species of *Leishmania*.

Results

Characteristic features of PCD in stationary phase cultures of promastigotes of *Leishmania donovani*

When promastigotes of *L. donovani* were cultured *in vitro* from a starting cell density of 1×10^6/ml, there was an exponential increase in cell number in the first 3–4 days, followed by a plateau in growth (Figure 1A). The latter part of the growth curve was described as stationary phase since there was no substantial increase in cell number. We investigated the nature of cell death occurring in stationary phase culture, which could be the result of either necrosis or PCD. A characteristic feature of PCD in multicellular organisms is the formation of a DNA ladder. In stationary phase promastigotes, we observed a DNA fragmentation pattern in multiples of oligonucleosomal length units (Figure 1B). The intensity of the DNA ladder was more pronounced in the latter stages of stationary phase (lane D10). Transmission electron microscopy showed that promastigotes also undergo morphological changes characteristics of PCD at day 7, which includes a certain degree of condensation of nuclear chromatin (Figure 2B *vs* A). Further, by TUNEL assay, an indicator of DNA nicking, we observed a significant number of TUNEL positive cells in stationary phase cultures (Figure 2D). However, only a few TUNEL positive cells were observed in late logarithmic phase cultures of promastigotes (Figure 2C, white arrowhead). Thus, *in vitro* grown *Leishmania donovani* exhibit nuclear changes similar to those associated with PCD in higher eukaryotes.

Decrease in mitochondrial membrane potential of *Leishmania donovani* promastigotes during stationary phase cultures

A reduction in mitochondrial membrane potential ($\Delta\Psi_m$) has been observed in a number of models of apoptosis.[21–26] However, it is not clear whether $\Delta\Psi_m$ is the primary event in PCD.[27–32] Little is known about the involvement of mitochondria in PCD in unicellular organisms such as *Leishmania*. Tetramethylrhodamine ethyl ester (TMRE), is a cationic lipophilic dye that enters cells and reversibly accumulates in the negatively charged mitochondrial matrix depending on mitochondrial membrane potential according to the Nernst equation potential.[33] Live cells that were either in late log phase or at different times in the stationary phase cultures were incubated with TMRE and fluorescence was measured by flow cytometry. To prove that *Leishmania* cells functionally respond to pharmacological alteration of $\Delta\Psi_m$, TMRE-loaded promastigotes were treated with different concentrations of the protonophore carbonyl cyanide p-trifluoromethoxy-phenylhydrazone (CCCP), which has been shown to induce loss of mitochondrial membrane potential in various cell types.[34,35] We first examined the effect of CCCP on promastigotes at day 3 of culture showing no hallmarks of PCD. Results show that addition of CCCP (50 μM) resulted in maximal decrease of TMRE fluorescence indicating loss of mitochondrial membrane potential (Figure 3). We next assessed whether a change in mitochondrial membrane potential occurs in these cells during stationary phase culture. When promastigotes at day 6 of culture were loaded with TMRE, two populations of cells were observed (Figure 4B). One population representing 46.8% of cells (labeled M2, Figure 4B) had a mean TMRE fluorescence similar to day 3 cells (Figure 4A). The second population representing 53% of cells (labeled M1, Figure 4B) had a substantial reduced mean TMRE fluorescence. By day 7 the low TMRE fluorescence M1 population increased to 77% of total cells and the high TMRE (M1) population decreased to 21.4% (Figure 4C). Treatment of cells with CCCP (50 μM) showed that the high TMRE (M2) population responded to membrane potential changes whereas, the low TMRE (M1) population was insensitive (Figure 4D,E,F). These results suggest that, upon aging, *Leishmania* promastigotes loose mitochondrial membrane potential.

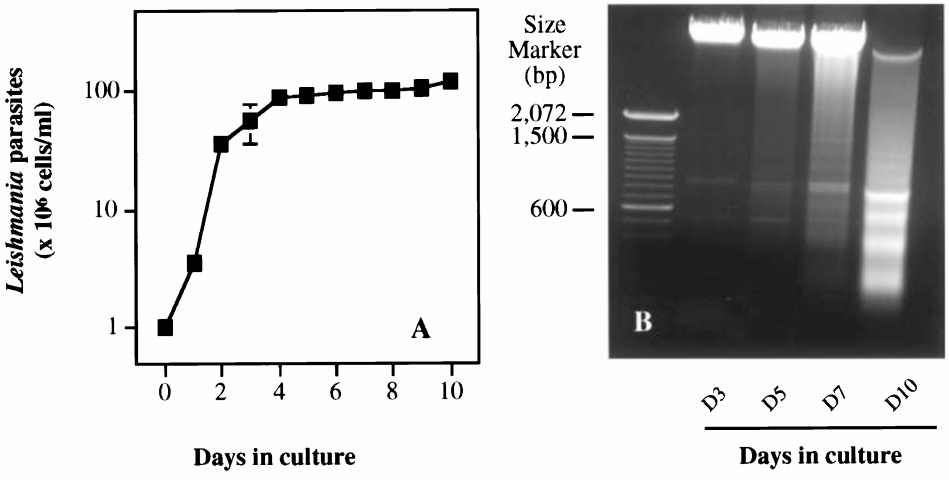

Figure 1 Cell growth in culture and the appearance of DNA ladder formation. *L. donovani* promastigotes were cultured *in vitro* at an initial concentration of 1×10^6 cells/ml. The cells were kept in culture for 10 days and counted at indicated days (**A**). DNA was isolated at each time point, run through an agarose gel, and visualized by ethidium bromide (**B**). DNA size markers are shown in base pairs (bp)

