Cryptosporidium parvum Activates Nuclear Factor κB in Biliary Epithelia Preventing Epithelial Cell Apoptosis

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Background & Aims: **Our previous studies have shown that *Cryptosporidium parvum* induces biliary epithelial cell apoptosis in vivo and causes apoptosis in bystander uninfected biliary epithelia in vitro. We analyzed *C. parvum*–induced nuclear factor kappa B (NF-κB) activation in human biliary epithelial cells and assessed its relevance to epithelial cell apoptosis.** ***Methods:*** **In vitro models of cryptosporidial infection using a human biliary epithelial cell line were used to assay *C. parvum*–induced NF-κB activation and associated apoptosis.** ***Results:*** **Degradation of IκB and nuclear translocation of the NF-κB family of proteins (p65 and p50) were observed in the biliary epithelial cell cultures directly exposed to the parasite. Activation of NF-κB was found only in directly infected cells (but not in bystander uninfected cells). A time-dependent secretion of a known NF-κB gene product, interleukin 8, from infected cell cultures was detected. *C. parvum*–induced biliary epithelial cell apoptosis was limited to bystander uninfected cells. In contrast, inhibition of NF-κB activation resulted in apoptosis in directly infected cells and significantly enhanced *C. parvum*–induced apoptosis in bystander uninfected cells.** ***Conclusions:*** **These observations support the concept that, while *C. parvum* triggers host cell apoptosis in bystander uninfected biliary epithelial cells, which may limit spread of the infection, it directly activates the NF-κB/IκB system in infected biliary epithelia thus protecting infected cells from death and facilitating parasite survival and propagation.**

Cryptosporidium parvum is an intracellular protozoan parasite and an important causative agent of human gastrointestinal disease worldwide. Although infection in immunocompetent hosts usually results in diarrhea, which is self-limited, in immunocompromised patients, particularly those with acquired immunodeficiency syndrome (AIDS), the infection may be life-threatening.[1] Patients with AIDS infected with *C. parvum* also develop extraintestinal disease, most frequently of the biliary tract, resulting in sclerosing cholangitis and cholecystitis in some patients.[2,3] Indeed, *C. parvum* is the single most common identifiable pathogen infecting the biliary tract in AIDS patients, has been found in up to 65% of patients with so-called AIDS cholangiopathy,[4,5] and is associated with a poor prognosis. The nature of the immune response to *C. parvum* is not completely clear, although a low CD4 T-cell count in AIDS patients is associated with chronic severe infection.[2] Despite the prevalence and poor outcome of cryptosporidiosis in patients with AIDS, the pathogenesis is poorly understood, and there is currently no fully effective medical therapy.[1]

Histologic changes associated with intestinal cryptosporidiosis are relatively nonspecific, with blunting of villi, hyperplasia of intestinal crypt cells, and infiltration of inflammatory cells into the lamina propria. Neutrophilic infiltrate, epithelial cell apoptosis, villus blunting, cryptitis, and reactive epithelial changes in the intestine in AIDS patients with cryptosporidiosis have been shown to be associated with intensity of *C. parvum* infection.[6] Biliary cryptosporidiosis is also associated with a nonspecific inflammatory response. Histologically, there is periductal inflammation with interstitial edema, mixed inflammatory cell infiltrates, and hyperplasia and dilatation of the periductual glands.[7] In previous studies, we reported that *C. parvum* causes biliary epithelial cell apoptosis in AIDS patients.[3,8] Using an in vitro model of biliary cryptosporidiosis using a human biliary epithelial cell line, we further observed that *C. parvum* infection of biliary epithelial cells induces bystander-uninfected epi-

Abbreviations used in this paper: AIDS, acquired immunodeficiency syndrome; DAPI, 4,6-diamidino-2-phenylindole; EGTA, ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; ELISA, enzyme-linked immunosorbent assay; FasL, Fas ligand; FITC, fluorescein isothiocyanate; IAP, inhibitor-of-apoptosis; IL, interleukin; NF-κB, nuclear factor kappa B; PBS, phosphate-buffered saline; TNF, tumor necrosis factor.

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thelial cells to undergo apoptosis by a Fas/Fas ligand (FasL)-dependent apoptotic mechanism.[9] However, the molecular mechanisms by which *C. parvum* does not kill directly infected cells, while causing apoptosis in bystander uninfected cells, are unclear.

Activation of nuclear transcription factors plays a central role in the gastrointestinal epithelial cell response induced by infection with enteroinvasive pathogens including parasitic protozoans such as *Theileria parva*.[10] The nuclear factor kappa B (NF-κB) family of transcription factors regulates the activation of a wide variety of genes that respond to immune or inflammatory signals in epithelial cells.[11] NF-κB is also known to activate a number of intracellular survival signals such as the c-Myc protooncogene (c-Myc), inhibitor-of-apoptosis (IAP) proteins c-IAP1 and c-IAP2, and Bcl-x_L, and to play a critical role in protecting cells from apoptosis induced by tumor necrosis factor (TNF)-α, Fas agonistic antibody, radiation, and chemotherapeutic drugs.[12–15] Although expression of NF-κB gene products such as TNF-α and interleukin (IL)-8 in *C. parvum* infection of intestinal epithelial cells has been documented,[16,17] whether *C. parvum* can activate nuclear transcription factors such as NF-κB is unclear, and nothing is known about the potential relationships among *C. parvum*–induced pathology and nuclear transcription factor activation in any epithelial cell.

Given that *C. parvum*–induced apoptosis is limited to bystander uninfected cells in vivo[8] and that NF-κB has been shown to suppress apoptosis induced by a range of stimuli,[12–15] we investigated the specific role of parasite-induced NF-κB activation in the survival of *C. parvum*–infected biliary epithelial cells. The data reported here show that in directly infected biliary epithelia, *C. parvum* activates NF-κB resulting in IL-8 secretion and inhibition of epithelial cell apoptosis. We propose that, in vivo, these events both account for the inflammatory response characteristic of *C. parvum* infection and facilitate parasite survival and propagation.

