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Interaction of *Cryptosporidium hominis* and *Cryptosporidium parvum* with Primary Human and Bovine Intestinal Cells

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Cryptosporidiosis in humans is caused by the zoonotic pathogen *Cryptosporidium parvum* and the anthroponotic pathogen *Cryptosporidium hominis*. To what extent the recently recognized *C. hominis* species differs from *C. parvum* is unknown. In this study we compared the mechanisms of *C. parvum* and *C. hominis* invasion using a primary cell model of infection. Cultured primary bovine and human epithelial intestinal cells were infected with *C. parvum* or *C. hominis*. The effects of the carbohydrate lectin galactose-*N*-acetylgalactosamine (Gal/GalNAc) and inhibitors of cytoskeletal function and signal transduction mechanisms on entry of the parasites into host cells were tested. HCT-8 cells (human ileocecal adenocarcinoma cells) were used for the purpose of comparison. Pretreatment of parasites with Gal/GalNAc inhibited entry of *C. parvum* into HCT-8 cells and primary bovine cells but had no effect on entry of either *C. parvum* or *C. hominis* into primary human cells or on entry of *C. hominis* into HCT-8 cells. Both *Cryptosporidium* species entered primary cells by a protein kinase C (PKC)- and actin-dependent mechanism. Staurosporine, in particular, attenuated infection, likely through a combination of PKC inhibition and induction of apoptosis. Diversity in the mechanisms used by *Cryptosporidium* species to infect cells of different origins has important implications for understanding the relevance of in vitro studies of *Cryptosporidium* pathogenesis.

Cryptosporidium is an obligate enteric parasite of the phylum *Apicomplexa* and an important cause of diarrheal disease worldwide. The infection is transmitted by fecal, food, and waterborne routes and is initiated when sporozoites released from oocysts in the intestinal tract attach to and invade mucosal epithelial cells. In susceptible immunocompetent humans (mainly children), infection leads to a self-limiting diarrhea. In immunocompromised patients, especially AIDS patients and those with congenital immune deficiencies, chronic diarrhea frequently develops and leads to considerable fluid and weight loss and even death. In addition to the upper intestine, the parasite can also infect the stomach, pancreas, liver, and bile ducts of immunocompromised hosts. Although promising chemotherapeutic treatments against cryptosporidiosis are beginning to appear (22), elimination of the parasite is extremely difficult and recurrence is common.

The genus *Cryptosporidium* consists of various different species and genotypes that infect a wide range of hosts. Originally, almost all isolates from human patients were assigned to *Cryptosporidium parvum*. Within *C. parvum*, two genotypes were distinguished: the "human genotype," type I, and the "cattle genotype," type II, both of which are capable of initiating human infection. The two genotypes differ significantly in their host range and genetics (24, 31) and maintain separate reproductive cycles (27). They are now widely recognized as two separate species, *Cryptosporidium hominis* (formerly known as type I) and *C. parvum* (formerly known as type II). So far, natural infections of *C. hominis* have only been reported from humans, although experimental infections have been carried out in neonatal pigs (21). In contrast, *C. parvum* infects most, if not all, mammals, including humans, and is a major pathogen of calves. Our knowledge of how these species differ in relation to host range, infectivity, or pathogenicity is only beginning to evolve.

Generally, humans are infected with *C. hominis* in an anthroponotic cycle and with *C. parvum* in a zoonotic cycle. Most work to date on the biology and pathogenicity of *Cryptosporidium* in relation to human infection has been done using *C. parvum*. Therefore, compared to *C. parvum* little is known of the biology of invasion of the human-restricted *C. hominis*. In a recent study we compared the pathogenesis of *C. hominis* and *C. parvum* using both HCT-8 cells and primary culture of both bovine and human intestinal cells. *C. hominis* infection of HCT-8 cells differed from *C. parvum* infection (17). Entry of *C. hominis* into HCT-8 cells was less efficient than *C. parvum* and showed a distinct focal pattern of infection in the monolayer, whereas *C. parvum* infection of HCT-8 cells was evenly distributed throughout the monolayer. In contrast to HCT-8 cells, there was no difference between the two species of *Cryptosporidium* during infection of primary human intestinal cells. However, only *C. parvum* was capable of infecting primary bovine cells. These data suggested that the species restriction of *C. hominis* is due to host tissue tropism of the infecting isolate and that different adhesin-receptor interactions may promote entry of these organisms in cells of different origins.

Identification of the parasite and host molecules that mediate the initial host-parasite interactions during host-cell invasion is crucial for designing preventive and interventional strategies to combat cryptosporidiosis. A number of sporozoite glycoproteins are known to act as mediators of *C. parvum*

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TABLE 1. Inhibitors used to study signal transduction pathways involved in *C. parvum* and *C. hominis* entry of HCT-8 cells and primary human and bovine intestinal cells

Inhibitor	Mode of action	Concn(s) used
Genistein	Specific inhibitor of tyrosine phosphorylation	25, 250, 500 μM
Herbimycin A	Specific inhibitor of tyrosine phosphorylation	1, 10 μM
Staurosporine	General protein kinase inhibitor	0.5, 1, 1.5 μM
Gö6976	PKC inhibitor, selectively inhibits Ca^{2+}-dependent PKCα	0.08 μM
Bisindolymaleimide	Inhibitor for the ATP binding site of PKC	10 μM
Chelerythrine chloride	PKC inhibitor; acts on the catalytic domain of PKC	0.66 μM
Calphostin C	PKC inhibitor; competes at the binding site of diacylglycerol and phorbol esters	0.66 μM
PKC	An N-terminal myristoylated membrane-permeable inhibitor of PKC	8 μM
Rö-32-0432	PKC inhibitor; displays about 10-fold greater selectivity for PKC_{α} and 4-fold greater selectivity for $PKC_{\beta I}$ over PKC_{ε}	0.18 μM
KN-93	Serine/threonine kinase inhibitor	5 μM
H-89 dihydrochloride	Serine/threonine kinase inhibitor; potent inhibitor of PKA (K_i = 48 nM)	30 μM
ML-7	Serine/threonine kinase inhibitor; inhibitor of myosin light chain kinase	42 μM
PKGI	Serine/threonine kinase inhibitor; a more specific inhibitor of PKG relative to PKA	86 μM
Suramin sodium salt	Uncouples G proteins from receptors; inhibits phospholipase D and blocks the binding of ligands to several growth factor receptors	1, 10 μM
Wortmannin	Inhibits phosphatidylinositol 3-kinase, mitogen-activated protein kinase, and myosin light chain kinase	1, 10, 100 nmol

attachment in vitro including a galactose-*N*-acetylgalactosamine (Gal/GalNAc)-specific sporozoite epitope (7, 18, 19, 26). Studies have shown that sporozoite motility and invasion are dependent on parasite and host cytoskeletal elements (7, 14, 15). In the present study we have investigated the role of a Gal/GalNAc-specific lectin carbohydrate interaction in mediating colonization of primary human cells by *C. hominis* and *C. parvum* and colonization of primary bovine cells by *C. parvum*. In addition, we have studied the cytoskeletal and signal transduction events that occur following invasion of primary human and bovine intestinal cells by *C. hominis* and *C. parvum*.