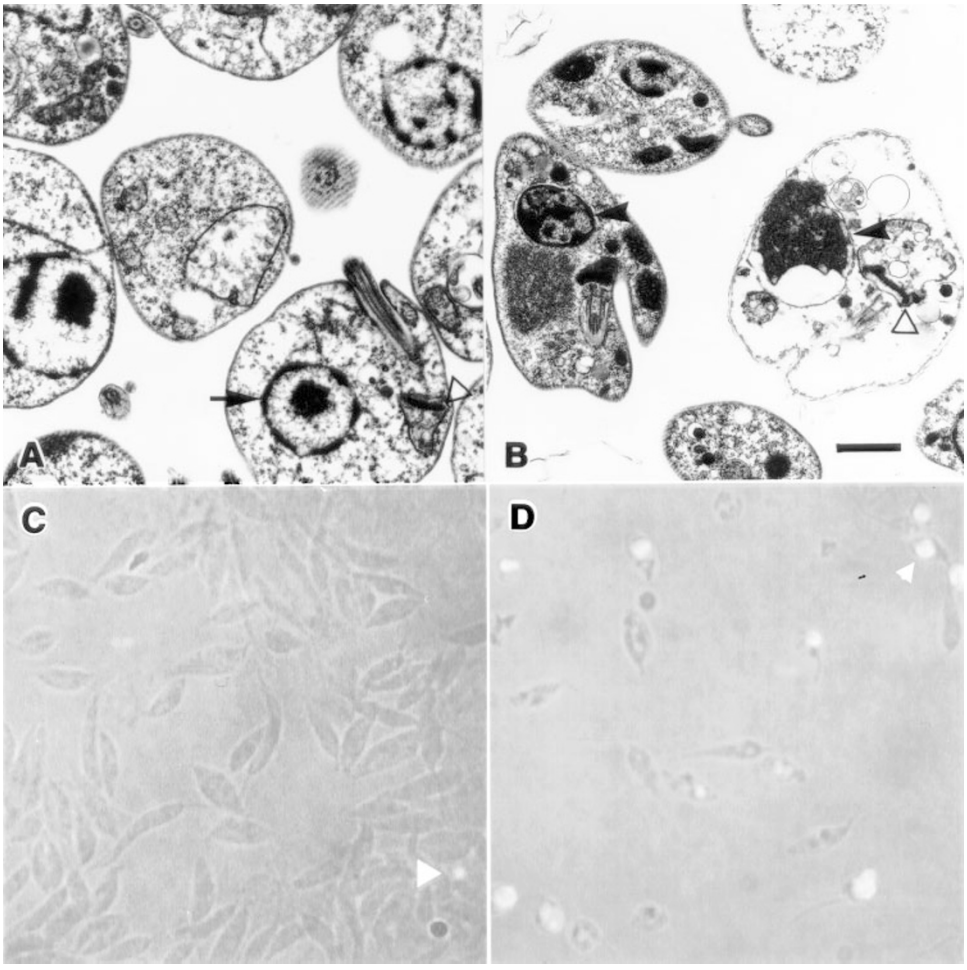

Figure 2 Transmission electron micrographs and TUNEL assay of promastigotes. Transmission electron micrographs of (**A**) late log (3 day old) and, (**B**) stationary (10 day old) *L. donovani* promastigotes. Arrows point to intact nuclei. Solid arrowheads point to condensed nuclei and empty arrowheads point to kinetoplasts. The bar in panel B indicates 1 μm. Merged phase contrast and fluorescent micrographs of the same field of late log (**C**), and stationary *L. donovani* promastigotes (**D**). Green nuclei resulting from the incorporation of FITC-labeled dUTP into nicked DNA are shown and are present predominantly in stationary phase cells. To the right of the white arrowhead is FITC-labeled nucleus in C

Induction of *in vivo* PPL-cleavage activity in *Leishmania donovani*

In multicellular organisms, the effector phase of PCD involves the activation of a class of cysteine proteases called caspases.[36] To assess whether a unicellular organism like *Leishmania* possesses similar effector molecules, we measured *in vivo* caspase-like activity in *L. donovani* promastigotes at various stages of growth *in vitro*. To that end, live cells were incubated with the cell permeable caspase-specific substrate PhiPhiLux (PPL) and the resulting intracellular fluorescent product was measured by flow cytometry. By day 4, at the beginning of the stationary phase, no significant ($\sim$5%) PPL-cleavage activity was observed (Figure 5A, left panel). However, by day 7 up to $\sim$66% of the cells were PPL positive (Figure 5A, right panel), and remained constant thereafter (Figure 5B). This induction of activity coincided with the appearance of DNA ladder formation in promastigotes (Figure 1B). These results suggest that a PPL-cleavage activity is induced in stationary phase cultures of promastigotes.

We next investigated the sequence of events of PCD occurring at various stages of growth in promastigotes by measuring mitochondrial membrane potential, PPL-cleavage activity and plasma membrane integrity. To that end, fluorescence of cells treated with TMRE, PPL and propidium iodide (PI) was measured by FACS analysis. Each cell population exhibiting either low (M1) or high (M2) TMRE fluorescence was analyzed for PPL and PI fluorescence (Figure 6). The results show that in a culture having a high TMRE population (M2) representing 63.6% of cells, approximately 95% of these cells were double negative for PI fluorescence and PPL-cleavage activity (Figure 6B). In the subsequent 2 days, this M2 population dropped to 53.5% and 37.4% of total cells while remaining greater than 90% double negative (Figure 6D,F), suggesting that the high TMRE cells are not undergoing PCD. In contrast, the low TMRE (M1)

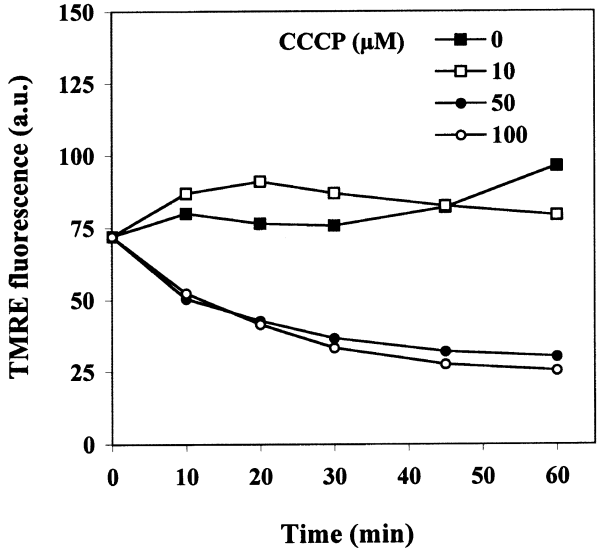

Figure 3 Effect of protonophore CCCP on the mitochondrial membrane potential as measured by fluorescence of TMRE. TMRE-loaded promastigotes at day 3 of culture (showing no hallmarks of PCD) were treated with different concentrations (0–100 μM) of CCCP. The mean TMRE fluorescence was measured at different time points after addition of CCCP and is expressed as arbitrary units (a. u.)

population initially representing 36.7% of total cells at the first time point (Figure 6A), showed fewer double negative cells (82.4%) and a corresponding increase in cells that were stained with PI and had PPL-cleavage activity (double positive, 7.8%) (Figure 6A). Forty-eight hours later the low TMRE population rose to 46.7% of total cells and within that population 17.5% were double positive (Figure 6C). Seventy-two hours after the initial reading, the low TMRE population continued to increase (62.7% of total cells) with a concomitant increase in double positive cells (26.7%) (Figure 6E). At each of these time points in the low TMRE population, there were a number of PI positive cells lacking PPL-cleavage activity equal to the number of double positive cells. On the other hand, during this time course, only a limited number ($<$5%) of low TMRE cells were PI negative and PPL-cleavage positive. These results show that during aging cells lose their mitochondrial membrane potential, which is followed by a simultaneous induction of plasma membrane permeability and PPL-cleavage activity. Taken together, from the above studies various features of a PCD can be demonstrated in *Leishmania*.

