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Cryptosporidium parvum at Different Developmental Stages Modulates Host Cell Apoptosis In Vitro

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We studied apoptosis in a human ileocecal adenocarcinoma tumor cell line (HCT-8) infected with *Cryptosporidium parvum*, from 2 to 72 h postinfection (h.p.i.). At 2 h.p.i., the percentage of annexin V-positive cells in the cell culture had increased to 10% compared to 2.5% in noninfected control culture; sorted infected cells expressed mRNA of FasL, the active form of caspase 3, and high caspase 3 activity, whereas the noninfected neighboring cells sorted from the same culture showed no signs of apoptosis. At 24 h.p.i., the percentages of early (annexin V positive) and late (DNA fragment) apoptotic cells were 13 and 2%, respectively, in the entire cell culture, and these percentages were not statistically significant in comparison with those from noninfected control cultures. At this time, sorted infected cells expressed the inactive form of caspase 3, a low caspase 3 activity, and the antiapoptotic protein Bcl-2. Noninfected cells sorted from the same culture showed expression of the active form of caspase 3, a moderate caspase 3 activity, and no Bcl-2 expression. At 48 h.p.i., the percentages of early and late apoptotic cells and caspase 3 activity had increased in the total cell culture, and both sorted infected and noninfected cells showed the active form of caspase 3. These results show that *C. parvum*, depending on its developmental stage, can inhibit (at the trophozoite stage) or promote (at the sporozoite and merozoite stages) host cell apoptosis, suggesting that it is able to interact with and regulate the host-cell gene expression.

Cryptosporidium parvum is an intracellular yet extracytoplasmic protozoa, and because of this peculiar niche, it is a particularly useful model for studying immunopathological mechanisms in both the host cell and in neighboring cells (10, 28, 30). The primary site of *C. parvum* infection is the epithelium of the intestine, although epithelial cells in extraintestinal sites, including the stomach and the biliary and respiratory tracts, can also be colonized (18).

Infection of the intestinal epithelium with *C. parvum* can result in epithelial apoptosis in addition to other histological changes, such as blunting of the intestinal villi, crypt hyperplasia, and infiltration of inflammatory cells in the lamina propria (19, 21). Apoptosis has been established as a crucial modulator of host-parasite interactions. Apoptosis of parasite-infected host cells may exert parasiticidal activity, but several protozoan parasites have been shown to modulate host cell death by direct or indirect mechanisms. However, the mechanism through which apoptosis in specific host cells is induced by parasites (not only *C. parvum* but also *Entamoeba histolytica*, *Trypanosoma cruzi*, and *Schistosoma mansoni*) (6, 17, 25, 35) remains obscure, especially for epithelial cells. Candidate pathways for apoptosis in epithelial cells include, but are not limited to, microbial-induced increases in host cell protease activity (e.g., caspases and up regulation of Fas [CD95]/FasL), up-regulation of proapoptotic cytokines (e.g., tumor necrosis factor alpha), and down-regulation of antiapoptotic proteins (e.g., Bcl-2) (14).

Although these studies show that *C. parvum* exerts its influence on host cell apoptosis in both infected cells and neighboring noninfected cells, no information is available on whether this influence changes according to the developmental stage of the parasite or on whether the effect on infected cells differs from that on the neighboring noninfected cells. To address these issues, we measured markers of apoptosis in a *C. parvum*-infected cell culture from the invasive stage of the sporozoite to that of gamont development and, furthermore, we measured the apoptotic markers separately in infected and noninfected cells.

The results show that host cell apoptosis is modulated from the developmental stages of the parasite, i.e., apoptosis of the host cell is induced by the sporozoite invasion for up to 6 h postinfection (h.p.i.) and then the *C. parvum* trophozoite inhibits the apoptosis of the host cell at 24 h.p.i. Apoptosis is again active in both infected and uninfected neighboring host cells at 48 h.p.i.

MATERIALS AND METHODS

Infection of cells with *C. parvum* sporulated oocysts. HCT-8 cells were cultured in RPMI 1640 (GIBCO/Invitrogen, Paisley, United Kingdom) supplemented with 5% fetal calf serum (FCS; GIBCO), 200 mM L-glutamine (Sigma, St. Louis, Mo.), 1% sodium pyruvate (Sigma), 5% penicillin, and 5% streptomycin. The cells were maintained in tissue culture flasks in a 5% CO_2 atmosphere at 37°C and 85% humidity (32). *C. parvum* oocysts, genotype C (isolate code ISSC6), were obtained from experimentally infected calves after feces purification by sucrose and Percoll density gradients (1). For excystation, oocysts were resuspended in 10 mM HCl and incubated at 37°C for 10 min, in accordance with the method of Gut and Nelson (11). In brief, the suspension was centrifuged at 3,000 × *g* for 5 min; the pellet was resuspended in 2 mM sodium taurocholate (Sigma) in phosphate-buffered saline (PBS) and incubated at 15°C for 10 min and at 37°C for 5 to 8 min. The maximum level of excystation (i.e., 90%) was obtained when using 2- to 3-week-old oocysts; thus, 2- to 3-week-old oocysts were used throughout the study. Sporulated oocysts were resuspended, aliquoted in the growth medium, and transferred to a petri dish. Before infection with sporulated oocysts, HCT-8 cells were plated in petri dishes at 10^6 cells/78

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cm^2, the FCS concentration was increased to 10%, and the cells were grown to 60 to 70% confluence. The cells were infected with 1 sporulated oocyst per cell (ratio 1/1), to obtain an infection level of about 80% after 24 h (23).