Materials and Methods

C. parvum

C. parvum oocysts harvested from calves inoculated with a strain originally obtained from Dr. Harley Moon at the National Animal Disease Center in Ames, IA, were purchased from a commercial source (Pleasant Hill Farms, Troy, ID). Oocysts were purified using a modified ether extraction technique, then suspended in phosphate-buffered saline (PBS) and stored at 4°C. Before infecting the cells, oocysts were treated with 1% sodium hypochlorite on ice for 20 minutes and subjected to an excystation solution consisting of 0.75% taurodeoxycholate and 0.25% trypsin for 30 minutes at 37°C. The viability of oocysts and the excystation rate were determined as previously described by others.[18,19] Oocysts used in the experiments were 70%–90% viable depending on the lot and period of storage. Therefore, an optimal dose of oocysts was used from each new batch of oocysts, and usually 3–5 × 10^6 oocysts could release 5 × 10^6 sporozoites.

Cell Culture and Infection

H69 cells (a gift of Dr. D. Jefferson, Tufts University, Boston, MA) are SV40-transformed human bile duct epithelial cells originally derived from normal liver harvested for transplant. These biliary epithelial cells continued to express biliary epithelial cell markers, including cytokeratin 19, gamma glutamyl transpeptidase, and ion transporters consistent with biliary function and have been extensively characterized previously.[20] Stock cultures of these nonmalignant but immortalized cells were maintained in coculture with irradiated NIH3T3 mouse fibroblasts and were grown in a hormonally supplemented medium with 10% fetal bovine serum. For experiments, cells were maintained for 3 passages without coculture cells to ensure that the culture was free of fibroblasts. All experiments were performed with cells between passage 23 and 26. Infections were performed using a coculture system as described previously.[9] Briefly, H69 cells were grown to 70%–80% confluency in 6-well Costar tissue culture inserts (Becton Dickinson Labware, Parsippany, NJ) with cells both on the inserts (upper chamber) and on the plates below the inserts (lower chamber). The 2 cell populations were physically separated by a polycarbonate membrane with a high density of 0.4 μm pore size, which allows free exchanges of molecules (but not *C. parvum* sporozoites) between the upper and lower media reservoirs. Only the cells on the inserts (upper chamber) were exposed to the organism. Infection with *C. parvum* was done in a culture medium consisting of DMEM-F12 (Bio Whittaker, Walkersville, MD), 100 U/mL penicillin, and 100 μg/mL streptomycin (referred to hereafter as assay medium) and freshly excysted *C. parvum* sporozoites (1 × 10^6 sporozoites/slide well). Inactive organisms (treated at 65°C for 30 minutes) were used for sham infection controls.[21] In another control experiment, live sporozoites were added to the upper chamber of the inserts without H69 cells and then cocultured with H69 cells grown on the plates in the lower chamber. In some experiments, H69 cells were also seeded into 4-well chamber slides or 6-well Costar tissue culture plates and grown to 70%–80% confluence and then exposed to *C. parvum*. To reduce the effects of cell differentiation on *C. parvum* infection to a minimum, 70%–80% confluence of H69 cells (24 hours after seeding) were used for all experiments. Infection assay was carried out after 2 hours of incubation with the parasite using an indirect immunofluorescent technique as previously described.[22,23] About 15%–35% of biliary epithelial cells exposed to the parasite were infected directly by the organism, similar to other models using intestinal epithelial cells.[24,25]

In some experiments, infection was carried out in the presence of various specific inhibitors to NF-κB activation. A peptide-aldehyde proteasome (IkBα degradation) inhibitor,

MG-132 (Sigma, St. Louis, MO), and a specific NF-κB inhibitory peptide, SN50 (BIOMOL Research Laboratories, Inc., Plymouth Meeting, PA),[26] were used. Because these agents alone can induce cell death, H69 cells were cultured with various concentrations of these agents for 24 hours to determine the appropriate concentrations of MG-132 and SN50 for the study. Treated cells were then either incubated with fluorescein diacetate (50 μmol/L; Sigma) and propidium iodide (20 μmol/L; Sigma) for 5 minutes at room temperature to assess cell viability by fluorescence microscopy as previously reported[22] or assessed for apoptosis by 4,6-diamidino-2-phenylindole (DAPI) (Sigma) staining.[9] A concentration of 1 μmol/L of MG-132 or 50 μg/mL of SN50 showed no cytotoxic or apoptotic effects on H69 cells and were selected for study. For the inhibitory experiments, H69 cells grown on the inserts of the coculture system or 4-well chamber slides were washed with DMEM-F12 and then exposed to *C. parvum*. MG-132 or SN50 was added in the assay medium at the same time *C. parvum* was added.

Immunoblots of IκBα

H69 cells on the inserts of the coculture system were exposed to viable *C. parvum* sporozoites for 0, 12, and 24 hours, and cells both on the inserts and on the plates were harvested for protein extraction. After cell culture medium was removed, cells were lysed and quantitative immunoblots were performed as previously described.[9,27] Briefly, cells were lysed with the M-PER mammalian protein extraction reagent (Pierce, Rockford, IL) and protein concentrations were determined using Bradford reagent according to the instructions of the supplier (Sigma). Twenty micrograms of lysate protein per lane were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis under reducing conditions and blotted onto nitrocellulose membranes. Membranes were sequentially incubated with the IkBα antibody (C-21; Santa Cruz Biotechnology, Santa Cruz, CA), and then with 0.2 μg/mL of horseradish peroxidase–conjugated secondary antibody and revealed with enhanced chemiluminescence light substrate (ECL; Amersham, Buckinghamshire, England).

Gel Shift and Antibody Supershift Assays

H69 cells were seeded on 6-well plates and grown to 70%–80% confluence. Nuclear extracts were prepared from cells either before or after exposure to viable *C. parvum* sporozoites for various lengths of time (0, 12, 24 hours) according to a previously published technique.[28] Briefly, after aspiration of cell culture medium, cells were rinsed twice with ice-cold PBS. Cells were then lysed in cytoplasmic extraction buffer (10 mmol/L HEPES [pH 7.9], 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 0.5 μg/mL leupeptin) for 5 minutes at 4°C. The cells were removed by scraping and the nuclei separated from the cytosol by centrifugation at 2500 rpm for 3 minutes. The nuclear extracts were then snap frozen in dry ice and resuspended in nuclear extract buffer (20 mmol/L HEPES [pH 7.9], 25% glycerol, 1.5 mmol/L $MgCl_2$, 0.42 mol/L NaCl, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 0.5 μg/mL leupeptin), vortexed for 1 minute, and incubated on ice for 10 minutes. The nuclear extract was vortexed again and resuspended in a buffer (20 mmol/L HEPES [pH 7.9], 20% glycerol, 0.05 mol/L KCl, 0.2 mmol/L EDTA, 0.2 mmol/L phenylmethylsulfonyl fluoride, 1 μg/mL pepstatin, and 0.5 μg/mL leupeptin) after centrifugation at 12,000 rpm for 10 minutes. Protein concentration was determined with Bradford reagent (Sigma).