Induction of PCD by anti-*Leishmanial* drugs in *Leishmania donovani*

Human *Leishmania*sis is initially treated with pentavalent antimony (SbV, Pentostam). The 10–25% of cases that are not cured by Pentostam are treated with amphotericin B. In both cases, these drugs are leishmanicidal. We explored whether the killing of *Leishmania* by these two drugs involved the PCD pathway as illustrated above. To that end, late log phase *L. donovani* promastigotes and axenic amastigotes were incubated for 10 min with increasing concentrations of amphotericin B and assayed for PPL-cleavage activity as above. Results showed that PPL-cleavage activity was induced in amphotericin B treated axenic amastigotes and promastigotes with an IC_{50} of $\sim$0.9 μg/ml and $\sim$0.4 μg/ml respectively (Figure 7). DNA ladder formation was observed in these cells after 2 h of treatment, and longer treatment with the drug ($>$10 h) resulted in complete degradation of DNA (data not shown). Pentostam treatment of promastigotes for 48 h did not induce significantly higher PPL-cleavage activity than the untreated cells, whereas treatment of axenic amastigotes with increasing concentration (50–200 μg/ml) resulted in a significant induction, $\sim$80% of cells were PPL-cleavage activity positive (data not shown).

Decrease in mitochondrial membrane potential of *Leishmania donovani* promastigotes upon treatment with amphotericin B

In our earlier experiments with the stationary phase cells (Figure 4), we observed a loss of mitochondrial membrane potential prior to induction of PPL-cleavage activity. We explored whether a similar change in mitochondrial membrane potential also occurred prior to the induction of PPL-cleavage activity by amphotericin B treatment. The TMRE and PPL-cleavage fluorescence was measured in late log phase promastigotes by FACS analysis at different times after treatment with 0.4 μg/ml of amphotericin B. Results show that a significant decrease (80%) in mitochondrial membrane potential occurred within the first 2 min after treatment with amphotericin B which remained low during the 30 min of treatment (Figure 8A). However, the appearance of PPL-positive cells was observed only after 10 min of amphotericin B treatment and the percentage of PPL positive cells continued to increase with longer treatment times (Figure 8A), suggesting that PPL-cleavage activity follows alteration in mitochondrial membrane potential.

We next investigated the sequence of events during PCD by measuring mitochondrial membrane potential, PPL-cleavage activity and plasma membrane integrity in promastigotes at various times after 0.4 μg/ml amphotericin B treatment. To that end, fluorescence of cells treated with TMRE, PPL and propidium iodide (PI) was measured by FACS analysis (Figure 8B). Within 15 min of amphotericin B treatment there was an 88% decrease in TMRE fluorescence compared to untreated cells (Figure 8B). However, there was only a small increase ($<$4%) in the proportion of cells that exhibited both PPL-cleavage activity and PI staining. After 30 min of amphotericin B treatment a significant number of cells became either PI single positive (14.6%) or PI and PPL double positive (13.4%) with a concomitant decrease in double negative cells. These results again suggested that, similar to aging cells, amphotericin B treatment results in rapid decrease of mitochondrial membrane potential followed by a simultaneous induction of plasma membrane permeability and PPL-cleavage activity.

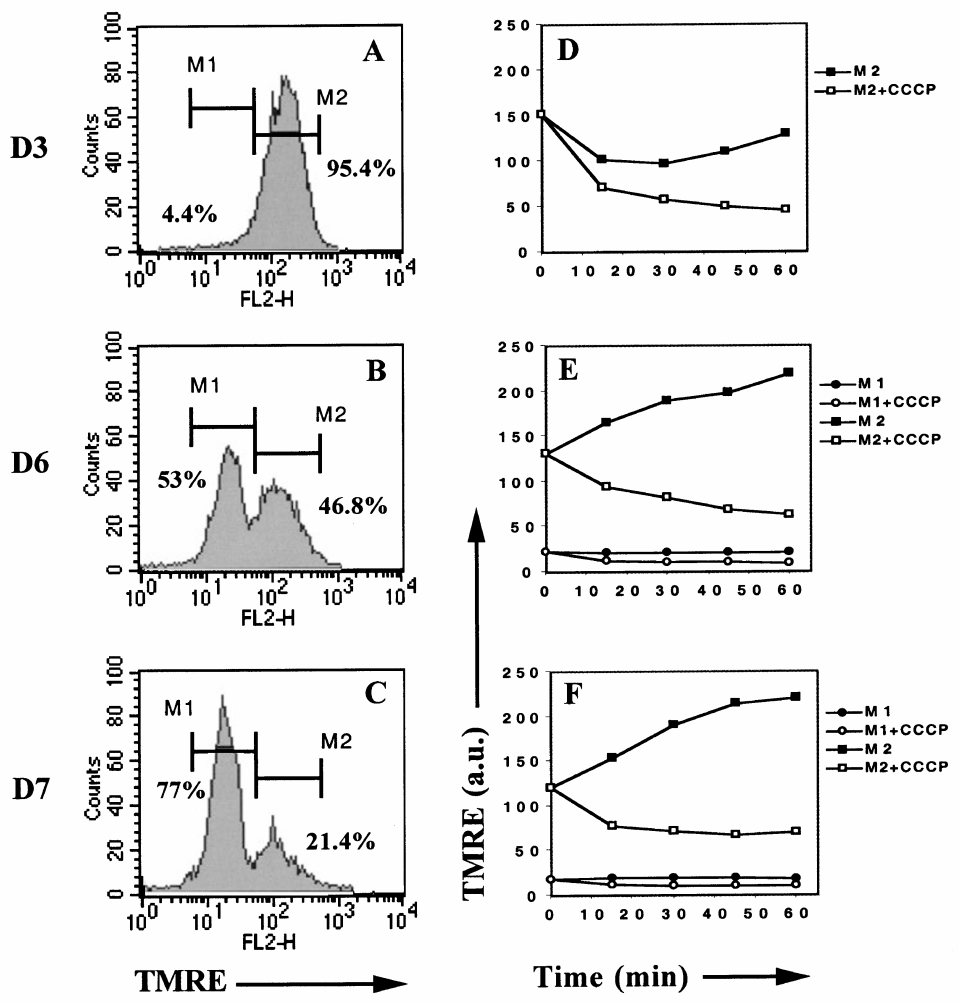

Figure 4 Change in the mitochondrial membrane potential of promastigotes during stationary phase in culture. (**A, B, C**) histograms showing fluorescence intensity of TMRE positive cells at days 3 (D3), 6 (D6), and 7 (D7) in culture. The high fluorescence intensity peaks are marked as M2, whereas the low fluorescence intensity peaks are marked as M1. (**D, E, F**) change in mitochondrial membrane potential of high (M2) and low (M1) TMRE fluorescent cells at different times after addition of 50 μM CCCP