Quantification of infected cells. HCT-8 cells were treated with a solution of 0.05% trypsin and 0.02% EDTA and centrifuged at 500 × *g* for 10 min. The cells were then fixed in 4% formaldehyde in PBS for 20 min and treated with 1% Triton X-100 in PBS for 10 min and with 2% bovine serum albumin (BSA) in PBS for 30 min. To evaluate the percentage of infected cells, a rabbit anti-*C. parvum* immunoglobulin G (IgG) polyclonal antibody which recognizes all stages of *C. parvum* was used (26). The fixed cells were incubated with 0.5 μg of the anti-*C. parvum* IgG/ml with 1% BSA in PBS at room temperature for 30 min. The cells were then washed twice in PBS and centrifuged at 500 × *g* for 10 min and resuspended with 1% BSA in PBS. The cell suspension was pipetted vigorously. One microgram of the anti-rabbit fluorescein (FITC) conjugate/milliliter was added to the cells, which were incubated at room temperature for 30 min. The cells were centrifuged at 500 × *g* for 10 min and resuspended in 500 μl of PBS. They were analyzed by flow cytometry (FACSCalibur; Becton-Dickinson, San Jose, Calif.) (22). The instrument was set up to measure the forward-angle light scatter, side-angle light scatter, and the fluorescence intensity of FITC on an FL-1 detector. The samples were analyzed with a threshold for forward-angle light scatter to select only the HCT-8 population, discarding debris, free oocysts, and sporozoites. Samples were examined at 2, 6, 24, 48, and 72 h.p.i.; for each point in time, four to six replicate experiments were performed.

Quantification of apoptotic cells. To detect cells in early apoptosis, a marker of phosphatidylserine on the external layer of the plasma membrane was used. HCT-8 cells were treated with a solution of 0.05% Trypsin and 0.02% EDTA, and aliquots of 10^6 cells were centrifuged at 500 × *g* for 10 min. The pellets were resuspended in 500 μl of the binding buffer (Clontech Laboratories Inc., Palo Alto, Calif.) with 5 μl of FITC-conjugated annexin V (Clontech Laboratories Inc.) and 5 μl of propidium iodide (PI) and incubated at room temperature in the dark for 15 min. The samples were then centrifuged at 500 × *g* for 10 min. The supernatant was removed, and the pellets were resuspended in 500 μl of binding buffer and analyzed by flow cytometry. Early apoptotic cells were those showing PI-negative staining and annexin V-positive staining, hereafter referred to as annexin V^+/PI^-, whereas necrotic cells were those showing PI-positive staining and annexin V-negative staining, hereafter referred to as annexin V^-/PI^+. As negative controls, we used noninfected HCT-8 cells. For each point in time following infection, five replicate experiments were performed.

To detect cells in late apoptosis, we used the APO-DIRECT apoptotic kit (Becton-Dickinson Pharmingen, San Diego, Calif.), a terminal deoxynucleotidyl transferase (TdT)-based end-labeling assay for DNA strand breaks (5). In brief, at 2, 6, 24, 48, and 72 h.p.i., HCT-8 cells were fixed with 4% formaldehyde in PBS and then incubated with bromodeoxyuridine triphosphate (Br-dUTP) and TdT, which binds the Br-dUTP to the 3′ hydroxyl end of the DNA fragment. Br-dUTP was detected with an FITC-labeled anti-Br-dUTP monoclonal antibody, and the DNA was counterstained with PI. Only when the cell membrane is strongly injured does PI incorporate itself into the cells, allowing the total quantity of DNA in single cells to be evaluated. Br-dUTP–FITC allows the cells to be counted in that it binds to 3′-OH DNA fragments of 200 bp, which are generated by apoptotic endonucleases. The late apoptosis is hereafter referred to as $Br\text{-}dUTP^+$. As negative controls, we used infected cells at different times postinfection, as above, but without TdT, and noninfected HCT-8 cells with TdT. As positive controls, Jurkat cells (clone E6-1; American Type Culture Collection, Manassas, Va.) treated with 1 μg of staurosporine (Sigma)/ml for 3 h were used. The data were analyzed with Cellquest software (Becton-Dickinson), and a histogram plot of the single fluorescence of Br-dUTP–FITC (FL1-H) was created to quantify positive cells, i.e., late apoptotic cells ($BrdUTP^+$). Three replicate experiments were performed for each point in time after infection.

Immunohistochemical staining of *C. parvum* intracellular stages. HCT-8 cells, plated in chamber slides (Falcon), were incubated with sporulated oocysts for 2, 6, 24, 48, and 72 h and then treated with 4% formaldehyde in PBS at room temperature for 20 min. The slides were treated twice with ethanol for 10 min and then placed in 3% H_2O_2 in methanol for 15 min to block the endogenous peroxidase activity. After washing with PBS, the slides were treated with 95, 70, and 50% ethanol and H_2O for 2 min each. The slides were maintained in a water bath at 98°C in Un-Masker buffer (Medite Italia Histotechnic, Cappella Cantone, Italy) for 40 min and then treated with a rabbit anti-*C. parvum* IgG polyclonal antibody at a 1:400 dilution with 1% BSA in PBS at room temperature for 30 min. The slides were washed twice with PBS and then treated with polyvalent biotinylated link anti-mouse and anti-rabbit Igs (Medite Italia Histotechnic) for 30 min and then with horseradish peroxidase-streptavidin label (Medite Italia Histotechnic) at room temperature for 30 min. After washing twice with PBS, the reaction was developed by 3,3-diaminobenzidine at room temperature for 5 min. The slides were observed using a Zeiss epifluorescence microscope at magnifications of ×400 and ×1,000. The various stages of *C. parvum* were identified by their morphological characters (i.e., size and form) in accordance with the method of Hijjawi et al. (12) by using Carl Zeiss Axio-Vision software to measure sizes. At each time postinfection, the total percentage of infected cells and the percentages of cells infected with the specific stages of the parasite were evaluated (23).