Gel shift assays were formed with the gel shift assay system (Promega, Madison, WI), as specified by the manufacturer. Sequence of double-stranded consensus oligonucleotide for NF-κB used in the gel shift reactions was 5′-AGT TGA GGG GAC TTT CCC AGG C-3′ (Promega). Probe labeling was carried out as specified by the manufacturer with [^{32}P] adenosine triphosphate (3000 Ci/mmol; 10 mCi/mL; DuPont NEN Research Products, Boston, MA). Competition studies were performed with a 10-fold molar excess of unlabeled oligonucleotide added to the reaction mixtures before the addition of radiolabeled oligonucleotide probe. Reaction mixtures were analyzed on 5% nondenaturing polyacrylamide gels prepared with 0.5× TBE buffer (89 mmol/L Tris-HCl [pH 8.0], 89 mmol/L boric acid, and 2 mmol/L EDTA) as the running buffer. The gels were electrophoresed at 100 V for 2–3 hours and subjected to autoradiographic exposure for 10–18 hours.[28] For antibody supershift assays, antibodies targeted against p65, p52, p50, and Rcl (100 μg/0.1 mL) (Santa Cruz Biotechnology) were used. Before the addition of radiolabeled oligonucleotide probe, 2 μg of antibodies was added to the gel shift reaction mixtures, and followed by incubation at 37°C for 20 minutes. Each reaction mixture was analyzed, as for the gel shift assays described above, on a 5% nondenaturing polyacrylamide gel.[28]

Confocal Microscopy for NF-κB Nuclear Translocation

H69 cells were grown on 4-well chamber slides to 70% confluence and then exposed to *C. parvum* sporozoite as described above. After 24 hours of incubation, cells were fixed (0.1 mol/L 1,4-piperazinediethanesulfonic acid [pH 6.95] and 1 mmol/L ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid [EGTA]) and permeabilized with 0.2% (vol/vol) Triton-X100 in PBS. Slides were incubated with a primary monoclonal antibody to p65 in its activated form (Boehringer Mannheim, Mannheim, Germany),[29] mixed with a polyclonal antibody against *C. parvum* sporozoite membrane proteins (a generous gift from Drs. Guan Zhu and Janet Keithly, Wadsworth Center, Albany, NY), and followed by rhodamine-labeled anti-mouse and fluorescein-labeled anti-rabbit antibodies. Slides were mounted with mounting medium (H-1000; Vector Laboratories, Burlingame, CA) and assessed by confocal laser scanning microscopy. With an analysis system of the LSM 310 provided by Carl Zeiss, Inc. (Oberkochen, Germany), fluorescence intensities of fluorescein isothiocyanate (FITC) for activated p65 in the cell nuclei were measured from 200 randomly selected cells for each group.

Images obtained from the confocal microscope were manipulated uniformly for contrast and intensity using the Adobe (Mountain View, CA) Photoshop software and printed with a Phase 400 color printer.

Enzyme-Linked Immunosorbent Assays

To analyze the production of NF-κB–associated proinflammatory cytokines by H69 cells in response to *C. parvum* infection, H69 cells were grown to subconfluence in 4-well chamber slides. After different times of incubation with assay medium containing freshly excysted sporozoites and various specific inhibitors, supernatants were collected to assay the cytokines and cells were harvested for protein determination. IL-8 concentration in the supernatants was determined by enzyme-linked immunosorbent assay (ELISA) using a commercial kit (R&D System Company, Minneapolis, MN). Cells were lysed with the M-PER mammalian protein extraction reagent (Pierce), and protein concentrations were assessed with Bradford reagent (Sigma). The concentrations of IL-8 detected were calculated from standard curves prepared with the recombinant proteins and expressed as picogram IL-8 per microgram cell protein.

Analysis of Apoptosis

H69 cells were grown on 4-well chamber slides to 70% confluence and then exposed to *C. parvum* sporozoites in the presence or absence of MG-132 and SN50. After 24 hours of incubation, cells were fixed and immunostained for *C. parvum* followed by DAPI staining for apoptosis.[9,30] Briefly, cells were fixed (0.1 mol/L 1,4-piperazinediethanesulfonic acid [pH 6.95], 1 mmol/L EGTA, 3 mmol/L $MgSO_4$, and 2% paraformaldehyde) and permeabilized with 0.2% (vol/vol) Triton-X100 in PBS. Slides were incubated with the primary polyclonal antibody against *C. parvum* membrane proteins followed by a rhodamine-labeled anti-rabbit antibody. After washing with PBS, slides were exposed to the nuclear staining dye, DAPI (2.5 μmol/L, 5 minutes). Slides were then mounted with mounting medium (H-1000; Vector Laboratories) and assessed by confocal laser scanning microscopy as described above.

Statistics

All values are given as mean ± SEM. Means of groups were compared with the Student (unpaired) *t* test or analysis of variance test where appropriate. $P < 0.05$ was considered statistically significant.

Results

C. parvum Infection Activates the NF-κB System and Induces IL-8 Production in Infected H69 Cell Cultures

To assess whether the NF-κB/IκB pathway is activated by *C. parvum* infection in H69 cells, IκBα protein in total cell lysate and NF-κB complexes in the nuclei of *C. parvum*–infected cell cultures were determined by immunoblotting and electrophoretic gel shift assay, respectively. As shown in Figure 1, by 12 hours after infection, the levels of IkBα protein in cells from the inserts (upper chamber), which were directly exposed to the parasite, had diminished, with an 80% decrease by 24 hours after infection. No decrease of IkBα in cocultured cells from the plates (lower chamber) was found at any time points. In conjunction with these experiments, electrophoretic gel shift assays showed only a small amount of NF-κB in the nucleus of sham-infected cells. In contrast, there was a consistent increase of NF-κB in the nuclei of cells from the inserts (upper chamber) 12 and 24 hours after infection, whereas cocultured cells from the plates (lower chamber) showed no increase of NF-κB nuclear translocation (Figure 2*A*). The nuclear complexes activated by *C. parvum* infection reacted in gel supershift assays with polyclonal antibodies to both p50 and p65 but not to p52 and Rcl (Figure 2*B*). Cells on the plates cocultured with *C. parvum* alone in the inserts (only parasite without cells in the insert) also showed no increase of NF-κB nuclear translocation (data not shown). These observations suggest that IκBα is being degraded to activate the NF-κB system in infected cells after *C. parvum* infection and the NF-κB activation requires direct parasite/host cell interaction.