Effect of various protease inhibitors on the PPL-cleavage activity, mitochondrial membrane potential and plasma membrane integrity in promastigotes

To assess the nature of the protease activity that cleaved the PPL substrate, promastigotes were pre-incubated with various cell permeable caspase inhibitors (Z-VAD-FMK, Z-DEVD-FMK, Boc-D-FMK) as well as with inhibitors of cathepsin (Mu-Phe-HPh-FMK) and Calpain (Calpeptin). After 60 min incubation with the inhibitor (100 μM), parasites were induced with amphotericin B and fluorescence of cells treated with TMRE, PPL and PI was measured by FACS analysis. Results showed that 21–34% inhibition of the amphotericin B inducible PPL-cleavage activity was observed in promastigotes treated with the three caspase inhibitors (Figure 9A) whereas no inhibition was observed in cells treated with either the cathepsin inhibitor or the calpain inhibitor or a control peptide (Z-FA-FMK) (data not shown). Similarly, when axenic amastigotes were treated with Z-VAD-FMK, only ~15% of the PPL-cleavage activity was inhibited compared to untreated cells (data not shown). When the promastigotes were analyzed for PI staining, only cells treated with the caspase inhibitors showed significant reduction in percent of PI positive cells (15–21%) (Figure 9B). The inhibitors did not prevent the loss of mitochondrial membrane potential ($\Delta\Psi_m$) as measured by TMRE fluorescence in promastigotes treated with caspase inhibitors suggesting that changes in $\Delta\Psi_m$ occurs upstream of induction of PPL-cleavage activity. The inhibition of the PPL-cleavage activity in promastigotes was observed only at low levels of induction by amphotericin B (when ~25% of cells were PPL positive). These results suggest that of the total PPL-cleavage activity induced by amphotericin B in *L. donovani* only 21–34% in promastigotes and 15% in axenic amastigotes may be comprised of caspase-like activity. Since other cysteine protease inhibitors did not inhibit the PPL-

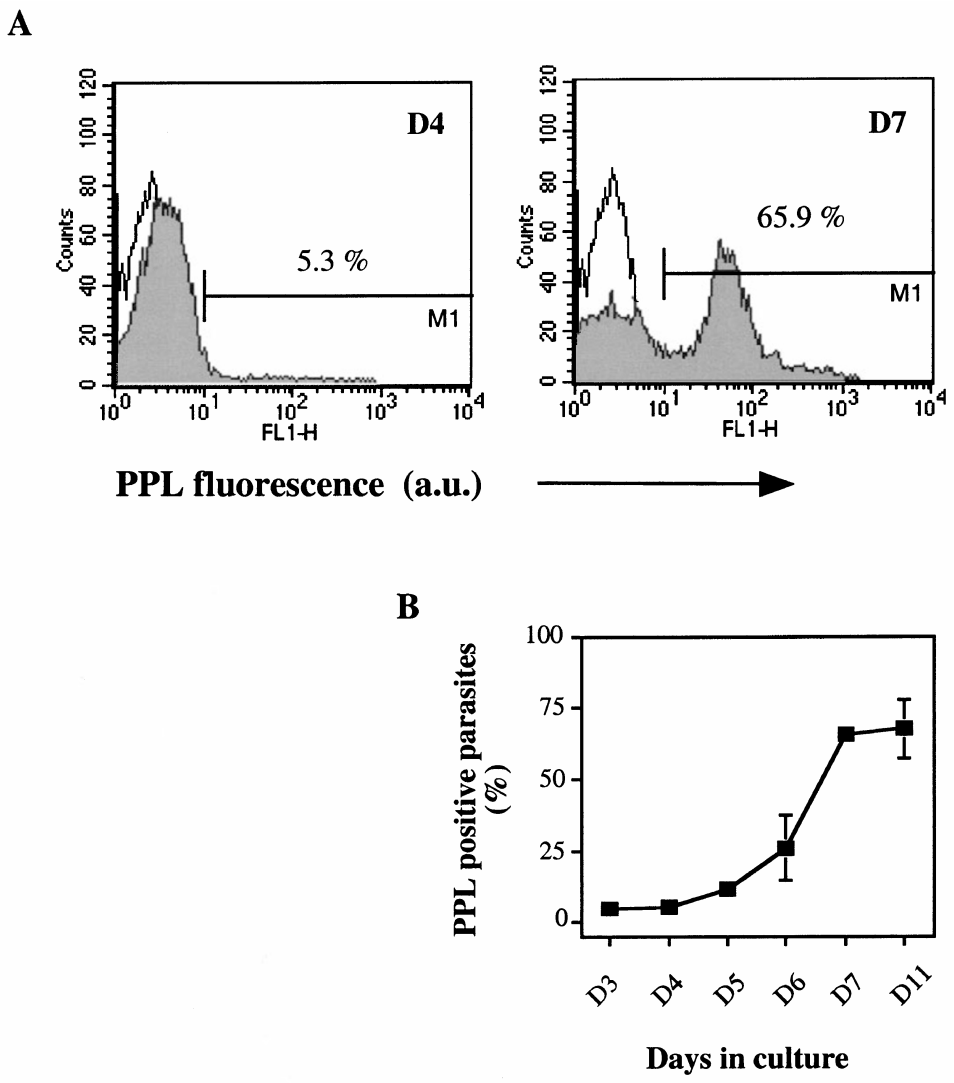

Figure 5 Analysis of PPL-cleavage activity *in vivo* in stationary phase promastigotes. (**A**) Fluorescence intensity of PPL loaded promastigote cells at day 4 and day 7 in culture (filled histograms). Open histograms represent basal cell fluorescence in the absence of PPL. Percent of PPL positive cells (M1 region) are indicated. (**B**) Increase of PPL positive promastigotes over time after initiation of the culture

cleavage activity, it is possible that there are either different proportions or different types of caspase-like activities induced by amphotericin B in promastigotes and axenic amastigotes of *L. donovani*.