Evaluation of caspase 3 activity. Caspase 3 activity was assayed in the entire cell culture (both infected and noninfected cells) at 2, 6, 24, 36, 48, 54, and 72 h.p.i. Caspase 3 activity was also determined in sorted infected and in sorted noninfected cells from the same cultures at 2 and 24 h.p.i. The cells were incubated for 30 min on ice and vortexed at 10-min intervals. Cellular debris was eliminated by centrifuging the cell lysate at 10,000 × *g* for 10 min. Cell lysate samples were diluted 1:2, 1:10, or 1:20 in an assay diluent buffer (Becton-Dickinson Pharmingen). Caspase 3 activity was assayed by using the cytometric bead array kit according to the manufacturer's instructions (Becton-Dickinson Pharmingen).

Cell sorting of infected and noninfected cells. At 2, 6, 24, and 48 h.p.i., HCT-8 cells were treated with 0.05% trypsin and 0.02% EDTA in PBS. The cells were centrifuged at 500 × *g* for 10 min, treated with 1% Triton X-100 in PBS for 10 min, and then incubated with 0.5 μg of the anti-*C. parvum* IgG/ml in 1% BSA in PBS at room temperature for 30 min. The cells were washed twice in PBS, centrifuged at 500 × *g* for 10 min, and then resuspended with 1% BSA in PBS. One microgram of the anti-rabbit FITC conjugate/milliliter was added to the cells, which were incubated at room temperature for 30 min. From 5×10^6 to 10×10^6 cells were sorted by flow cytometry based on anti-*C. parvum* FITC antibody labeling. After sorting, the noninfected and infected cells were examined with an epifluorescence microscope to confirm whether or not they were infected and then they were counted. About 10^6 (each) noninfected and infected cells were resuspended in 300 μl of PBS containing 1:200-diluted protease inhibitor cocktail (Sigma) and used for the Western blot assay and the assay for evaluating caspase 3 activity.

Measurement of FasL expression by RT-PCR. FasL expression was evaluated in HCT-8 cells at 2, 6, 24, 48, and 72 h.p.i. Total RNA was extracted from 10^6 cells in an extraction buffer (Qiagen GmbH, Hilden, Germany) by following the manufacturer's instructions. mRNA was subsequently isolated from 250 μg of the total RNA with an Oligotex mRNA mini kit (Qiagen). cDNA was synthesized from 50 ng of mRNA with *Taq* polymerase (Invitrogen) with an oligo(dT) primer and a cDNA cycle kit (Invitrogen) in a 20-μl reaction mixture. The forward and reverse primers used to amplify FasL and to produce a 419-bp product were 5′-GGAAAGTGGCCCATTTAACAG-3′ and 5′-CTCTTAGAGCTTATATAA GCCG-3′, respectively (29). For reverse transcriptase PCR (RT-PCR) amplification, 2 μl of the RNA-DNA template was used. Amplifications consisted of 40 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 2 min, and elongation at 72° for 1 min. As a loading control for the RT-PCR amplification, human β-actin cDNA was checked on 2 μl of template with the following primers: 5′-ATCTGGCACCACACCTTCTACAATGAGCTGCG-3′ and 5′-CG TCATACTCCTGCTTGCTGATCCACATCTGC-3′. Amplification consisted of 22 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and elongation at 72° for 1 min.

As positive and negative controls, we used, respectively, HCT-8 cells treated with gamma interferon (100 U/ml) for 24 h and cells without polymerase (13, 33). The PCR products were separated by electrophoresis on a 2% agarose gel and visualized by ethidium bromide staining and UV.

Western blotting to detect Fas, caspase 3, and Bcl-2 expression. Proapoptotic signals are mediated by extracellular specific ligands and/or surface death receptors and can activate the process of apoptosis in the cell cytoplasm and organelles. We used Fas, FasL, and caspase 3 as proapoptotic markers and a Bcl-2 protein as an antiapoptotic marker. To evaluate whether or not *C. parvum* is capable of regulating the expression of apoptotic markers in the cell, the total cell lysate was prepared from infected and noninfected HCT-8 cells. These two populations, which were in the same petri dish, were separated by FACSorter at 2, 6, 24, and 48 h.p.i. In brief, about 10^6 infected and noninfected cells were centrifuged at 500 × *g* for 10 min, resuspended in 300 μl of PBS containing a protease inhibitor cocktail (Sigma), and sonicated five times for 30 s on ice. The cells were boiled in Laemmli sodium dodecyl sulfate buffer (Bio-Rad, Hercules, Calif.) for 2 min and frozen at −20°C until use. The proteins were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 10% resolving gel and transferred to nitrocellulose membranes. The nonspecific binding of antibodies was blocked by incubating the membranes with 2% FCS in TNT (Tris-HCl [pH 8.0] with 0.05% Tween 20) at room temperature for 1 h. After blocking, the membranes were processed for immunoblotting with a 1:200-diluted anti-Fas monoclonal antibody (Sigma), a 1:1,000-diluted polyclonal anti-