MATERIALS AND METHODS

***C. parvum* and *C. hominis*.** *C. parvum* oocysts (Iowa strain) were obtained from a commercial source (Pleasant Hill Farms, Troy, ID). This strain was originally isolated from a calf by Harley Moon. It has been passaged through calves and purified from the fecal material by ether extraction, followed by a one-step sucrose gradient.

C. hominis TU502 oocysts were a gift from Donna Akiyoshi, Tufts University School of Veterinary Medicine. Boston, Mass. *C. hominis* strain TU502 is a well-characterized isolate that has been passaged through gnotobiotic piglets (2), and its genome has been sequenced (32). Before host cells were infected, oocysts were decontaminated. Briefly, they were washed twice with distilled water and then incubated with freshly prepared 10% (vol/vol) Clorox bleach (Sigma-Aldrich) for 10 min on ice. Following two further washes with ice-cold distilled water and one with RPMI 1640 medium (Bio-Whittaker), oocysts were resuspended at a concentration of 2×10^5 oocysts/ml and then used to infect cell monolayers.

Cell culture. Human ileocecal adenocarcinoma cells (HCT-8) (ATCC CCL 244) were obtained from the American Type Culture Collection. Cells were maintained in 75-cm^2 tissue culture flasks in RPMI 1640 medium and 10% (vol/vol) fetal bovine serum (Sigma). The cells were grown as adherent monolayers at 37°C in a 5% CO_2–95% air humidified incubator. Twenty-four hours prior to infection, the monolayers were trypsinized with trypsin-EDTA (Bio-Whittaker) for 15 min at 37°C. The cells were grown on 25-mm Thermonox coverslips in six-well Costar tissue culture plates (Gibco) for 24 h, at which time they were 80 to 85% confluent.

Isolation and culture of primary human and bovine intestinal epithelial cells. Human and bovine intestinal epithelial cells were isolated as described previously (17). Following approval by the ethics committee at the hospital and parental consent, human small-bowel biopsy tissue was obtained from children undergoing endoscopy at Our Lady's Hospital for Sick Children. Only children undergoing endoscopy for clinical reasons were recruited (e.g., investigation of abdominal pain, gastroesophageal reflux, and failure to thrive). Only grossly normal tissue was sampled. Bovine small-duodenal tissue was removed from cattle under 30 months of age immediately after slaughter at a local abattoir. Cells from both tissues were then isolated using similar procedures. Briefly, the tissue was washed with Hank's balanced salt solution (Bio-Whittaker) with Ca^{2+} or Mg^{2+} containing 0.1 mM EDTA (Sigma) and 0.1 mM dithiothreitol (Sigma) at 37°C for 10 min with vigorous shaking. The crypts and cells were isolated using 0.05% (wt/vol) collagenase (Sigma). The isolated cells and crypts were kept on ice until ready for use. A total of 500 μl was plated in 24-well Costar culture plates on 13-mm plastic coverslips using Dulbecco's modified Eagle's medium–Ham's F-12 with 10% fetal bovine serum, 8 μg/ml insulin, 10 μg/ml gentamicin, 50 μg/ml hydrocortisone, 100 μg/ml streptomycin, 100 U/ml penicillin, and 2.5 μg/ml amphotericin B. Cells were grown for 24 to 48 h at 37°C in a 5% CO_2–95% air humidified incubator, at which time isolated cells and crypts attached to the coverslips. Cells grew as isolated epithelial colonies and propagated by growing out from the crypts. The epithelial origin of the cultured cells was demonstrated by immunofluorescence staining with a monoclonal anti-pan cytokeratin (Sigma) as previously described (17).

Infection of HCT-8 cells and primary human and bovine intestinal cells. Maintenance medium was removed from the cells, and 2×10^5 *Cryptosporidium* oocysts in 2 ml of growth medium were added to the cells. The plates were placed at 37°C in a 5% CO_2–95% air humidified incubator. After incubation for 3 h, the infected cells were washed with phosphate-buffered saline (PBS) to remove unexcysted oocysts, empty oocyst walls, and toxic materials that may have been liberated from the oocysts. Then, 2 ml of growth medium containing 100 U of penicillin/ml and 100 μg of streptomycin/ml was added. Primary cells were infected for 24 h, and HCT-8 cells were infected for up to 72 h. During this time period the cells remained viable and did not appear to be damaged by infection.

Lectin VVL staining. Parasites were detected in the monolayers using lectin VVL (a plant lectin from *Vicia villosa*) staining, as previously described (16). Monolayers were fixed with 4% formaldehyde in PBS for 30 min, permeabilized with 1% Triton X-100 in PBS for 10 min, and blocked with 0.5% (wt/vol) bovine serum albumin for a further 10 min. They were then incubated with 1 μg/ml of conjugated lectin VVL-biotin (B1235; Vector Laboratories) in 0.5% (wt/vol) bovine serum albumin followed by 1 μg/ml of conjugated fluorophore streptavidin-CY3 (Sigma). The coverslips were mounted using fluorescence mounting medium (Dako) and examined by fluorescence microscopy using a 50× water immersion lens. The numbers of infected cells in 10 high-power fields were counted and the percent rate of infection ([number of infected cells/total number of cells] × 100) was calculated. All experiments were performed in triplicate and expressed as the mean percentage of cells infected ± the standard deviation of the mean. Uninfected monolayers were used as a control.