To further confirm *C. parvum*–induced activation of the NF-κB system, secretion of IL-8, a well-known gene product of NF-κB, from biliary epithelial cells in response to infection was assessed. H69 cells were incubated with *C. parvum* for 0, 12, 24, and 48 hours and supernatants were collected to measure IL-8 by ELISA. As shown in Figure 3, although both H69 cells from normal and sham-infected controls consistently secreted low levels of IL-8, a time-dependent increase of IL-8 secretion was found in the supernatants from cells incubated with *C. parvum*. Increase of IL-8 release was first noted at 12 hours after infection and was found to increase continuously up to about 3-fold compared with sham-infected controls at 48 hours after infection. Both MG-132, a well-known potent specific proteasome inhibitor that prevents IkBα degradation, and SN50, a specific cell-permeable inhibitory peptide that blocks NF-κB DNA binding in the nucleus, significantly decreased IL-8 production (Figure 3*B*).

C. parvum Induces NF-κB Activation Only in Directly Infected, not Bystander-Uninfected, Biliary Epithelial Cells Observed by Immunofluorescent Microscopy

Because only 15%–35% of the cells exposed to the parasite are directly infected by the organism,[22]

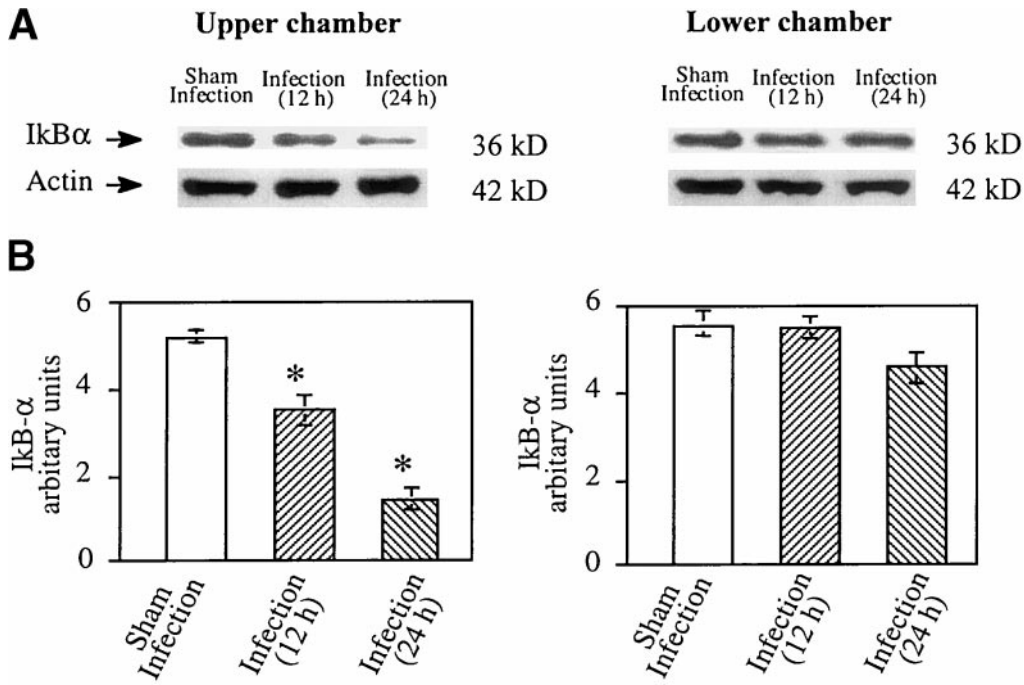

Figure 1. Immunoblotting of *C. parvum*–induced IκBα degradation in H69 cells. H69 cells were grown on the upper and lower chambers of the coculture system and only the cells in the upper chambers were exposed to *C. parvum*. After 12 and 24 hours of incubation with *C. parvum* sporozoites, cells from both the upper and lower chambers were lysed and subjected to quantitative immunoblotting. (*A*) Representative immunoblots for IκBα in H69 cells. (*B*) Densitometric analysis of IκBα expression for 3 separate experiments. Degradation of IkBα was found in cell cultures directly exposed to the organism in the upper chambers, but not in cells cocultured in the lower chambers. β-actin was also immunoblotted to ensure equal loading of proteins to each lane. $*P < 0.05$ compared with sham infection.

NF-κB activation might occur in directly infected and/or bystander uninfected cells in the monolayers directly exposed to the organism in the coculture system. To address this question, H69 cells were grown on 4-well chamber slides and then exposed to *C. parvum* and fixed for immunofluorescent microscopy. Cells that showed NF-κB nuclear translocation and were directly infected by the parasite were identified by a primary monoclonal antibody to p65 in its activated form mixed with a primary polyclonal *C. parvum* antibody followed by rhodamine-labeled anti-mouse and fluorescein-labeled anti-rabbit antibodies. As shown in Figure 4*B*, cells from sham-infected controls did not show any *C. parvum* direct infection of cells, and these cells showed only a very weak reaction to p65 in the nuclei similar to the reaction found in normal control cells (Figure 4*A*). However, after a 24-hour incubation with the parasite, cells directly infected by *C. parvum* showed a significant increase of reaction to p65 in the nuclei (Figure 4*C*). In contrast, bystander-uninfected cells showed a similar reaction as sham infection controls. Semiquantitative analysis showed a significantly higher FITC fluorescent intensity for p65 in the nuclei of directly infected cells than in those of bystander-uninfected or sham-infected cells (Figure 4*D*).

