Host Cell Fate on Cryptosporidium parvum Egress from MDCK Cells

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Cryptosporidium parvum is an intracellular protozoan parasite that causes a severe diarrheal illness of unclear etiology. Also unclear is the fate of the host cell upon parasite egress. We show in an MDCK cell model that the host cell is killed upon parasite egress; this death is necrotic, rather than apoptotic, in nature.

The obligate intracellular protozoan parasite Cryptosporidium parvum causes a diarrheal illness in infected hosts (5, 7). While the life cycle of the parasite is understood, it is unclear what aspect of the host-parasite interaction causes disease. During the invasion process, Cryptosporidium establishes an intracellular compartment within the host cell from which it ruptures after its intracellular development (12, 14, 21, 22). It has been reported that Cryptosporidium infection induces caspase-dependent apoptosis of infected cells (2, 4, 15, 16, 23), but it is unclear if these observations are unique to the in vitro system. In one study of intestinal biopsy samples from AIDS patients with cryptosporidiosis, there was no association of the intensity of small bowel infection with apoptosis (13). Supporting this in vivo observation is the finding that Cryptosporidium infection induces apoptosis in infected cells but that there is increased apoptosis in uninfected neighboring cells, explaining the previously observed increase in vitro (4). Most of these investigations examined populations of infected cells several hours after infection; thus, the effects could not be correlated with specific events in the parasite life cycle. Our goal was to gain a better understanding of Cryptosporidium pathology by examining the fate of individual C. parvum-infected cells during parasite egress.

In order to investigate the fate of the individual Cryptosporidium-infected host cell over time, we first designed and tested an in vitro system that identifies changes in cell permeability and death upon Cryptosporidium egress. Human intestinal epithelial cell lines (HCT-8, Caco-2, and HT29) have been extensively used for in vitro apoptosis (4). Most of these investigations examined populations of infected cells several hours after infection; thus, the effects could not be correlated with specific events in the parasite life cycle. Our goal was to gain a better understanding of Cryptosporidium pathology by examining the fate of individual C. parvum-infected cells during parasite egress.

To identify characteristic features of apoptosis in this system, MDCK cells were exposed to 360 kJ shortwave UV radiation for 3 min (Stratalinker 2400) to induce apoptosis (18). The nuclear fragmentation that is characteristic of apoptosis was highlighted by the nuclear stain (Fig. 1B and C). There was no staining of the DNA with the membrane-impermeable dye SYTOX green (1), indicating that the plasma membrane remains intact during early apoptosis. By using deconvolution microscopy, an apoptotic MDCK cell in our system that had been extruded from the monolayer was demonstrated, as shown in Fig. 1D and E (Delta Vision microscope and SoftWoRx version 2.5; Applied Precision, Issaquah, Wash.). The fragmentation of its nucleus was highlighted by the Hoechst and SYTOX dyes.

To evaluate the integrity of host cell plasma membranes in this system, saponin was added to the MDCK cells to selectively permeabilize the plasma membrane. In cells with intact plasma membranes, SYTOX green is excluded from cells, preventing nuclear staining, but when the plasma membrane is disrupted, SYTOX green enters the cell and stains the nucleus. Figure 2A demonstrates the exclusion of SYTOX green from intact MDCK cells but rapid nuclear staining after exposure to saponin (Fig. 2B). The nuclear morphology of the intact MDCK cells is stable for at least 8 h after permeabilization, unlike that of apoptotic cells. Also unlike the apoptotic cells,
FIG. 1. Fluorescence microscopy of MDCK cells showing apoptosis at different stages following UV irradiation. Staining was done with MitoTracker, Hoechst stain for DNA, and SYTOX green, which stains DNA in permeable cells. At 60 min after the induction of apoptosis by UV irradiation (B), the nucleus assumes the characteristic fragmented appearance associated with apoptosis. Also note that 90 min after UV irradiation (C), there is no staining with the SYTOX green stain, indicating that the plasma membrane of the cell is intact. Panel D shows a late-stage (time [T] ≈ 120 min) apoptotic cell (outlined) being extruded from the monolayer. Panel E shows the same cell, but in cross section (an xz view), clearly demonstrating that the cell has been extruded from the monolayer. Note the fragmented nuclear staining by Hoechst stain and SYTOX green, typical of apoptosis. Original magnification, ×400.
FIG. 2. Fluorescence microscopy of an MDCK cell monolayer showing permeabilization of the cells by saponin (A and B) and MitoTracker staining of parasites (C and D). At 15 min after saponin addition (B), the nuclei are brightly stained with the SYTOX green dye, indicating plasma membrane disruption that allows nuclear staining. Note that there is an associated decrease in brightness of the MitoTracker staining, indicating a decrease in the membrane potential of the mitochondria. Panel C shows a *C. parvum*-infected MDCK cell monolayer stained with MitoTracker and Hoechst stain. Note the parasite in panels C and D (arrows). It is not known how the Hoechst nuclear dye is excluded from the parasite. Panel D shows the same field in cross section (an xz view), clearly demonstrating that the parasite (arrow) is above the monolayer. These images are projections of a deconvolved stack of images. T, time in minutes. Original magnification, ×400.
the mitochondria lose their electrical potential, as demonstrated by loss of MitoTracker staining (Fig. 2B).