Colocalization of host cell actin and *Cryptosporidium*. In order to stain cells for both actin and parasite, cells were stained with lectin VVL as described above with a slight modification. After permeabilization with Triton X-100, cells were incubated with 5 μg/ml phalloidin conjugated to fluorescein isothiocyanate (FITC) (Sigma) together with 1 μg/ml lectin VVL for 30 min. Slides were examined using a laser scanning confocal microscope (Bio-Rad MRC 1024).

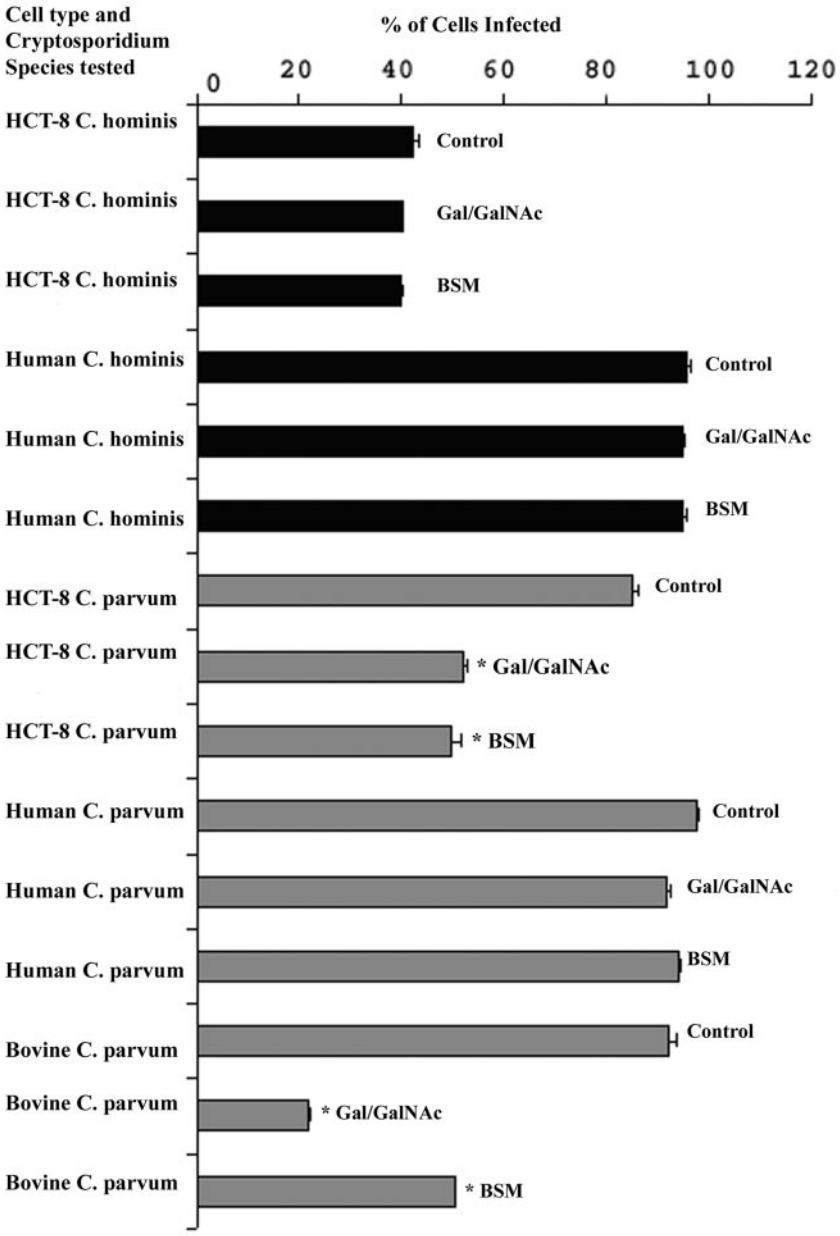

FIG. 1. Effect of Gal/GalNAc and BSM on invasion of HCT-8 cells and primary human and bovine cells by *C. hominis* and *C parvum*. Gal/GalNAC and BSM significantly (∗, $P = 0.0002$) reduced entry of *C. parvum* into HCT-8 cells and primary bovine cells but had no effect on entry of *C. parvum* into primary human cells or on entry of *C. hominis* into HCT-8 and primary human cells. The inhibitor used in each experiment is indicated at right.

Effect of Gal/GalNAc on *C. hominis* and *C. parvum* entry of HCT-8 cells and primary human and bovine intestinal cells. The effect of Gal/GalNAc and bovine submaxillary mucin (BSM; BSM contains Gal/GalNAc) on entry of sporozoites of *C. hominis* and *C. parvum* to HCT-8 cells and primary human and bovine intestinal cells was tested. Sporozoites were prepared by excystation of oocysts using a previously described method (28). Oocysts were suspended in 1 ml of PBS and incubated for 60 min at 37°C using a water bath. The suspension was pelleted by centrifugation at 5,000 × *g* for 5 min at 4°C using a microcentrifuge (Sigma). The supernatant was discarded, and the pellet was resuspended in 1 ml of isotonic Percoll solution and centrifuged at 5,000 × *g* for 5 min at 4°C. The pellet contained some sporozoites and intact oocysts. Immediately above the pellet was a buffy coat consisting of highly enriched sporozoites, while oocyst walls lay on top of the Percoll. By using a micropipette tip, the sporozoites were aspirated into a 1.5-ml Eppendorf tube. They were resuspended in four times their volume with PBS and were spun at 5,000 × *g* for 5 min at 4°C. Sporozoites were then incubated with 10 μM and 5 μM Gal/GalNAc and 0.1 mg/ml and 0.01 mg/ml BSM for 1 hour at 37°C. The sporozoites were then washed twice with PBS and used to infect HCT-8 cells and primary human and bovine intestinal cells. Infected HCT-8 cells and primary human and bovine intestinal cells were stained with lectin VVL as described above, and the number of infected cells in a total of 250 cells was counted and expressed as the infection rate. Untreated sporozoites were used as controls.