Discussion

We have demonstrated that PCD exists in the unicellular protozoan parasite *Leishmania donovani* promastigotes and axenic amastigotes. The characteristic features of PCD observed in multicellular organisms such as nuclear condensation, nicked DNA in the nucleus, and DNA ladder formation were observed in promastigotes of *L. donovani*. In addition, we also observed induction of PPL-cleavage activity, which was partially, inhibited by a caspase inhibitors, in both axenic amastigotes and promastigotes upon initiation of PCD. The induced PPL-cleavage activity was also not inhibited by inhibitors of other cysteine proteases such as cathepsin or calpain, which have been implicated to function in caspase-independent manner to induce PCD.[37,38] Therefore, suggesting that a certain fraction of total PPL-cleavage activity in *Leishmania* is caspase-like. However, the exact nature of the PPL-cleavage activity remains to be determined. The induction of PPL-cleavage activity, which coincided with increase in plasma membrane permeability, was observed after a drop in the mitochondrial membrane potential. The PCD was initiated when the parasites either reached stationary phase in culture or by exposure to anti-*Leishmanial* drugs.

PCD can be initiated by a number of different stimuli. However, the signaling mechanism by which these stimuli induce PCD is not clear. In the present study we tested the

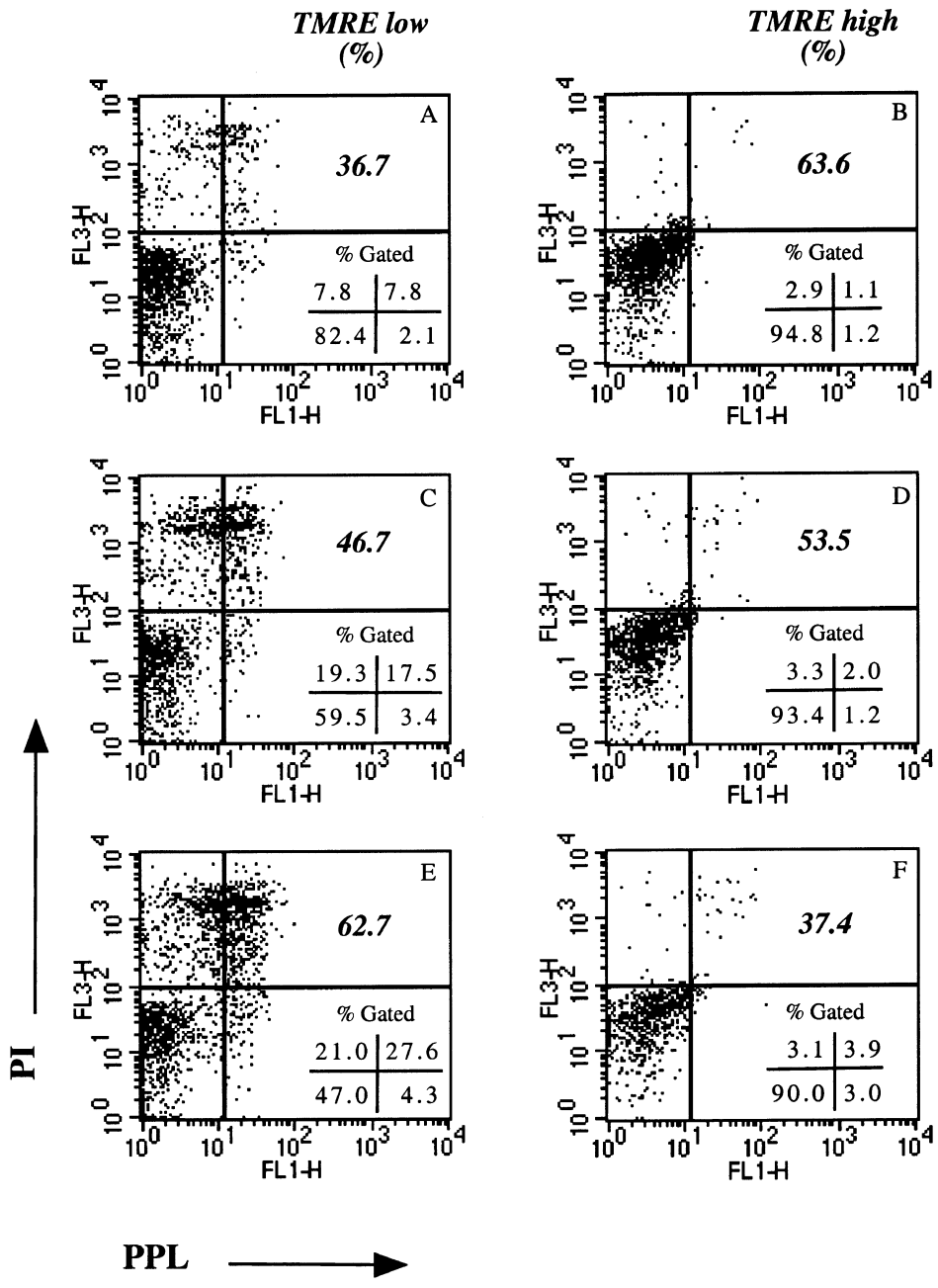

Figure 6 Analysis of mitochondrial membrane potential, PPL-cleavage activity and plasma membrane integrity in promastigotes during stationary phase in culture. Fluorescence of cells treated with TMRE, PPL and PI was measured by FACS analysis. (**A, C, E**) cells from different days in culture gated for low level of TMRE fluorescence, (**B, D, F**) cells gated for high level of TMRE fluorescence. Both populations were analyzed for PPL (FL-1) and PI (FL-3) fluorescence. The bold numbers represent the percentage of cells in each TMRE population. The percentage of cells gated for TMRE in each quadrant represent; lower left (double negative), upper left (PI, single positive), lower right (PPL, single positive), and upper right (PI, PPL double positive)

hypothesis whether *Leishmania* is amenable to death from various stimuli such as anti-*Leishmanial* drugs, which have been shown to kill intracellular parasites.[39,40] We observed that the killing of *Leishmania* either by Pentostam or by amphotericin B is associated with markers of PCD. However, there were differences in the induction of PCD in promastigotes by Pentostam as compared to axenic amastigotes. Previously, similar differential sensitivity of *L. donovani* promastigotes to Pentostam has been observed.[41] Thereby, suggesting that the two cell types of *Leishmania* respond differently to PCD signals, which could mean that there are either differences in the PCD machinery between these two cell types or differences in the sensitivities to the leishmanicidal activities of the drug. Interestingly, some of the features of PCD such as changes in the membrane integrity, increased electron density of the