The egress of merozoites from three meronts is shown over time in Fig. 3. It is clear that in less than 15 min after the parasites exit (Fig. 3A), the nucleus of the cell is strongly stained with SYTOX green, indicating that the cell has been permeabilized. Six minutes after egress, the mitochondrial staining has decreased by 41%, which suggests a loss of mito-
chondrial function. After 5 h, the nuclear morphology still appears normal even though the cell is dead, as indicated by the permanent loss of mitochondrial staining. Even at this late time point, the cell has not been extruded from the monolayer, as is commonly seen within 1 to 2 h after the induction of apoptosis. (The movie from which these images have been taken is available at http://homepage.mac.com/fitzrobert/PaperFigure.mov). These results suggest that C. parvum egress induces a defect in the host plasma membrane, followed by nonapoptotic cell death. These are consistent findings that have been documented in 12 parasite egress events during seven independent experiments.

Apoptotic and necrotic cell death can be easily distinguished by morphologic features and specific biochemical changes. Apoptosis involves a highly regulated pathway that leads to the controlled destruction of the cell (17). This process involves fragmentation of the DNA, loss of mitochondrial function, and cytoskeletal disruption manifested as cell shrinkage, nuclear fragmentation, and membrane blebbing. Necrotic cell death is a contrasting form of cell death resulting from cellular injury that is associated with an early loss of plasma membrane integrity; this loss results in leakage of cell contents, which can initiate an inflammatory response. In contrast to necrotic cells, apoptotic epithelial cells maintain their plasma membrane integrity until late in the death process (18).

In a system using the MDCK cell line, we have shown that upon its egress from a host cell, Cryptosporidium induces a defect in the plasma membrane of that cell. This is demonstrated by staining the host cell nucleus with a membrane-impermeable dye within 6 min of parasite egress. Over the next several hours, the nucleus remains morphologically intact, without evidence of apoptotic changes. Also, during this time, the mitochondria cease functioning, as demonstrated by a dye that recognizes electrochemical gradients. Taken together, these findings indicate that Cryptosporidium induces a non-apoptotic death in host cells upon egress.

If this conclusion is true in vivo, this nonapoptotic death may play several roles in the pathogenesis of the diarrhea induced by Cryptosporidium. It has previously been shown that Cryptosporidium induces a decrease in resistance across epithelial monolayers and mucosa, indicative of damage to the tight junctions between cells (6) or holes in the monolayer resulting from missing cells. It seems likely that the necrotic cell death induced by Cryptosporidium would contribute to this lack of epithelial integrity. Interestingly, even high levels of apoptosis do not alter the resistance of epithelial cell monolayers (18). It is also possible that nonapoptotic cell death plays a role in triggering the host immune reaction to Cryptosporidium (6). Future studies may further elucidate the relationship between the parasite life cycle and the pathogenesis of cryptosporidiosis.

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REFERENCES