TABLE 1. Percentage of infected HCT-8 cells with a given stage of the parasite detected in 100 microscopic fields[a]

Time (h.p.i.)	% of infected cells	% of parasite stage in infected cells				
		Sporozoite	Trophozoite	Meront	Merozoite	Microgamont
2	8	100				
6	25	100				
24	82		76	21	3	
48	73			65	35	
72	53			2	66	32

[a] Percentages of cells were determined according to the method of Mele et al. (23).

caspase 3 antibody (Sigma), a 1:1,000-diluted anti-Bcl-2 monoclonal antibody (Sigma), or a 1:5,000-diluted anti-β-actin monoclonal antibody (Oncogene Research Products, San Diego, Calif.) at room temperature for 1 h. After immunoblotting, the membranes were rinsed three times for 10 min each in TNT and incubated with peroxidase-conjugated secondary antibodies (either anti-rabbit or anti-mouse) at a 1:3,000 final dilution in PBS for 1 h. Fas, caspase 3, and Bcl-2 were detected by using a chemiluminescence system followed by xerography on CL-X Posure film (Pierce, Boston, Mass.). A HeLa cell line (7) and Jurkat cells treated with 1 μg of staurosporine/ml for 3 h (31) were used as positive controls for the expression of Bcl-2 and caspase 3, respectively.

Statistical analysis. The Student *t* test was used to analyze the data. A *P* value of <0.05 was considered statistically significant.

RESULTS

Quantification of *C. parvum* infection level in HCT-8 cultures. The percentage of infected cells increased from 8% at 2 h.p.i. to 82% at 24 h.p.i., whereas it decreased to 73% at 48 h.p.i. and to 53% at 72 h.p.i., because many infected cells had ruptured (Table 1).

Detection of intracellular stages of *C. parvum* in infected HCT-8 cultures by immunohistochemistry. Immunohistochemical labeling revealed the following: at 6 h.p.i. (Fig. 1A),