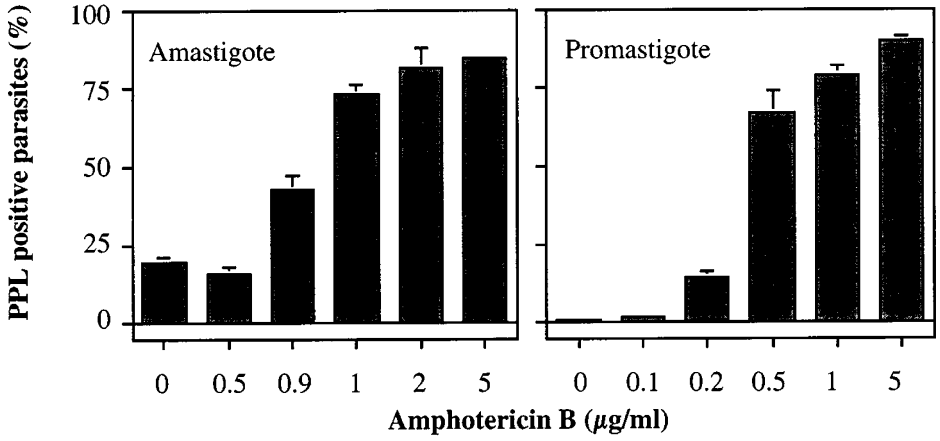

Figure 7 Induction of PPL-cleavage activity in axenic amastigotes and promastigotes by amphotericin B. Per cent of PPL positive cells in 3-day-old cultures of axenic amastigotes and promastigotes after 10 min of treatment with various concentrations of amphotericin B. The concentration of amphotericin B (μg/ml) is indicated

cytoplasm, and nuclear condensation also have been observed in *L. donovani* inside macrophages isolated from patients as well as in *L. tropica* inside *in vitro* infected macrophages upon treatment with either Pentostam or amphotericin B.[39,40] In addition, we also have demonstrated that PCD exists in both *L. donovani* and *L. major* that were freshly isolated from infected animals and grown *in vitro* as promastigotes for 2–5 passages, as shown by the amphotericin B induced PPL-cleavage activity (data not shown). These observations suggest that PCD observed in laboratory adapted parasites, is also observed in parasites during infection.

Existence of PCD also has been described previously in *Trypanosomatids* such as T. *brucei rhodesiense*,[17] T. *cruzi*,[13] and *Leishmania amazonensis*.[18] In addition, PCD has been demonstrated in other unicellular organisms such as *Tetrahymena*[42–44] and *Dictyostelium discoideum*.[15] Further, bacteria and yeast, which do not undergo PCD and do not possess proteins homologous to any of the apoptotic regulators such as Bcl-2 or Bax, were shown to undergo cell death upon expression of proapototic proteins in either cell type.[45–50] Similarly, a *S. cerevisiae* mutant that has a mutation in the cell division gene CDC48 showed typical markers of apoptosis.[51,52] These studies therefore imply the presence of molecular machinery, which is able to perform the basic steps of apoptosis in lower eukaryotes and prokaryotes. The induction of PCD initiated in unicellular organisms such as *Trypanosomatids, Tetrahymena, Dictyostelium* by external signals or in bacteria and yeast by expression of proapoptotic proteins leads us to believe that the PCD pathway is present in unicellular as well as in multicellular organisms. However, the PCD pathways in the two types of organisms could be different. The definition of apoptosis has been used interchangeably with the term PCD. Recently, an alternative nonapoptotic form of PCD, parapoptosis, has been described, which is distinct from apoptosis by the criteria of morphology, biochemistry, and response to apoptosis inhibitors.[19] Whether the PCD pathway observed in unicellular organisms is similar to parapoptosis remains to be seen. However, it has been postulated that PCD in *Dictyostelium discoideum* could resemble parapoptosis.[15] *Leishmania* shares some of the features of PCD observed in multicellular organisms, including a change in mitochondrial membrane potential and induction of PPL-cleavage activity, which has some features of caspase-like activity, that has not been as yet reported in other unicellular organisms. However, there are also differences between PCD in *Leishmania* and that observed in multicellular organisms: (1) The PCD associated PPL-cleavage activity is only partially inhibited by several caspase inhibitors and is not inhibited by other cysteine protease inhibitors such as cathepsin or calpain, suggesting proteases other than or different from caspases may be involved; (2) The permeability of the plasma membrane to PI occurs simultaneously with activation of PPL-cleavage activity rather than following the activation of this activity; (3) There is no formation of apoptotic bodies. Thus the form of PCD in the unicellular parasite may be more similar to parapoptosis. Some of the differences may arise because the PCD in unicellular organisms is not adapted to lead to phagocytosis of the dead cells as in multicellular organisms. However, until the effector and regulatory molecules of PCD in *Leishmania* are identified and characterized, it is premature to conclusively define the type of cell death pathway in *Leishmania*.

Having established that PCD exists in *Leishmania*, the question arises with regards to the significance of this pathway in this parasite or in other unicellular organisms because each cell exists as an individual. This is in opposition to multicellular organism where controlling the number of certain cell types such as those in the immune, haemopoietic, and nervous systems is important for survival of the whole organism. Further, the failure to trigger PCD in multicellular organisms may lead to uncontrolled growth resulting in various abnormalities such