C. parvum–Associated Apoptosis in H69 Cells

To clarify the relationship of *C. parvum*–induced NF-κB activation and associated epithelial apoptosis in biliary epithelial cells, apoptosis in H69 cells was assessed by nuclear binding dye DAPI staining after incubation with the parasite for 24 hours in the presence of various reagents to inhibit NF-κB activation. As shown in Figure 5*A*, cells from normal and sham-infected controls, as well as cells treated with 1 μmol/L of MG-132 or 50 μg/mL of SN50 alone, showed only a few cells undergoing apoptosis. Consistent with our previous report,[9] 10%–14% of cells exposed to *C. parvum* for 24 hours exhibited nuclear condensation and segmentation, characteristic nuclear changes associated with apoptosis of epithelial cells. MG-132 and SN50 significantly increased *C. parvum*–induced apoptosis in H69 cells (Figure 5*A*).

To identify those cells that were undergoing apoptosis and those that were directly infected by the organism, H69 cells grown on 4-well chamber slides were exposed to *C. parvum* for 24 hours and then observed by immunofluorescent microscopy. When stained with nuclear binding dye DAPI for apoptosis and a primary polyclonal

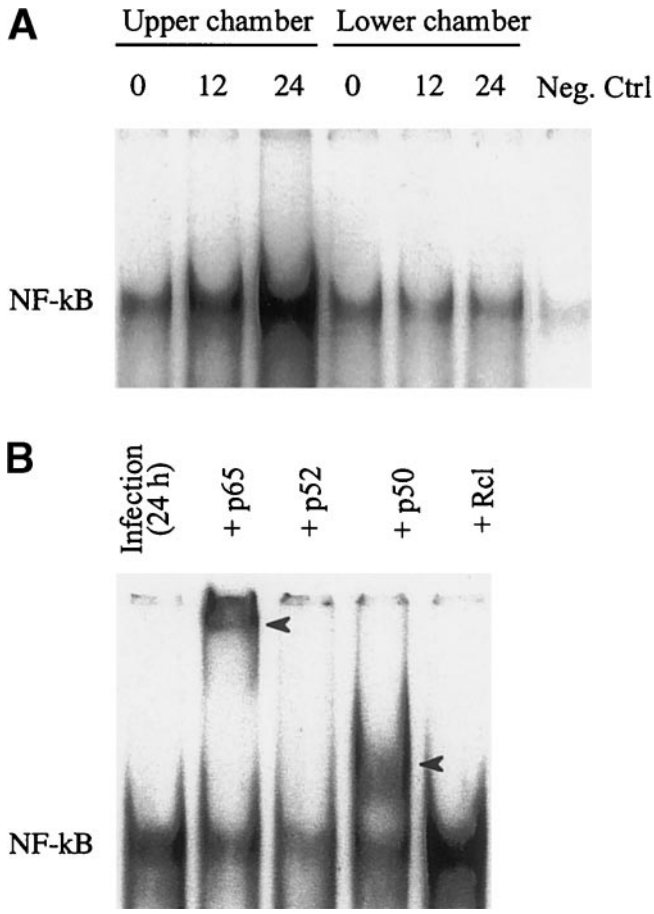

Figure 2. Gel shift and antibody supershift assays of *C. parvum*–induced NF-κB nuclear translocation in H69 cells. H69 cells were grown on the upper and lower chambers of the coculture system and only the cells in the upper chambers were exposed to *C. parvum*. After 12 and 24 hours of incubation with *C. parvum* sporozoites, nuclear extracts were made from cells of both the upper and lower chambers and then subjected to gel shift or antibody supershift assays. (*A*) Gel shift assay of NF-κB in the nuclear extracts of H69 cells. A consistent increase of shift complexes for NF-κB was found in the nuclear extracts from cell cultures directly exposed to the organism for 12 and 24 hours in the upper chambers, but not in cells cocultured in the lower chambers. The specificity of complex formation was confirmed by addition of a 10-fold molar excess of unlabeled oligonucleotide (Neg. Ctrl). (*B*) Antibody supershift analysis of activated NF-κB species. Nuclear extracts from H69 cells in the upper chambers after a 24-hour incubation with the parasite were subjected to gel supershift analysis using antibodies against the NF-κB subunits p65, p52, p50, and Rcl. Supershifted complexes were found for antibodies against p65 and p50 (*arrowheads*), but not for p52 and Rcl.

antibody to *C. parvum* followed by FITC-conjugated secondary antibody, cells undergoing apoptosis were found to be limited to bystander-uninfected ones; cells directly infected by the organism rarely exhibited apoptotic nuclear changes (Figure 5*B*, panel b). In contrast, in the presence of MG-132 (Figure 5*B*, panel c) or SN50 (Figure 5*B*, panel d), not only bystander-uninfected cells but also directly infected cells were found to exhibit nuclear changes characteristic of apoptosis. These results are consistent with our previous in vivo observation in patients with AIDS with biliary cryptosporidiosis,[8] suggesting that *C. parvum*–induced biliary epithelial apoptosis is limited to bystander-uninfected cells. These observations also suggest that NF-κB activation in directly infected cells might be responsible for their resistance to apoptosis.

Discussion

The major findings described here relate to the interaction of *C. parvum* with biliary epithelia. Our data show, for the first time, the following: (1) *C. parvum*