Effect of inhibitors of cytoskeletal components on *C. parvum* and *C. hominis* entry of HCT-8 cells and primary human and bovine intestinal cells. Stock solutions of 1 mg/ml of cytochalasin D and 5 mg/ml of cytochalasin B (Sigma) (actin inhibitors) were prepared and diluted to their final concentrations using culture medium. Three different experiments were performed. In the first set of experiments, cells were incubated for 1 h in an assay medium containing either

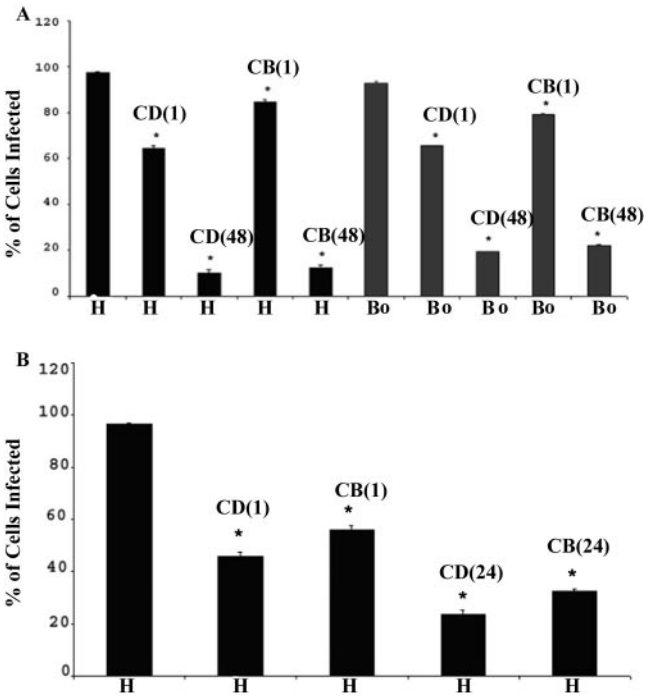

FIG. 2. Effect of cytochalasin D (CD) and cytochalasin B (CB) on invasion of human and bovine primary intestinal cells by *C. parvum* (A) and human cells by *C. hominis* (B). Pretreatment of cells prior to infection and infection of cells in the presence of inhibitors significantly reduced *C. parvum* entry into human and bovine cells and *C. hominis* entry into human cells. *, $P < 0.005$ compared to control (no inhibitor). The inhibitor used in each experiment is shown above the bars. Numbers in brackets indicate the time that cells were exposed to the inhibitors. H, human cells; Bo, bovine cells.

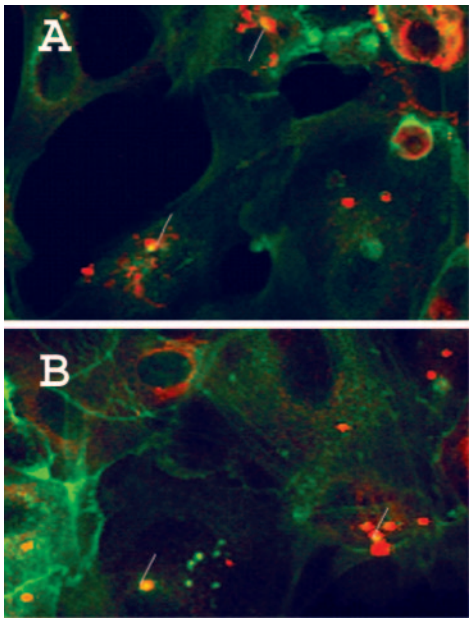

FIG. 3. Phalloidin-FITC and lectin VVL staining of primary human cell monolayers infected with *C. parvum* (A) and *C. hominis* (B). Green represents phalloidin-FITC-stained actin, and red represents the intracellular stages of the parasite. Colocalization of the actin and parasite appears yellow (indicated by white lines). The slide was examined using confocal laser scanning microscopy and a 40× lens. Original magnification, ×400.

1 μg/ml cytochalasin D or 10 μg/ml cytochalasin B (7) for 1 h at 37°C (30). The treated cells were washed twice with PBS and infected with 2×10^5 *C. parvum* oocysts for either 48 h or 22 h. In a second set of experiments, cells were infected as previously described, but the cytoskeletal inhibitors were present throughout the infection period. Finally, in order to assess the effect of cytoskeletal inhibition on the parasite, oocysts were incubated with 1 μg/ml cytochalasin D or 10 μg/ml cytochalasin B for 15 min at 37°C. Oocysts were then washed twice with PBS before being used to infect monolayers. Untreated cells or oocysts were used as controls.

Effect of signal transduction inhibitors on *C. hominis* and *C. parvum* invasion of HCT-8 cells and primary human and bovine intestinal cells. A number of signal transduction inhibitors were used to assess the importance of various host signal transduction pathways during invasion of *C. hominis* and *C. parvum* into host cells. Stock solutions of different inhibitors (Table 1) were prepared according to the manufacturers' recommendations and diluted to their final concentrations using culture medium. The final concentrations tested were the concentrations recommended by the manufacturers for inhibition of cellular function. Cells were incubated with the different inhibitors for 1 h. The cells were then washed twice with PBS and were infected with oocysts for up to 48 h. Untreated monolayers were used as controls.

Statistical analysis. The above results were expressed as the mean percentage of cells infected ± standard deviation of triplicate experiments. Means were compared using a nonparametric Mann-Whitney U test. A P value of <0.05 was considered statistically significant.

Annexin V and PI staining. Annexin V and propidium iodide (PI) staining was used to detect apoptotic cells. Cells were treated with different concentrations of staurosporine for 1 h, trypsinized with trypsin-EDTA from the bottom of 24-well plates, washed twice in ice-cold PBS, and resuspended in binding buffer (10 mM HEPES–NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) at a concentration of 10^6 cells/ml. One hundred microliters of cells was transferred to a FACScan tube to which were added 10 μl of FITC-conjugated annexin V solution (10 μg/ml) (IQ Corp., Groningen, The Netherlands) and 10 μl of PI (50 μg/ml). Annexin V is a phosphatidylserine binding protein that detects phosphatidylserine on the surface of cells undergoing apoptosis, whereas PI associates with nuclear DNA, indicating necrotic cell death. Cells were vortexed gently and then incubated at room temperature for 15 min in the dark. Then, 400 μl of binding buffer was added to the cells, and they were analyzed by flow cytometry within 1 h of staining. The data from 10,000 cells were collected and analyzed using LYSIS II software (Becton Dickinson). The signals for green (FL1; annexin V) and orange fluorescence (FL2; PI) were measured by logarithmic amplification.