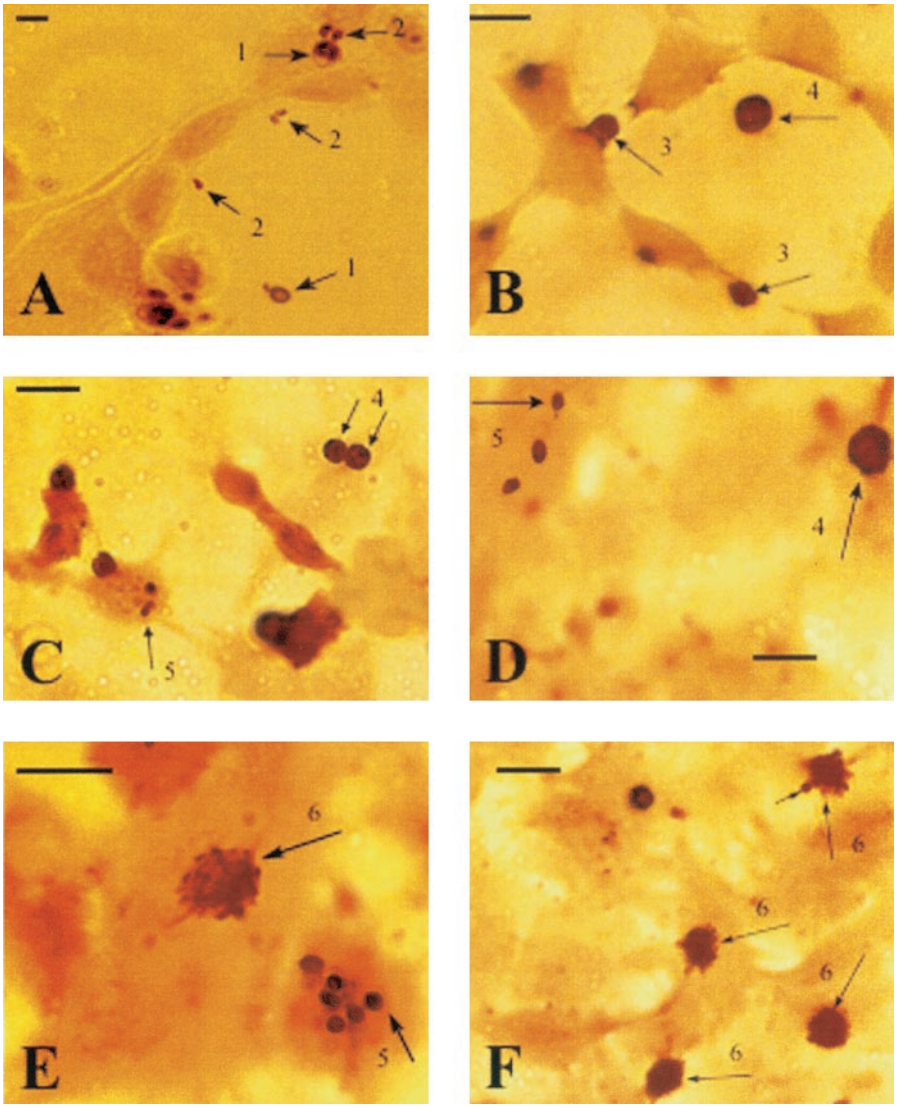

FIG. 1. Immunohistochemistry of *C. parvum*-infected HCT-8 cells labeled with anti-*C. parvum* biotin-streptavidin horseradish peroxidase. Bars, 6 μm. (A) *C. parvum*-infected cells at 6 h.p.i.; (B) *C. parvum*-infected cells at 24 h.p.i.; (C and D) *C. parvum*-infected cells at 48 h.p.i.; (E and F) *C. parvum*-infected cells at 72 h.p.i. The arrows indicate the stages of development: 1, oocyst; 2, sporozoite; 3, trophozoite; 4, meront; 5, merozoite; 6, microgamont.

sporozoites (1.7 to 3.0 μm in length) attached to the plasma membrane; at 24 h.p.i. (Fig. 1B), trophozoites (round or oval intracellular form of 3 by 2 to 3 by 3 μm) and meronts (intracellular form of 3.5 by 4 μm) containing either 4 or 6 merozoites (threadlike in form with rounded ends); at 48 h.p.i. (Fig. 1C and D), meronts and free merozoites; at 72 h.p.i. (Fig. 1E and F), meronts, free merozoites, and other stages, which could be microgamonts (intracellular form of 5 by 4.5 to 6 by 5.5 μm) containing many nonflagellated microgametes. The percentages of infected cells, by specific developmental stage of the parasite, are shown in Table 1.

Quantification of early (annexin V^+/PI^-) and late (Br-$dUTP^+$) apoptotic cells in infected HCT-8 cultures. In the noninfected culture, the percentage of annexin V^+/PI^- and $BrdUTP^+$ apoptotic cells and annexin V^-/PI^+ necrotic cells ranged from 2 to 8% at 24 h. At 48 h, the percentage of annexin V^+/PI^- cells increased to 14%, whereas the percentage of $BrdUTP^+$ apoptotic cells and annexin V^-/PI^+ necrotic cells remained at 2 to 8% (Fig. 2A). At 72 h, the percentage of annexin V^-/PI^+ necrotic cells increased to 16% and apoptotic cells were no longer detected.

In the infected cultures, before and at 24 h.p.i. (Fig. 2B), the percentage of annexin V^+/PI^- cells was 10 to 13% and the percentage of $BrdUTP^+$ apoptotic cells and annexin V^-/PI^+ necrotic cells was 1 to 8%. At 48 h.p.i., the percentage of annexin V^+/PI^- cells was 30% and the percentage of Br-$dUTP^+$ apoptotic cells was 23%. At 72 h.p.i., the percentage of annexin V^-/PI^+ necrotic cells was 42% and no apoptotic cells were detected. Apoptotic bodies containing fragmentized DNA labeled with Br-dUTP–FITC were detected at 48 h.p.i. (data not shown).

According to the statistical analysis, performed at different times postinfection, at 24 h.p.i., there were no significant differences between infected and noninfected cultures in terms of the percentage of annexin V^+/PI^- or $BrdUTP^+$ apoptotic cells, whereas significant differences were observed at 2 and 6 h.p.i. ($P < 0.05$) and at 48 h.p.i. ($P < 0.001$).

Effect of an apoptotic inducer on infected HCT-8 cultures. The finding that, at 24 h.p.i., there were no significant differences in terms of the percentage of apoptotic cells between infected cultures and noninfected control cultures (Fig. 2) suggests that most of the infected cells did not undergo apoptosis. To test this hypothesis, we used staurosporine as an apoptotic inducer in the infected culture. Staurosporine treatment increased the percentage of apoptotic cells at 2 and 6 h.p.i., whereas at 24 h.p.i., staurosporine had no effect. At 48 h.p.i., staurosporine again increased the percentage of apoptotic cells (Fig. 3).

Evaluation of caspase 3 activity in infected HCT-8 cultures. At 2 h.p.i., caspase 3 activity had greatly increased in the entire culture (135 IU/ml); it then decreased up to 24 h.p.i. (22 IU/ml) and increased again at 48 h.p.i. (150 IU/ml), peaking at 54 h.p.i. (300 IU/ml) (Fig. 4). At 2 h.p.i., sorted infected cells showed high caspase 3 activity (190 IU/ml), whereas they did not at 24 h.p.i. (22 IU/ml) (Fig. 4). At 2 h.p.i., sorted noninfected neighboring cells showed low caspase 3 activity (15 IU/ml), with increased activity at 24 h.p.i. (80 IU/ml) (Fig. 4).

Expression of proapoptotic and antiapoptotic signals in sorted infected and sorted noninfected cells. In the sorted infected cells, FasL mRNA, which was the first proapoptotic marker detected, was expressed at 2 and 24 h.p.i. but not at 48 and 72 h.p.i. (Fig. 5); in the sorted noninfected cells, FasL mRNA was not detected at any time (data not shown). Fas expression did not differ when comparing the sorted infected cells with the sorted noninfected cells (data not shown), although Fas is known to be expressed at a low level in HCT-8 cells (16). This finding, together with the observation that FasL was expressed only in infected cells and only early in the culture period, suggests that the parasite regulates apoptosis by interfering with effector enzymes downstream of the receptor.