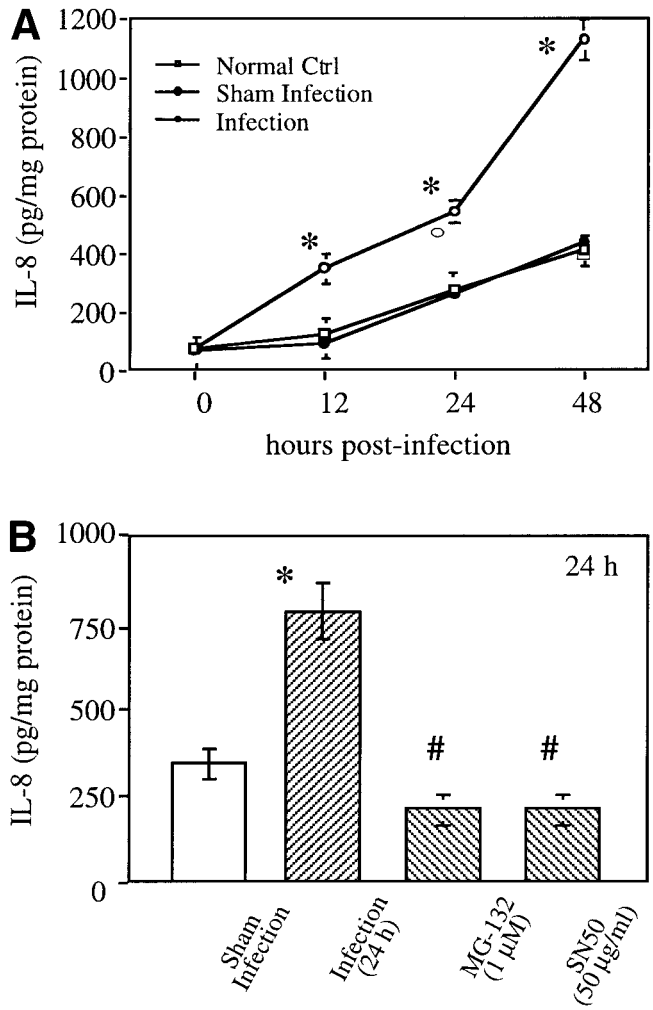

Figure 3. *C. parvum*–induced IL-8 secretion and its blockage by NF-κB inhibition in H69 cells. H69 cells were grown on 4-well chamber slides and exposed to *C. parvum* for various lengths of time. The supernatants were collected and subjected to ELISA for IL-8 and cells were collected for protein determination. (*A*) A time-dependent increase of IL-8 secretion was found in the supernatants from cell cultures exposed to the organism compared with the normal and sham infection controls. (*B*) Both MG-132 and SN50 significantly blocked IL-8 secretion induced by *C. parvum* in H69 cells. Data are from 3 separate experiments. $^{*}P < 0.05$ compared with sham infection controls; $^{\#}P < 0.05$ compared with infection without any inhibitors.

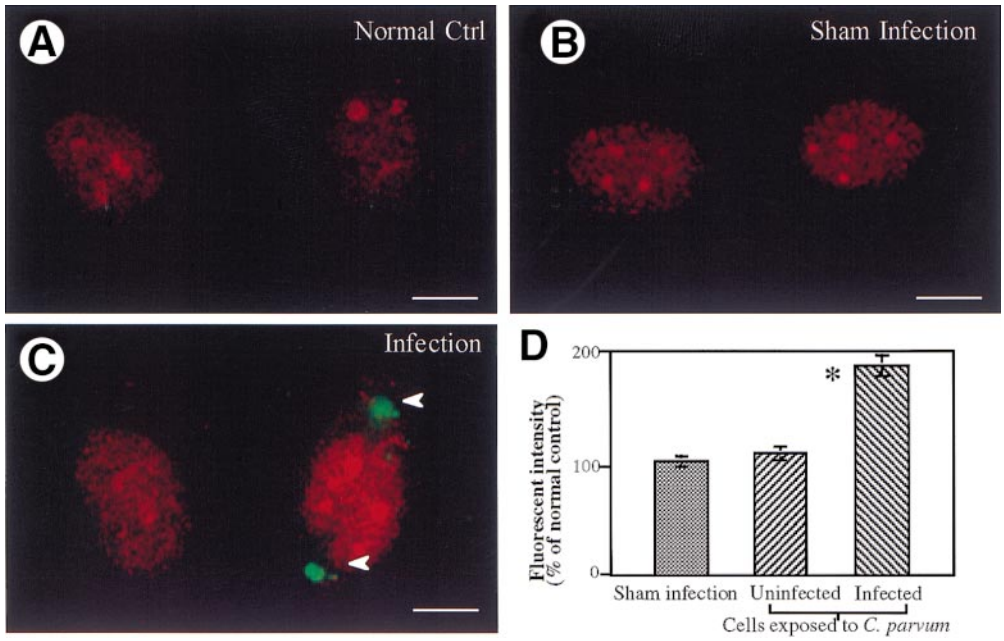

Figure 4. *C. parvum*–induced NF-κB nuclear translocation in directly infected H69 cells by immunofluorescent confocal microscopy. H69 cells grown on 4-well chamber slides were exposed to *C. parvum* for 24 hours. The slides were then fixed and analyzed by double staining using a monoclonal antibody against p65 in its activated form and a polyclonal antibody against *C. parvum* followed by secondary rhodamine/fluorescein-labeled antibodies. P65 in its activated form in the nuclei was stained *red* and *C. parvum* stained *green* (*arrowheads*). Very weak staining of p65 was found in the nuclei of cells from both (*A*) normal and (*B*) sham-infected controls. (*C*) Whereas bystander uninfected cells showed no increase of staining of p65, cells directly infected showed very strong staining of p65 in their nuclei. (*D*) Semiquantitative analysis showed a significant increase of FITC fluorescent intensity of p65 in the nuclei of directly infected cells compared to that of bystander uninfected cells and sham infection control. $*P < 0.05$ compared with sham infection control and uninfected bystander cells. *Bar* = 5 μm.