RESULTS

Role of Gal/GalNAc-specific lectin carbohydrate interaction in mediating attachment of *C. hominis* and *C. parvum* to HCT-8 cells and primary human and bovine intestinal cells. In a recent study (17) we have shown that restriction of *C. hominis* to human hosts reflects species-specific tissue tropism of this species. To investigate the basis for this tropism, the role of a Gal/GalNAc-specific lectin-carbohydrate interaction in mediating attachment to host cells was examined. Pretreatment of sporozoites with both BSM and Gal/GalNAc itself reduced invasion of primary bovine cells and HCT-8 cells by *C. parvum* but did not affect entry into primary human cells. In addition, pretreatment of *C. hominis* sporozoites with both BSM and Gal/GalNAc did not affect entry into either HCT-8 cells or into primary human cells (Fig. 1). These data suggest that *C. parvum* enters HCT-8 cells and bovine cells using a Gal/GalNAc-specific lectin-carbohydrate interaction. However, entry of *C. parvum* and *C. hominis* into primary human cells is Gal/GalNAc independent. In addition, *C. hominis* enters HCT-8 cells via a Gal/GalNAc-independent mechanism.

Role of cytoskeletal components during infection of primary intestinal cells with *C. parvum* and *C. hominis*. Previous studies

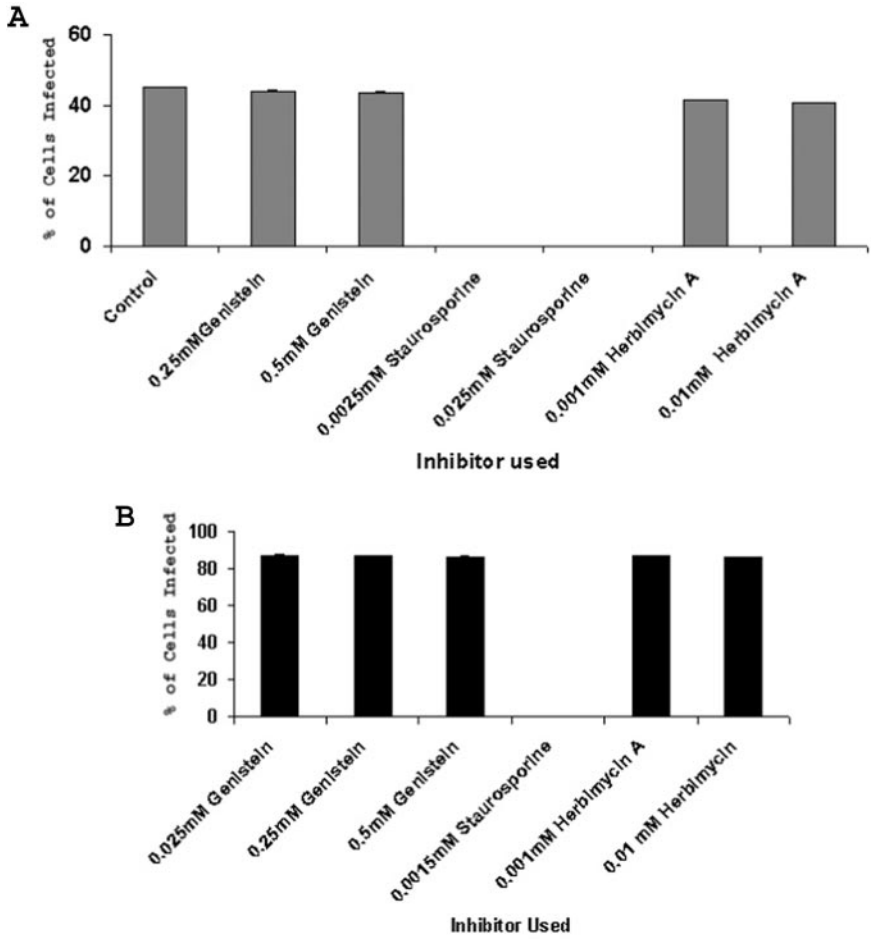

FIG. 4. The effect of tyrosine phosphorylation inhibitors on invasion of HCT-8 cells by *C. hominis* (A) and *C. parvum* (B).

using cultured cell lines have demonstrated that host actin polymerization occurs at the host-parasite interface during *C. parvum* (12) and *C. hominis* (17) invasion and that inhibition of polymerization inhibits parasite invasion. To investigate whether host or parasite actin are involved during entry of *C. hominis* and *C. parvum* into primary human and bovine intestinal epithelial cells, cells or parasites were treated with actin inhibitors prior to infection. Pretreatment of host cells with cytochalasin D and cytochalasin B significantly reduced invasion of *C. parvum* into primary human and bovine cells. The inhibitory effect of cytochalasin D and cytochalasin B on *C. parvum* invasion into primary human cells and bovine cells was more pronounced when both inhibitors were left throughout the infection period (Fig. 2). Similarly, pretreatment of host cells with cytochalasin D and cytochalasin B significantly reduced invasion of *C. hominis* into primary human cells (Fig. 2). Treatment of cells with cytochalasins did not affect cell viability or induce any gross morphological changes in the cells. However, pretreatment of oocysts of both species with cytochalasin D or cytochalasin B for 15 min prior to infection did not affect invasion (results not shown). Double staining of *C. parvum*- or *C. hominis*-infected primary human cells with phalloidin conjugated to FITC and lectin VVL demonstrated host actin accumulation in association with internalized sporozoites (Fig. 3).