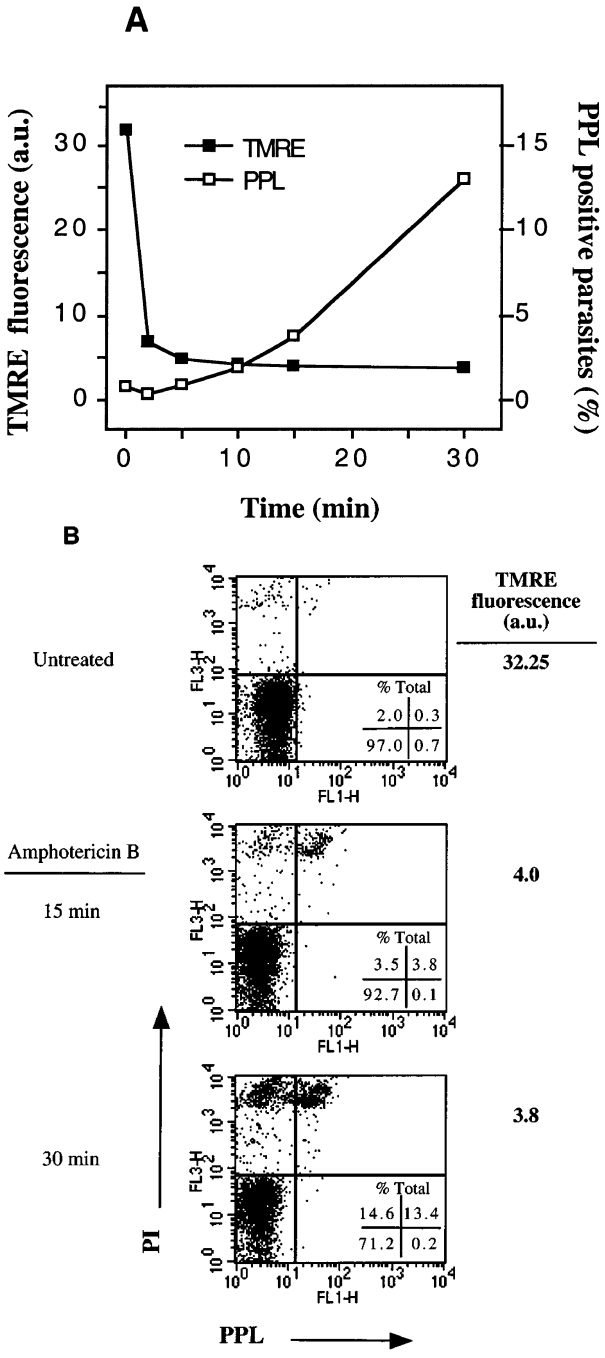

Figure 8 Analysis of mitochondrial membrane potential, PPL-cleavage activity and plasma membrane integrity in promastigotes treated with amphotericin B. (**A**) TMRE fluorescence and per cent of PPL positive promastigote cells at different times of treatment with amphotericin B (0.4 μg/ml). (**B**) Fluorescence of cells loaded with TMRE, PPL, and PI was measured by FACS analysis in untreated and amphotericin B (0.4 μg/ml) treated promastigotes after 15 and 30 min. Corresponding mean TMRE fluorescence of the cells is indicated in bold. The percentage of cells in each quadrant represent lower left (double negative), upper left (PI single positive), lower right (PPL single positive), and upper right (double positive)

as cancer, and autoimmune disease. One of the proposed functions of the PCD pathway in unicellular organisms is to control the cell population, as is the case in multicellular organisms.[14,53–55] This has been substantiated previously by demonstration of PCD in dividing epimastigotes of *T. cruzi* undergoing differentiation to stationary phase trypomastigotes,[16] in dying trypanosomes inside the tsetse fly mid gut,[20] and after triggering of the differentiation process in *Dictyostelium discoideum*.[15] In the present study we have observed that as soon as either promastigotes or axenic amastigotes enter stationary phase in culture there is a significant induction of PCD, and this induction can be delayed by transferring these parasites to fresh media (unpublished observations). The induction of PCD in stationary phase cultures *in vitro* could be due to the number of reasons such as differentiation; deprivation of nutrients essential for growth or due to increased cell density. Similarly it is conceivable that the PCD pathway could play a role *in vivo* in controlling the growth of *Leishmania* inside the sand fly gut, and could be triggered because of the limited resources in the sand fly gut. Alternatively, the PCD pathway could be a response to signals from the host such as growth factors or cytokines.[56] In addition, PCD may be beneficial to the organism to maintain clonal characteristics by self-destruction of some cells and ensuring the propagation of other cells for the overall benefit of the parasite survival.[18,57] For example, in the case of *Leishmania* promastigotes, which spend a significant time in the insect vector and differentiate into metacyclic forms before being transmitted to the mammalian host.[58,59] However, it is not clear what happens to the rest of the undifferentiated procyclic parasite population during this time. Does PCD sort out the metacyclic form from the procyclic form and thus ensure the selection of the infectious form? Further studies using *Leishmania* isolated from the sandfly gut are essential to answer this question. In this regard, it is interesting to note that during transformation to infectious form the majority of *T. brucei rhodesiense* parasites fail to establish infection in the mid gut of tsetse fly and die with the characteristic apoptotic morphology.[20]

In conclusion, we have demonstrated that a type of PCD exists in *Leishmania donovani*, which is induced when the cells enter stationary phase in culture, and in response to anti-*Leishmanial* drugs. We also have evidence that PCD exists in freshly isolated parasites as well, thereby suggesting that it is not a phenomenon of *in vitro* culturing. Induction of PPL-cleavage activity in *L. major* leads us to believe that PCD is not restricted to *L. donovani*. Further, we argue that this is an adapted process that serves the long-term survival of some members of the population by eliminating unwanted individuals. The process is dependent on expression of cellular proteins such as the ones responsible for PPL-cleavage activity. However, it remains to be determined whether similar induction of PCD takes place in *Leishmania* inside the insect gut or vertebrate macrophages. In addition, to understand the mechanism of *Leishmania* PCD pathway, both regulatory and effector molecules involved in this pathway have to be established in *Leishmania*. In that regard, we have shown in this study the protease activity induced in parallel with nuclear changes is comprised partly by the caspase-like proteases.

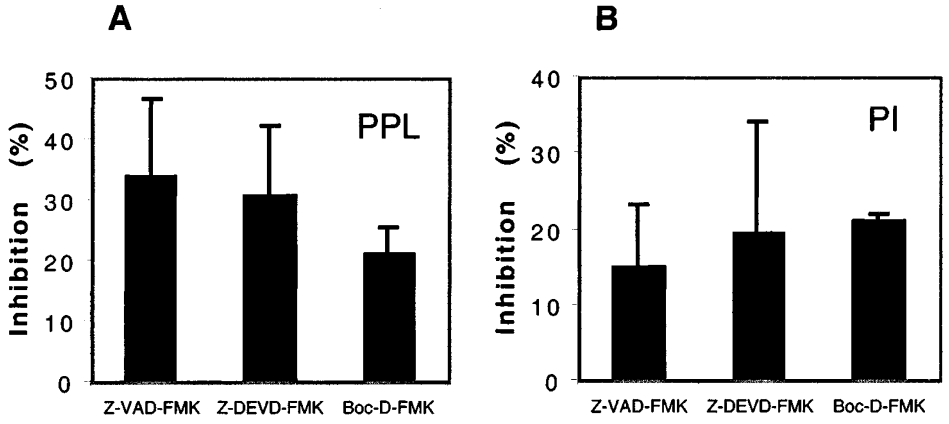

Figure 9 Inhibition of PPL-cleavage activity and plasma membrane integrity by different caspase inhibitors in promastigotes treated with amphotericin B. (**A**) Per cent of inhibition of PPL-cleavage activity in promastigotes treated with amphotericin B, after incubation with different caspase inhibitors (100 μM). Mean with standard error bar results from three independent experiments is shown. (**B**) Per cent of inhibition of PI positive cells in promastigotes treated with amphotericin B after incubation with different caspase inhibitors (100 μM). Mean with standard error bar results from three independent experiments is shown

Further studies are needed to characterize these and other molecules involved in PCD in *Leishmania*. A detailed understanding of the mechanism of PCD will be helpful in devising therapeutic strategies to design drugs that can induce preferential killing of *Leishmania*.