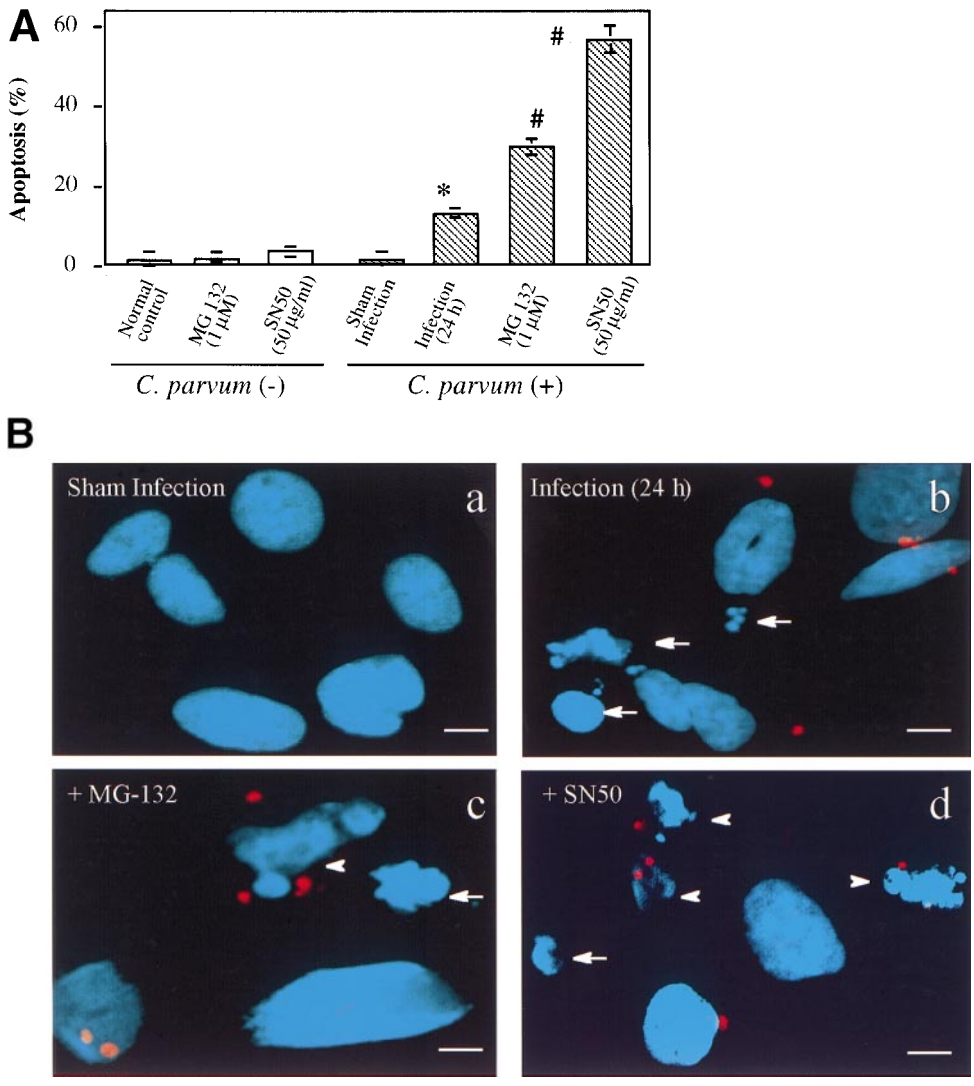

Figure 5. *C. parvum*–induced epithelial cell apoptosis in H69 cells. H69 cells were grown on 4-well chamber slides. After 24 hours of incubation with *C. parvum*, cells were fixed and subjected to DAPI staining (nuclei stained *blue*) for apoptosis or costaining of DAPI with a polyclonal antibody against *C. parvum* (stained *red*). (*A*) Quantitative analysis of apoptosis. Only a few apoptotic cells were found in cells from sham infection controls or those treated with MG-132 or SN50 alone. Although 10%–15% of cells were found undergoing apoptosis after 24 hours of incubation with the parasite by DAPI staining, a significant increase of apoptosis was found in cells exposed to the parasite in the presence of MG-132 or SN50 compared with cells incubated with *C. parvum* alone. (*B*) Apoptosis observation by confocal microscopy. Whereas nuclei of cells directly infected with the organism showed (*B*, panel b) similar nucleus staining as (*B*, panel a) normal controls, (*B*, panel b) nuclei of bystander-infected cells show condensation and segmentation (*arrows*) of nucleus with fluorescent DNA binding dye DAPI. (*B*, panels c and d) In contrast, apoptosis was found in both bystander-uninfected (*arrows*) and directly infected (*arrowheads*) cells in the presence of MG-132 or SN50. $*P < 0.05$ compared with sham infection controls; $^{\#}P < 0.05$ compared with infection without any inhibitors. *Bar* = 5 μm.

infection of cultured human biliary epithelia activates the NF-κB/IκB pathway in directly infected cells, but not in bystander-uninfected cells; (2) *C. parvum*–induced biliary epithelial cell apoptosis is limited to bystander-uninfected cells in vitro; and (3) inhibition of NF-κB activation results in apoptosis in directly infected cells and enhances *C. parvum*–induced apoptosis in bystander uninfected cells. The data suggest that *C. parvum* activates NF-κB in directly infected biliary epithelia, a process that induces IL-8 secretion and inhibits apoptosis in these cells. As a result, the organism's ability to survive and propagate is enhanced by the NF-κB–induced resistance to apoptosis of infected cells.

Epithelial cells that line the intestine and biliary tree are the initial site of interaction between the host and enteroinvasive microbial pathogens. After microbial infection, epithelial cells rapidly express a number of genes, the products of which activate mucosal inflammatory and immune responses and alter epithelial cell functions.[31] Nuclear transcription factors such as NF-κB play a central role in the gastrointestinal epithelial cell response to infection with pathogens including *Shigella flexneri, Listeria monocytogenes, Salmonella typhimurium*, many viruses, and parasitic protozoans such as *Theileria parva*.[10,32–34] For some pathogens (e.g., *Helicobacter pylori*), the NF-κB system is activated via indirect pathways involving soluble factors released from infected cells and is not limited to cells directly infected by the pathogen.[35] For other pathogens (e.g., *Rickettsia rickettsii*), the activation of NF-κB requires direct pathogen/host cell interactions and is limited to directly infected cells.[36] In most cells, NF-κB exists in a latent state in the cytoplasm bound to inhibitory proteins (collectively called IκBs) that mask its nuclear localization signal. This latent form can be activated by a variety of agents that signal phosphorylation and subsequent degradation of IκBs. Activated NF-κB is then free to translocate to the nucleus and by its nuclear translocation and binding to the NF-κB binding motif, NF-κB acts synergistically with other nuclear factors such as AP-1, NF–IL-6, etc., to activate a variety of genes involved in the immune or inflammatory epithelial cell response to infection.[11] In certain epithelia, NF-κB activation may also activate intracellular survival signals to prevent the infected cell from dying.[12]

Secretion of NF-κB–regulated gene products such as prostaglandin E2, TNF-α, and IL-8 in epithelial cells in response to *C. parvum* infection has been documented in vivo and in vitro.[16,17,37] However, whether *C. parvum* can activate nuclear transcription factors such as NF-κB is unclear and nothing is known about the potential relationships among *C. parvum*–induced pathology and nuclear transcription factor activation in any epithelial cell. Using a coculture system, we found that NF-κB activation only occurs in cells directly exposed to the parasite. Activation of NF-κB in directly infected biliary epithelia (but not in bystander-uninfected or cocultured cells) was further confirmed by immunofluorescent costaining and confocal microscopy using a monoclonal antibody against p65 in its activated form and a polyclonal antibody to *C. parvum*. Thus, a direct parasite/host cell interaction is a prerequisite for NF-κB activation; soluble factors from the parasite or the infected cells are not involved.