Signal transduction pathways involved during infection of HCT-8 cells by *C. parvum* and *C. hominis*. To assess the signal transduction pathways involved during infection of HCT-8 cells by *C. parvum* and *C. hominis*, cells were treated with a variety of protein kinase inhibitors for 1 h prior to infection. Exposure of cells to staurosporine (a general protein kinase inhibitor that inhibits tyrosine kinase, protein kinase C [PKC] and serine/threonine kinases) completely abolished entry of *C. parvum* and *C. hominis* into HCT-8 cells (Fig. 4). Two PKC inhibitors, calphostin C and Gö6976, had an inhibitory effect on invasion of *C. hominis*. In addition the PKC inhibitors chlerythrine chloride and Gö6976 and the serine/threonine kinase inhibitor KN-93 had inhibitory effects on *C. parvum* invasion of cells (Fig. 5). However, it was noteworthy that none of these inhibitors reduced *C. parvum* or *C. hominis* invasion of HCT-8 cells to the levels seen following staurosporine treatment. Moreover, combining PKC inhibitors with genistein or with serine/threonine kinase inhibitors could not reproduce the complete inhibition seen with staurosporine (Tables 2 and 3). These data suggested that staurosporine was affecting invasion by an additional or alternative mechanism. As *C. parvum* has been shown not to invade apoptotic cells (8, 29) and staurosporine is a known apoptosis-inducing agent (10), we examined if it could induce apoptosis in HCT-8 cells. The number of

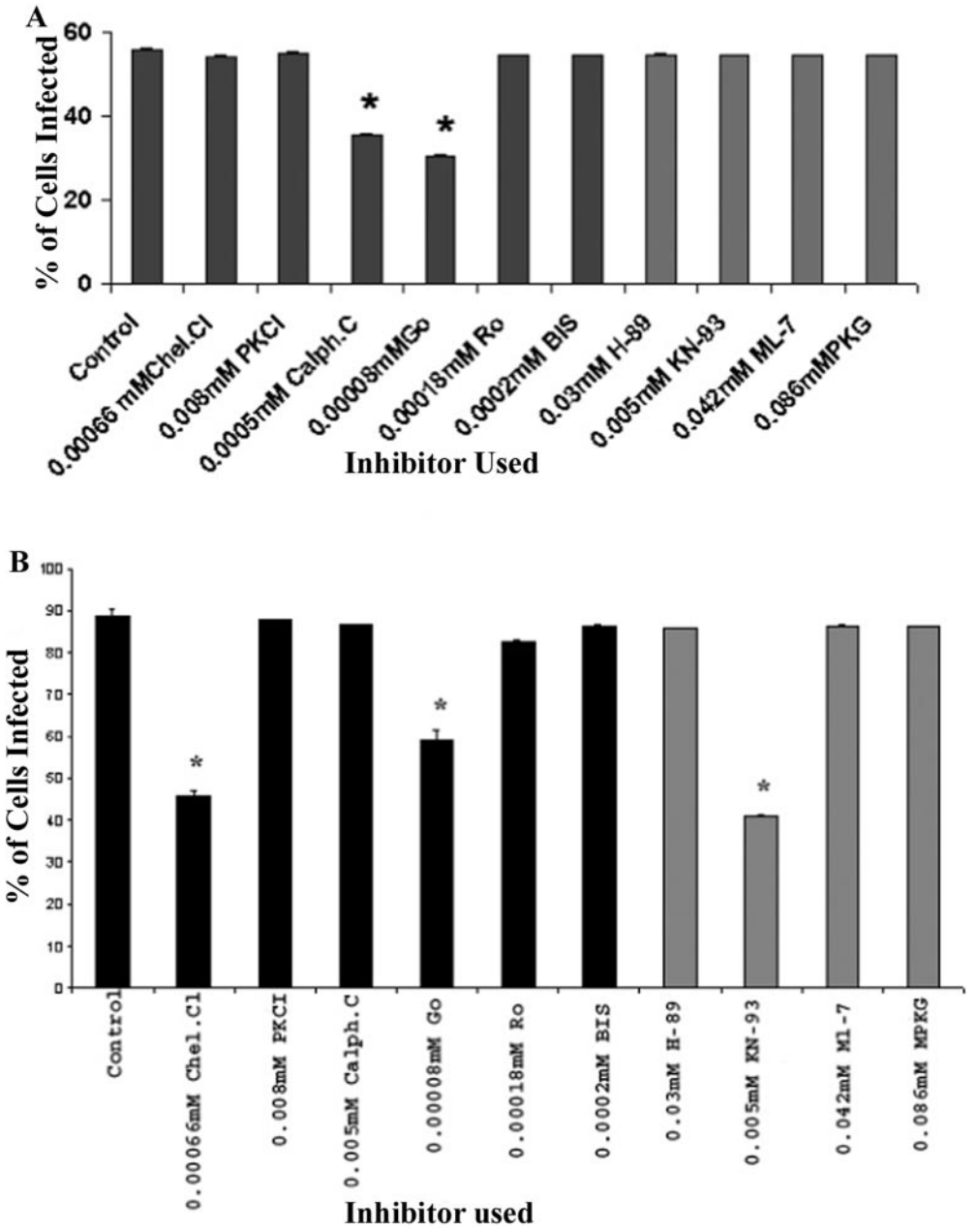

FIG. 5. Effect of individual PKC and serine/threonine kinase inhibitors on invasion of HCT-8 cells by *C. hominis* (A) and *C. parvum* (B). *, $P < 0.005$ compared to control. Chel.Cl, chelerythrine chloride; Calph. C., calphostin C; Gö, Gö6976; Ro, Ro-32-0432; Bis, bisindolylmaleimide I; PKCI, PKC inhibitor.

apoptotic cells increased from 19.5% for control (untreated cells) to a maximum of 67.4% as the concentration of staurosporine was increased to 2.5 μM (Fig. 6).

Signal transduction pathways involved during infection of primary intestinal cells by *C. parvum* and *C. hominis*. Primary cells were more resistant to the effect of staurosporine than HCT-8 cells. A concentration of 5 μM was required to completely abolish invasion of all primary cells by either *C. parvum* or *C. hominis*. Staurosporine at a concentration of 2.5 μM reduced *C. parvum* infection of primary human cells from 97.3% ± 0.2% to 60.4% ± 0.4% and of bovine cells from 93.6% ± 0.2% to 51.3% ± 0.1%, while *C. hominis* entry into primary human cells was reduced from 95.2% ± 0.2% to 60.0% ± 0.2%. This inhibitory effect was mimicked by the PKCα inhibitor Gö6976 that reduced *C. parvum* infection of human cells from 97.4% ± 0.2% to 49.6% ± 1.6% and of bovine cells from 92.7% ± 0.7% to 48.3% ± 1.5%, while *C. hominis* infection of human cells was reduced from 95.2% ± 0.2% for control cells to 60.0% ± 0.2% (Fig. 7). None of the other tyrosine phosphorylation inhibitors, PKC inhibitors, serine/threonine kinase inhibitors, wortmannin, or suramin sodium salt had any effect on entry of *C. parvum* into primary