In sorted infected cells, a product of about 25 kDa of caspase 3 was expressed from 2 to 6 h.p.i. and then disappeared at 24 h.p.i. At this time, two products of about 71 and 32 kDa were expressed; these products, which were also detected in the noninfected control culture, correspond to the inactive products of the enzyme (2). At 48 h.p.i., these inactive products

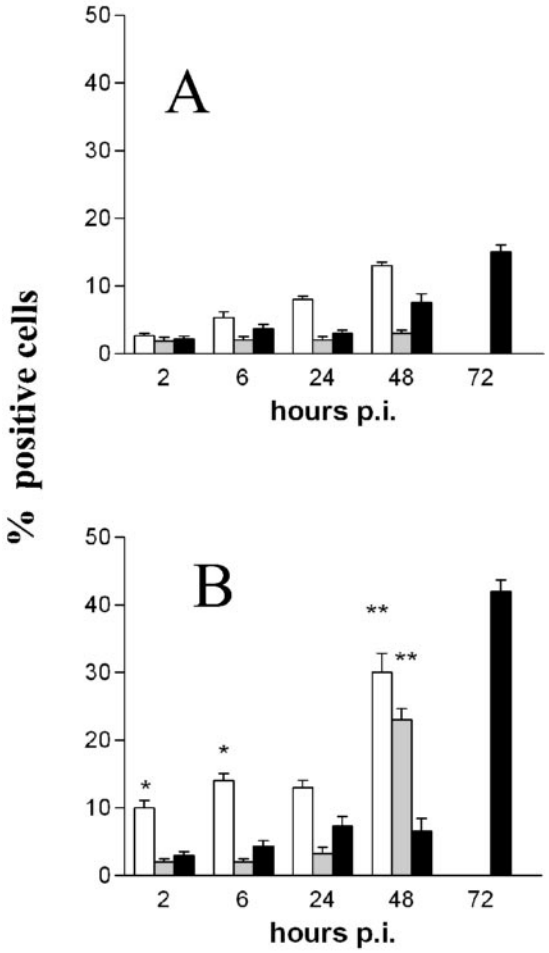

FIG. 2. Percentage of apoptotic noninfected control HCT-8 cells (A) and *C. parvum*-infected HCT-8 cells (B) at different times. Apoptotic cells were quantified by flow cytometry with annexin V-FITC and BrdUTP-FITC labeling. Necrotic cells were quantified by flowcytometry as PI-positive cells and annexin V-FITC- and BrdUTP-FITC-negative cells. Black bars, PI-positive cells; white bars, annexin V-FITC-positive cells; gray bars, BrdUTP-FITC-positive cells. Data are presented as means ± standard errors of three values. Asterisks denote *P* values of <0.05 (*) and <0.001 (**) for *C. parvum*-infected cells versus noninfected cells.

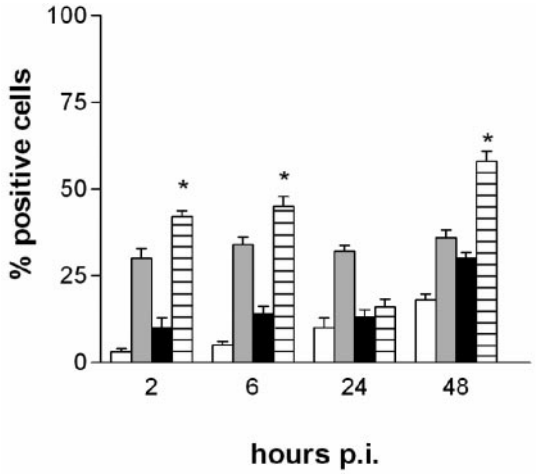

FIG. 3. Percentage of apoptosis in *C. parvum*-infected HCT-8 cells from entire infected cultures after treatment with staurosporine. Cells were treated with 1 μg of staurosporine/ml for 3 h. Apoptotic cells were quantified by flow cytometry as annexin V-FITC-positive cells. White bars, HCT-8 cells; gray bars, HCT-8 cells treated with staurosporine; black bars, *C. parvum*-infected cells; striped bars, *C. parvum*-infected cells treated with staurosporine. Data are presented as means ± standard errors of three values. *, $P < 0.05$ for *C. parvum*-infected cells treated with staurosporine versus nontreated *C. parvum*-infected cells.

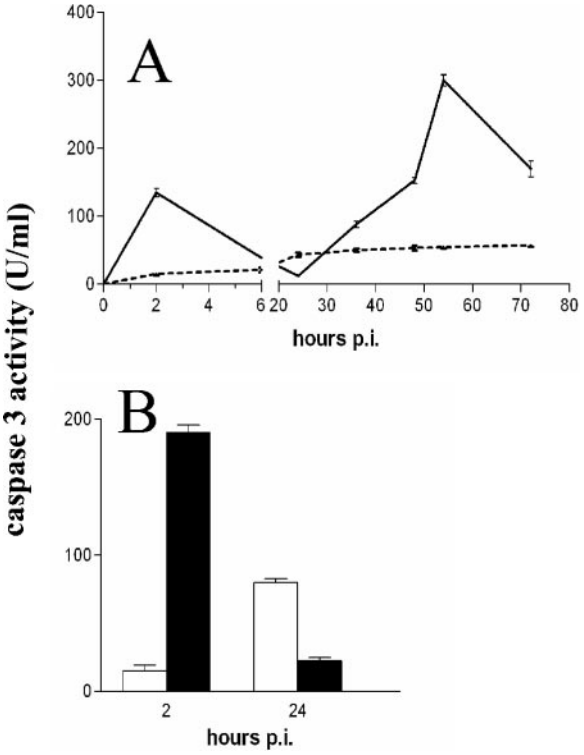

FIG. 4. (A) Caspase 3 activity in entire *C. parvum*-infected HCT-8 cell cultures (solid line) and in noninfected control HCT-8 cell cultures (dotted line) at various times after infection. (B) Caspase 3 activity in sorted infected cells (black bars) and in sorted noninfected cells (white bars) at 2 and 24 h.p.i. Caspase 3 activity was quantified by flow cytometry with the cytometric bead array caspase 3 assay kit. Data are presented as means ± standard errors of three values.