Materials and Methods

In vitro culture of parasites

A cloned line of *L. donovani* (WHO designation: MHOM/SD/62/1S-$C1_{2D}$) was used in all experiments.[60] Promastigotes and axenic amastigotes were grown, and harvested as described previously.[60] *L. donovani* [WHO designation MHOM/SD/00/IS-2D] and *L. major* [WHO designation MHOM/IL/80/Friedlin] parasites, freshly isolated from infected hamster and BALB/C mice, respectively,[61] were kindly provided by Dr. David Sacks (NIH, Bethesda, MD, USA) and were maintained as promastigote cultures as above for less than five passages.

Electron microscopy

Cells were fixed with 2% glutaraldehyde-2% paraformaldehyde in 0.1 M sodium cacodylate buffer, pH 7.3, for 3 h and stored in PBS at 4°C. Cells were post-fixed for 1 h with 2% osmium tetraoxide, dehydrated with graded alcohols, and embedded in epoxy resin. Thin sections were stained with uranyl acetate and lead citrate and examined with a Zeiss EM 912 Omega electron microscope.

TUNEL assay

L. donovani promastigote cells were grown for different lengths of time in chamber slides, washed with PBS, fixed in 75% methanol/2.5% acetic acid, rinsed twice with PBS, layered with TUNEL reaction mixture (Boehringer Mannheim) containing FITC-labeled dUTP and terminal deoxynucleotidyl transferase, and incubated for 1 h at 37°C in a moist chamber. Cells were visualized on a fluorescent microscope, and images captured with a digital video camera and processed with Adobe PhotoShop software.

DNA gel analysis

Total genomic DNA was isolated from parasites as described in the G NOME DNA kit protocol (Bio-101, Inc). Usually 5×10^8 to 1×10^9 cells were used for extraction of DNA. Approximately 10 μg of genomic DNA was run on 1% agarose gels in 1% Tris-Acetate-EDTA (TAE) buffer.[60] The gels were stained with ethidium bromide and visualized under UV light. DNA ladder data presented are representative of at least three independent experiments.

FACS analysis of PPL-cleavage activity in live *Leishmania*

A PhiPhiLux G1D2 (PPL, Calbiochem, La Jolla, CA, USA) substrate was used to measure caspase-like activity by flow cytometry in living cells. This peptide substrate contains a caspase-3 specific G*DEVD*GI sequence with the cleavage site located between D and G and is conjugated to two fluorophores (G1D2). The quenched fluorophores fluoresce upon cleavage of the peptide.[33] 2×10^6 cells were resuspended into 25 μl medium (RPMI 1640, 10% FBS, 20 mM HEPES pH 7), incubated with additional 25 μl PPL for 15–30 min at 37°C, washed and resuspended into 500 μl cytometry dilution buffer (Caspase-3 intracellular activity assay kit I; Calbiochem). The cells were dispersed by several passings through a 26-gauge needle and analyzed using a FACScan flow cytometer (Becton Dickinson). FL1-H fluorescence was recorded on 10 000 events gated according to forward and side scatter and analyzed using CellQuest software. Data are expressed as per cent of PPL positive cells and are representative of three independent experiments. Induction of PCD by amphotericin B (Sigma) or pentavalent antimony (SbV, Pentostam, The Wellcome Foundation Ltd., London, UK) was done by incubating 3-day-old (late log phase) parasite cultures for the indicated periods of time with different concentrations of inducer prior to processing cells for PPL-cleavage activity as above.

Measurement of mitochondrial membrane potential ($\Delta\Psi_m$) in live *Leishmania* promastigotes

Tetramethylrhodamine ethylester perchlorate (TMRE, Molecular Probes, Inc., Eugene, OR, USA) is a cationic lipophilic dye that accumulates in the negatively charged mitochondrial matrix according to the Nernst equation potential.[33] A stock of TMRE was prepared at a

concentration of 4 mg/ml in DMSO and stored at -20°C. For estimation of ($\Delta\Psi_m$), *L. donovani* promastigotes were washed once in PBS and resuspended at 2×10^6 cells/ml in PBS containing 100 nM TMRE. Cells were dispersed by several passings through a 26-gauge needle, incubated for 15 min at room temperature and analyzed by flow cytometry on the FL2-H channel. Baseline TMRE was recorded and expressed as mean fluorescence in arbitrary units (a.u.). Subsequently, carbonyl cyanide m-chlorophenylhydrazone (CCCP, Sigma) was added to the cells (10–200 μM final concentration) and TMRE fluorescence was measured at various times up to 60 min. CCCP depolarizes mitochondria by abolishing the proton gradient across the inner mitochondrial membrane.[62,63] In some experiments, *L. donovani* promastigotes were first incubated with PPL for 15 min as above and subsequently resuspended in PBS containing 100 nM TMRE and 4 μg/ml propidium iodide (PI, Biosciences, Inc., La Jolla, CA, USA). After 15 min incubation at room temperature, cells were analyzed by flow cytometry as above. PI fluorescence was recorded on the FL3-H channel.

Inhibition of the PPL-cleavage activity in live *Leishmania*

For inhibition studies, the following protease inhibitors were used: Z-Val-Ala-Asp (OMe)-CH_2F (Z-VAD-FMK, Alexis Biochemicals, San Diego, CA, USA); Z-Asp (OMe)-Glu (OMe)-Val-Asp (OMe)-CH_2F (Z-DEVD-FMK, Calbiochem); Boc-Asp (OMe)-CH_2F (Boc-D-FMK, Calbiochem); Z-Phe-Ala-CH_2F (Z-FA-FMK, Enzyme Systems Products, Livermore, CA, USA); Mu-Phe-HPh-CH_2F (Mu-Phe-HPh-FMK, Enzyme Systems Products); Calpeptin (Calbiochem). 2.4×10^7 promastigotes from a 3-day-old culture were incubated at 26°C for 60 min in 400 μl of culture medium containing either 100 μM of each protease inhibitors described above or equivalent amount of DMSO and further treated for an additional 10 min with different concentrations of amphotericin B to induce PCD. An aliquot of 2×10^6 cells was subsequently washed in 1 ml RPMI 1640 (Gibco) and resuspended in 25 μl medium containing either 200 μM of the corresponding protease inhibitor or equivalent amount of DMSO, and an equal volume (25 μl) of PPL substrate. After 15 min incubation at 37°C, cells were washed in 1 ml PBS, resuspended in PBS containing 100 nM TMRE and 4 μg/ml propidium iodide and analyzed by flow cytometry as above.

Acknowledgements

Authors would like to thank Drs. Dwyer and Sacks (NIAID, NIH) for critical reading of the manuscript. We thank Drs. Navdeep Chandel and Gerry Melino for providing helpful suggestions in performing mitochondrial membrane potential experiments. We also thank Dr. Sacks for providing the two strains of *Leishmania* that were freshly isolated from infected animals.

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