TABLE 2. Effect of different combinations of genistein and PKC or serine/threonine kinase inhibitors on invasion of HCT-8 cells by *C. parvum*

Inhibitor used	Effect with genistein (500 μM)
PKC inhibitor	No effect
Gö6976	Reduced invasion from 91.4% ± 0.5% to 59.9% ± 2.6%
Bisindolylmaleimide	No effect
Chelerythrine chloride	Reduced invasion from 91.4% ± 0.5% to 30.3% ± 0.4%
Calphostin C	No effect
Ro-32-0432	No effect
KN-93	Reduced invasion from 91.4% ± 0.5% to 40.6% ± 1.9%
ML-7	No effect
PKGI	No effect
H-89 dihydrochloride	No effect

TABLE 3. Effect of different combinations of PKC and serine/threonine kinase inhibitors on invasion of HCT-8 cells by *C. parvum*

Inhibitors used	Effect on invasion
Gö6976 and KN-93	Reduced invasion from 91.4% ± 0.5% to 20.4% ± 0.4%
Calphostin C and KN-93	Reduced invasion from 91.4% ± 0.5% to 22.6% ± 0.2%
Gö6976 and ML-7	Reduced invasion from 91.4% ± 0.5% to 44.8% ± 0.7%
Gö6976 and H-89	Reduced invasion from 91.4% ± 0.5% to 43.9% ± 0.9%
KN-93 and bisindolylmaleimide	Reduced invasion from 91.4% ± 0.5% to 21.5% ± 0.6%
Bisindolylmaleimide and ML-7	No effect
PKC inhibitor and H-89 dihydrochloride	No effect
PKGI and calphostin C	No effect
Ro-32-0432 and H-89 dihydrochloride	No effect
Bisindolylmaleimide and PKGI	No effect
Ro-32-0432 and ML-7	No effect

human or bovine cells or on entry of *C. hominis* into primary human cells (data not shown).

DISCUSSION

In this study a primary cell model was used to study the interactions of *C. parvum* and *C. hominis* with host primary intestinal epithelial cells. Primary intestinal cells have a number of advantages over immortalized tissue culture cell lines for the study of host-parasite interactions in vitro. They may more accurately reflect in vivo conditions than immortalized cells. In particular, primary cells allow direct and meaningful examination ex vivo of species tropism and the importance of specific-receptor ligand interaction, as they are likely to occur in vivo.

In order to investigate the basis for species tropism, the mechanisms by which *C. parvum* and *C. hominis* enter human and bovine cells were examined. It has been shown previously that Gal/GalNAc-specific lectin-carbohydrate interactions play a role in mediating attachment of *C. parvum* to intestinal and biliary epithelial cells (7). Therefore, we wanted to investigate whether Gal/GalNAc and BSM could be used to block the ligand on the parasite surface prior to infection of primary human and bovine intestinal cells. The results obtained showed that *C. parvum* entry into primary bovine and HCT-8 cells is dependent on the Gal/GalNAc-specific lectin-carbohydrate interaction. However, neither *C. parvum* nor *C. hominis* entry into human cells involved such an interaction. In addition, *C. hominis* entry into HCT-8 cells was Gal/GalNAc independent. These data clearly underline the differences between the invasion mechanisms used by the two species of parasite and the differences between the different cell types. These results also provide an explanation for the findings of our earlier study (17), where we showed that *C. hominis* did not invade primary bovine cells and that there was a different pattern of infection when HCT-8 cells were used (infection localized to certain cells) compared to when primary human cells were used (infection dispersed evenly throughout the monolayer). These results suggest that *C. hominis* may be using a pathway distinct from that of *C. parvum* for infection. Alternatively, both *C. parvum* and *C. hominis* may have evolved a specialized mechanism of host-parasite interaction specific for infecting human intestinal cells. Comparison of the genomes of *C. parvum* and *C. hominis* showed that the two genomes are very similar, exhibiting only 3 to 5% sequence divergence, with no large insertions, deletions, or rearrangements evident (32). Extensive arrays of potentially variant surface proteins were not observed in either the *C. hominis* or the *C. parvum* genome (1, 32). The identification of the surface molecules which interact with human cells and/or the receptor(s) on human cells for *C. parvum* and *C. hominis* could potentially be a significant development in the search for therapeutics to specifically treat infections.

Studies have shown that *C. parvum* induces host cell actin rearrangement upon invasion of different cell lines and that this process is necessary for invasion to occur (6, 9, 12, 13).We have previously shown that this also occurs when *C. hominis* enters HCT-8 cells (17). Our demonstration of the colocaliza-

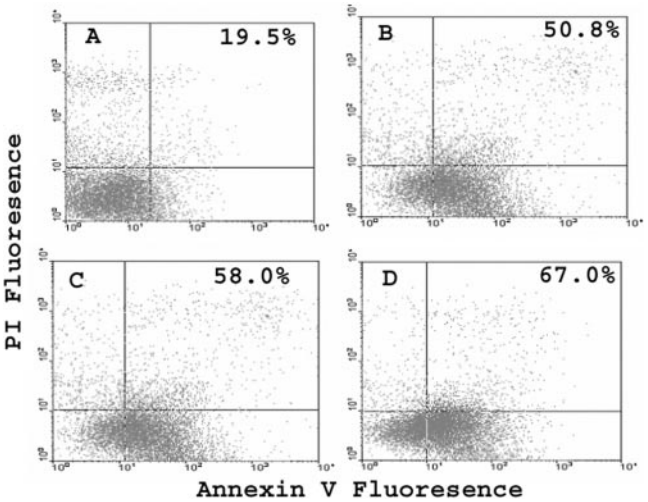

FIG. 6. Apoptotic effect of staurosporine on HCT-8 cells. Cellular apoptosis increased from 19.5% for nontreated cells (A) to 50.8% for 1.5 μM (B), 58.0% for 2.0 μM (C), and 67.0% for 2.5 μM (D) staurosporine-treated cells.