were expressed and the 25-kDa product reappeared, yet at a low level (Fig. 6A). In sorted noninfected cells, caspase 3 was not detected until 24 h.p.i., when the 25-kDa product appeared (Fig. 6B); at 48 h.p.i., this product was still present.

The presence of the active form of caspase 3 was confirmed by the detection of its activity. At 2 h.p.i., the high activity of caspase 3 (190 U/ml) in sorted infected cells (Fig. 4) and the presence of its active form (25 kDa) (Fig. 6A) indicated that the apoptotic cells, which represented 10% of the entire cell culture, consisted of infected cells. At 24 h.p.i., in the entire culture, only a small amount of noninfected cells was present (Table 1) and the caspase 3 activity was not appreciable (Fig. 4); however, in the sorted noninfected cells, the caspase 3 activity was 80 U/ml. This finding, together with the presence of the active form of caspase 3 at this time point in the noninfected cells, indicates that apoptotic cells were constituted of noninfected neighboring cells.

In the noninfected cells, Bcl-2 was never expressed (data not shown), whereas in infected cells a product of 26 kDa was expressed at 24 h.p.i.; at 48 h.p.i., the expression of the 26-kDa product had decreased and a product appeared with a higher molecular mass (Fig. 7) which could represent a ubiquitinated form (34).

DISCUSSION

At the beginning of the infection, both free sporozoites and sporozoites attached to the host cell membrane, inducing early apoptosis in the cells, were identified and 10 and 14% of the cells in the entire culture, at 2 and 6 h.p.i., respectively, were annexin V^+/PI^- (Fig. 2B). These percentages were significantly higher than the percentages of apoptotic cells in noninfected cultures at these times (Fig. 2A). In the sorted infected cells, at 2 and 6 h.p.i., we observed the proapoptotic signals of FasL expression (Fig. 5), the active form of caspase 3 (Fig. 6A), and a high caspase 3 activity (190 U/ml), whereas the neighboring noninfected cells sorted from the same culture showed no sign of apoptosis. These data show that, when *C. parvum* sporozoites bind to the host cell membrane and the parasitophorous vacuole begins to develop, the host cell undergoes

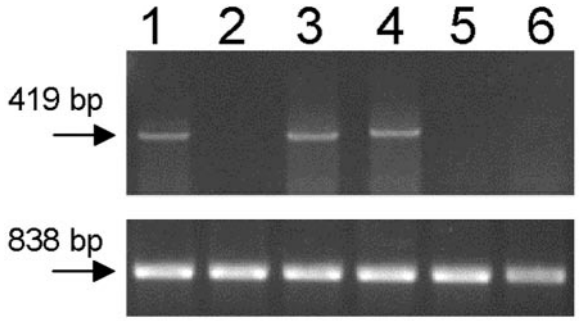

FIG. 5. RT-PCR analysis of gene expression of FasL in sorted *C. parvum*-infected HCT-8 cells at different times postinfection. Lanes: 1, HCT-8 noninfected cells with 100 U of gamma interferon/ml; 2, HCT-8 noninfected control cells; 3, *C. parvum*-infected HCT-8 cells at 2 h.p.i.; 4, *C. parvum*-infected HCT-8 cells at 24 h.p.i.; 5, *C. parvum*-infected HCT-8 cells at 48 h.p.i.; 6, *C. parvum*-infected HCT-8 cells at 72 h.p.i. Lower panel, RT-PCR of gene expression of β-actin.