The kinetics of the biliary epithelial cell NF-κB activation and IL-8 secretion in response to *C. parvum* infection is consistent with a previous report using an in vitro model of intestinal cryptosporidiosis.[16] However, these kinetics are markedly different from those seen after infection of epithelial cells with invasive bacteria or rapidly cytolytic pathogens such as *Entamoeba histolytica*.[38] We found that the NF-κB activation and secretion of IL-8 were delayed 12–24 hours after infection and for at least 48 hours. In contrast, epithelial cell infection with invasive pathogens (e.g., *Salmonella, Yersinia*, or enteroinvasive *Escherichia coli* strains) results in rapid (2–3 hours) but brief (4–10 hours) IL-8 secretion by epithelial cells.[39–41] The relatively delayed NF-κB activation in response to *C. parvum* in biliary epithelia suggests that *C. parvum*–induced NF-κB activation in biliary epithelia is not directly associated with the attachment to and invasion of the cell by the organism. It is more likely that interactions between the organism and the host cell during the late stage of infection (i.e., when the parasite already resides in the parasitophorous vacuole) triggers IkBα degradation and NF-κB nuclear translocation. Although the attachment of *C. parvum* to the biliary epithelial cell membrane depends on a Gal/GalNAc–associated parasite protein,[22] entry of the organism into the epithelial cell involves active host cell plasma membrane spreading to form a parasitophorous vacuole, a organelle that keeps the parasite intracellular but extracytoplasmic.[8,42]

Increasing numbers of microbial pathogens mediate apoptosis in vivo, and host cell apoptosis could represent a host defense reaction to limit microbial production and eliminate spread of organisms. Inhibition of host cell apoptotic death, therefore, could be advantageous for some pathogens, particularly intracellular pathogens, which depend on host cells to survive and propagate. Indeed, several pathogens, including herpes-, pox-, and baculoviridae, have evolved or acquired genes that specifically inhibit the apoptotic machinery[43]; NF-κB activation has been shown to guarantee cell survival during

Rickettsia rickettsii infection.[36] Intestinal epithelial cell apoptosis is a conserved response to bacterial invasion; many bacteria (e.g., *Salmonella* and *Escherichia coli*) can trigger apoptosis in intestinal epithelia.[44] However, whether pathogens can inhibit epithelial cell apoptosis is obscure. *C. parvum* has a monoxenous life cycle, i.e., all stages of development (asexual and sexual) occur in one host. Parasite survival therefore depends on persistent epithelial cell infection. While we have previously shown that *C. parvum* infection of biliary epithelial cells induces bystander-uninfected cells to undergo apoptosis,[8,9] it is unclear how directly infected epithelial cells can survive while *C. parvum* kills bystander-uninfected cells via apoptosis. Using our in vitro model of biliary cryptosporidiosis, we show here that only the cells directly infected with the parasite display NF-κB nuclear translocation and that cells undergoing apoptosis are limited to bystander uninfected cells (i.e., those cells not directly invaded by *C. parvum*). Inhibition of NF-κB activation both by a proteasome inhibitor, MG-132, and the specific NF-κB inhibitory peptide, SN50, significantly enhanced *C. parvum*–induced apoptosis in uninfected bystander cells while simultaneously inducing apoptosis in directly infected cells. These original observations suggest that while *C. parvum* triggers apoptosis in bystander-uninfected epithelial cells (a process that may represent a host defense reaction to limit spread of the infection), NF-κB activation in directly infected epithelial cells by *C. parvum* makes these cells resistant to apoptosis, a process that would benefit the survival and propagation of the organism. Indeed, our observations are supported by a recent study that showed that inhibition of NF-κB activation enhances *C. parvum*–induced apoptosis in intestinal epithelial cells in vitro.[45] Protection against apoptotic cell death in directly infected cells by NF-κB activation has also been reported in other apicocomplexan parasitic infections such as *Theileria parva* and *Toxoplasma gondii*,[10,46] raising the possibility that this is a common characteristic of parasites belonging to the phylum Apicomplexa.

In conclusion, using an in vitro model of biliary cryptosporidiosis in an immortal but nonmalignant human bile duct epithelial cell line, we have shown for the first time that *C. parvum* activates the NF-κB system in directly infected biliary epithelial cells, an event that enhances survival of infected cells (Figure 6). Furthermore, *C. parvum* activation of the NF-κB system also results in secretion of IL-8 presumably contributing to the inflammatory reaction in the bile ducts caused by this microbe. Future studies should use in vivo infectious models and define the molecular mechanisms of NF-κB activation and antiapoptotic signals activated by NF-κB in directly infected cells and apoptosis in bystander-uninfected cells, respectively.

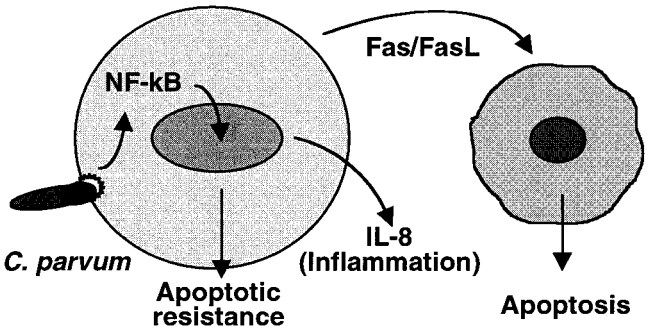

Figure 6. Schematic model of *C. parvum*–induced NF-κB activation and its relevance to IL-8 secretion from, and associated epithelial apoptosis in biliary epithelial cells. Although *C. parvum* causes apoptosis in uninfected bystander biliary epithelial cells from Fas activation, the organisms induce NF-κB activation in infected biliary epithelia, resulting in the release of IL-8 to stimulate an inflammatory reaction and inhibition of apoptosis in infected cells to facilitate parasite survival and propagation.

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