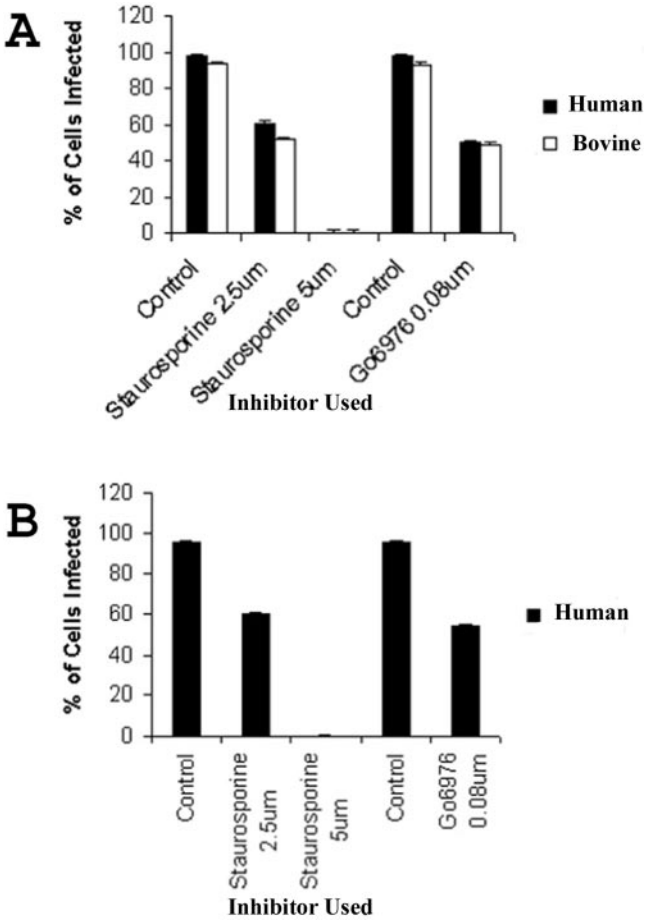

FIG. 7. The effect of staurosporine and PKCα inhibitor Gö6976 on *C. parvum* entry into primary human and primary bovine intestinal cells (A) and on *C. hominis* entry into primary human cells (B).

tion of *C. parvum* and *C. hominis* with host actin using primary human intestinal cells corroborates the importance of this cellular event previously observed in immortalized cells during infection with *Cryptosporidium*. Inhibition of entry of both species to primary cells by the cytoskeletal inhibitors cytochalasin B and cytochalasin D underlines the importance of actin rearrangement for successful invasion of the host cell.

A previous study has shown that treatment of primary bovine fallopian tube epithelial cells with genistein, staurosporine, suramin sodium salt, or wortmannin inhibited entry of *C. parvum* sporozoites (14). *C. parvum* sporozoites were shown to induce tyrosine phosphorylation of cortactin in a human bile duct epithelial cell line, and inhibition of c-Src, a host protein tyrosine kinase inhibitor, inhibited invasion (5). In contrast, Elliot and Clark infected HCT-8 cells with *C. parvum* oocysts and were unable to demonstrate tyrosine phosphorylation at the site of developing trophozoites and merozoites (12). We found that only staurosporine had an effect on entry of *C. parvum* or *C. hominis* oocysts into HCT-8 cells and into primary bovine or human intestinal epithelial cells. The effect of staurosporine was shown to be mediated in primary cells through inhibition of a PKC signaling pathway. However, the effect of staurosporine on HCT-8 cells could only be partly mimicked by a PKC inhibitor. A plausible mechanism to explain the potency of staurosporine compared with other PKC inhibitors is the induction of apoptosis (10). However, whether apoptosis or some other mechanism underlies these different effects remains to be proven. These results highlight the importance of the type of host cell used to study the invasion mechanisms of *Cryptosporidium* species and suggest that some of the conflicting data in the literature about the role of different signaling pathways in the invasion process may be due to the use of a variety of host cells for infection studies. The use of sporozoites in some assays and oocysts in others to initiate infections could possibly also explain some conflicting results. Some inhibitors may be more potent when sporozoites are used as they can adhere to and invade host cells faster than oocysts, which need to excyst prior to infection. In the time that it takes the oocysts to excyst, it is possible that the host cells may recover from the effect of the inhibitor.

Our results clearly point toward a role for the classical Ca^{2+}-dependent PKC isoenzyme PKCα and/or PKCβ in the invasion process by *C. parvum* of primary human and primary bovine intestinal cells and *C. hominis* entry into primary human intestinal cells. PKC is a ubiquitous phospholipid-dependent serine/threonine kinase involved in major signaling events that regulate a wide variety of biological responses to stimuli (11). It has been shown that the discharge of *C. parvum* sporozoite apical organelle contents is dependent on both intracellular Ca^{2+} and the cytoskeleton and is required for host cell invasion (9). We have also found that chelation of intracellular Ca^{2+} inhibits entry of *C. parvum* oocysts into HCT-8 cells (unpublished observation). Interestingly, PKCα has been shown to play an important role in the invasion of human brain microvascular endothelial cells by *Escherichia coli*. Like *Cryptosporidium*, *E. coli* induces host actin condensation at the site of infection when it invades host cells. Immunocytochemical studies indicated that activated PKCα is associated with actin condensation beneath the bacterial entry site (25). Further studies are required to investigate whether a similar situation occurs upon invasion of host cells by *Cryptosporidium* species. The PKCα inhibitor used in this study, Gö6976, has been shown to promote the formation of tight junctions in urinary bladder carcinoma cells. Specifically, Gö6976 was shown to induce the formation of adherens and desmosomal cell-cell junctions (20). Disruption of tight junctions is known to contribute to intestinal disorders caused by enteric pathogens (4). Infection of bovine and human epithelial cell lines with *Cryptosporidium andersoni* disrupted tight junctional zonula occludens 1 (3). PKCα expression has been correlated with tight junctional leakiness in renal epithelial cells (23). It is plausible that the induction of tight junctions in host cells and inhibition of disruption of those junctions is a mechanism that could prevent entry of *C. parvum* and *C. hominis*.

This study demonstrates the importance of the model system used to study *Cryptosporidium* invasion of host cells and suggests that the use of primary cells, although technically more difficult to work with, may provide a more biologically relevant model system than conventional cell lines. There is a need now to identify the potential receptor-ligand interactions that promote *C. hominis* invasion of human intestinal epithelial cells. The identification of such receptors and further characterization of the signaling pathways used by *C. parvum* and *C. homi-*

nis to enter primary human cells could lead to the development of new therapeutic agents for the treatment of infection.

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