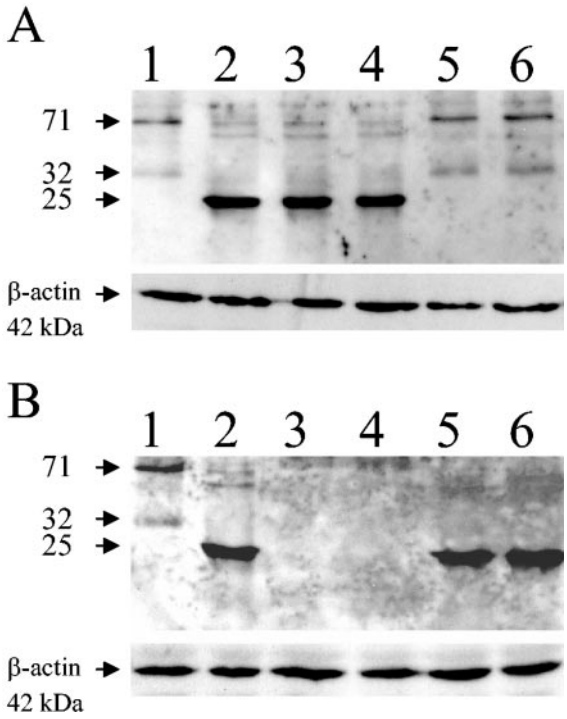

FIG. 6. Western blot analysis of caspase 3 in sorted HCT-8 cells at various times after *C. parvum* infection. (A) Sorted *C. parvum*-infected HCT-8 cells. Lanes: 1, negative control, noninfected HCT-8 cells; 2, positive control, Jurkat cells treated with 1 μg of staurosporine/ml for 3 h; 3, sorted *C. parvum*-infected HCT-8 cells at 2 h.p.i.; 4, sorted *C. parvum*-infected HCT-8 cells at 6 h.p.i.; 5, sorted *C. parvum*-infected HCT-8 cells at 24 h.p.i.; 6, sorted *C. parvum*-infected HCT-8 cells at 48 h.p.i. (B) Sorted noninfected cells. Lanes: 1, negative control, noninfected HCT-8 cells; 2, positive control, Jurkat cells treated with 1 μg of staurosporine/ml for 3 h; 3, sorted noninfected HCT-8 cells at 2 h.p.i.; 4, sorted noninfected HCT-8 cells at 6 h.p.i.; 5, sorted noninfected HCT-8 cells at 24 h.p.i.; 6, sorted noninfected HCT-8 cells at 48 h.p.i. Molecular masses in kilodaltons are shown on the left. The β-actin housekeeping protein was used as a loading control.

apoptosis. Similar results were obtained by Chen et al. (3), who reported the widespread occurrence of apoptosis in biliary epithelial cells beginning within 2 h of infection.

At 24 h.p.i., the trophozoites were the prevalent stage in the culture (Fig. 1B; Table 1) and no signal of apoptosis or expression of the antiapoptotic protein Bcl-2 (Fig. 7) were detected in the sorted infected cells, which constituted 82% of the cell culture. These data show that the intracellular stages of the parasite succeeded in reprogramming the gene expression of the host cell, inhibiting apoptosis at this time postinfection, when the parasite depends on the host cell for growth and development. The inhibition of apoptosis was evident even in the presence of staurosporine (Fig. 3). The finding that apoptosis was inhibited in the infected cells is consistent with the findings that, at 24 h.p.i., the percentage of apoptotic cells in the entire infected culture was not significantly different from that of the noninfected control culture (Fig. 2). At the same time, sorted noninfected cells from the same culture showed signs of moderate apoptosis. However, no statistically significant difference was observed between the percentage of apoptotic cells in the entire infected culture and in noninfected control cultures (Fig. 2), although it must be considered that there were too few noninfected cells in the entire infected culture to draw firm conclusions (Table 1). These results are in agreement with the results of Chen et al. (4), who showed that *C. parvum*-induced biliary epithelial cell apoptosis is limited to bystander noninfected cells at 24 h.p.i., possibly serving to limit the spread of the infection. In a natural infection, numerous parasites were attached to the apical surface of the gallbladder and apoptotic cells were found in the area adjacent to *C. parvum*-infected cells (3). Furthermore, it has been found that the activation of the NF-κB/IκB system at 24 h.p.i. in *C. parvum*-infected H69 cells protects infected cells from death and facilitates the survival and propagation of the parasite (4, 27). McCole et al. (21) found that, at 24 h.p.i., *C. parvum* induces a moderate level of apoptosis in cultured human intestinal epithelial cells, yet the parasite also attenuates the epithelial apoptosis induced by strongly proapoptotic agents.

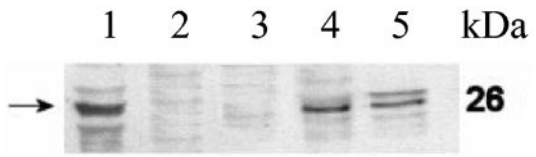

FIG. 7. Western blot analysis of Bcl-2 in sorted *C. parvum*-infected HCT-8 cells at different times postinfection. Lanes: 1, positive control, HeLa cells; 2, negative control, noninfected HCT-8 cells; 3, sorted *C. parvum*-infected cells at 2 h.p.i.; 4, sorted *C. parvum*-infected cells at 24 h.p.i.; 5, sorted *C. parvum*-infected cells at 48 h.p.i. The molecular mass in kilodaltons is shown on the right.

Moreover, it has been reported that other intracellular parasites, such as *Leishmania donovani* and *Toxoplasma gondii*, inhibit apoptosis in host cells to complete their development (8, 9, 24). Lau et al. (15) have shown that *Plasmodium yoelii* is able to regulate the expression of host cell molecules, and they found parasite-induced overexpression of several host transcripts (e.g., glutamylcysteine synthase). In various cell lines, overexpression of glutamylcysteine synthase has been reported to inhibit tumor necrosis factor alpha-induced apoptosis and to regulate the activation of NF-κB (20).

At 48 h.p.i., the development of merozoites (Fig. 4C and D) and their need to exit from the host cells may have induced apoptosis through an autocrine mechanism triggered by intracellular meronts. In noninfected cells, apoptosis is triggered by free stages of the parasite and/or by parasitized cells through a paracrine mechanism which could engage soluble signaling molecules (4).

The present results show that *C. parvum*, depending on its developmental stage, can inhibit or promote host cell apoptosis, suggesting that this parasite is able to interact with and regulate the host cell's gene expression.

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