Host Cell Tropism Underlies Species Restriction of Human and Bovine Cryptosporidium parvum Genotypes

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Received 31 March 2004/Returned for modification 22 May 2004/Accepted 15 July 2004

It has been recognized recently that human cryptosporidiosis is usually caused by Cryptosporidium parvum genotype I (“human” C. parvum), which is not found in animals. Compared to C. parvum genotype II, little is known of the biology of infection of the human-restricted C. parvum genotype I. The aims of the present study were (i) to explore and compare with genotype II the pathogenesis of C. parvum genotype I infection by using an established in vitro model of infection and (ii) to examine the possibility that host-specific cell tropism determines species restriction among C. parvum genotypes by using a novel ex vivo small intestinal primary cell model of infection. Oocysts of C. parvum genotypes I and II were used to infect HCT-8 cells and primary intestinal epithelial cells in vitro. Primary cells were harvested from human endoscopic small-bowel biopsies and from bovine duodenum postmortem. C. parvum genotype I infected HCT-8 cells with lower efficiency than C. parvum genotype II. Actin colocalization at the host parasite interface and reduction in levels of invasion after treatment with microfilament inhibitors (cytochalasin B and cytochalasin D) were observed for both genotypes. C. parvum genotype II invaded primary intestinal epithelial cells, regardless of the species of origin. In contrast, C. parvum genotype I invaded only human small-bowel cells. The pathogenesis of C. parvum genotype I differs from C. parvum genotype II. C. parvum genotype I does not enter primary bovine intestinal cells, suggesting that the species restriction of this genotype is due to host tissue tropism of the infecting isolate.

Cryptosporidium parvum, an intracellular parasite belonging to the phylum Apicomplexa, is an important cause of diarrheal disease worldwide (10, 13, 17). Although many C. parvum infections are self-limiting, severe manifestations may occur in immunologically incompetent and debilitated individuals (10, 16, 17). In the immunodeficient host, in particular those with AIDS, infection may be life-threatening, and the development of effective antimicrobial therapy for human cryptosporidiosis is only beginning to evolve (35, 44, 45).

To develop novel, effective treatments against cryptosporidiosis, a detailed understanding of the pathogenesis of the causative organisms is necessary. Traditionally, cryptosporidiosis in humans has been viewed as a zoonotic infection, with cattle being the principal environmental reservoir. However, recently it has emerged that humans are commonly infected with a C. parvum genotype (genotype I) only very rarely found in other species (45). Although C. parvum genotype I is restricted to humans, C. parvum genotype II can infect humans, cattle, and a wide variety of other animals (2, 33, 37, 40). A compelling case now exists for the redesignation of this subtype as a novel cryptosporidial species (Cryptosporidium hominis sp. nov.) based on molecular differences at several genetic loci and in terms of oocyst shedding, disease severity, and site of infection (28, 34). For clarity, we continue to use here the terminology “genotype I” and “genotype II.”

In most human epidemiological studies C. parvum genotype I isolates are identified in human stool samples more frequently than genotype II (21, 24, 25, 43, 47). However, understanding of Cryptosporidium pathogenesis to date has relied on studies with C. parvum genotype II isolates or isolates of unknown genotype. The emergence of a widely prevalent non-zoonotic C. parvum genotype has stimulated a major reassessment of our understanding of this disease in humans. In particular, the possibility that the mechanisms of transmission and pathogenesis of this organism differ from those associated with C. parvum genotype II has major implications for strategies aimed at controlling and treating human cryptosporidiosis. We have used here a combination of established in vitro tissue culture models and an ex vivo primary intestinal cell model of cryptosporidial infection to show that C. parvum genotype I differs in the pattern and severity of infection compared to C. parvum genotype II isolates. In addition, we show that bovine primary cells cannot be infected with C. parvum genotype I isolates, indicating that host cell tropism underlies the species restriction of this C. parvum subtype in vivo.

MATERIALS AND METHODS

C. parvum oocysts. C. parvum genotype II oocysts (Iowa strain) were obtained from a commercial source (Pleasant Hill Farms, Troy, Idaho). This genotype II
strain was originally isolated from a calf by Harley Moon. It has been passaged in the Maintenance Medium (Dako) and examined using a laser scanning confocal microscope (Bio-Rad MRC 1024).

Effect of cytoskeletal inhibitors on C. parvum invasion of gastrointestinal cells. To investigate the importance of cytoskeletal components during invasion, the actin-disrupting drugs cytochalasin D and cytochalasin B (Sigma) and microtubule depolymerizing drugs colchicine and vincristine (Sigma) were used. Stock solutions of the four drugs were prepared and diluted to their final concentration by using culture medium. Three separate experiments were performed. In the first set of experiments, HCT-8 cells and primary cells were incubated for 1 h in an assay medium containing either 1 μg of cytochalasin D/ml, 10 μg of cytochalasin B/ml, or a range of concentrations of colchicine and vincristine (48 h) for 1 h at 37°C. The treated monolayers were washed twice with PBS and infected with 2 × 10⁵ Cryptosporidium oocysts/ml for either 48 h (HCT-8 cells) or 22 h (primary cells). In a second set of experiments cell monolayers were infected as described above but without removing the cytoskeletal inhibitor during the infection assay. Finally, in order to assess the effect of cytoskeletal inhibition on the parasite, oocysts were incubated with a range of concentrations of either colchicine, vincristine, cytochalasin D, or cytochalasin B for 15 min at 37°C. Oocysts were then washed twice with PBS before being used to infect monolayers. Untreated monolayers or oocysts were used as a control.

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

RESULTS

Invasion of HCT-8 cells by Cryptosporidium isolates. The patterns and degrees of invasiveness of C. parvum genotypes I and II were compared. Both genotypes invaded HCT-8 cells. However, genotype I entered HCT-8 cells with significantly lower efficiency than did genotype II. Specifically, 55.8% ± 1.01% of cells were infected by genotype I, and 88.9% ± 1.54% of cells were infected by genotype II (P = 0.002). The invasion efficiency of HCT-8 cells by both isolates of genotype I was similar. The pattern of infection differed according to the or-
igin of the isolates. Whereas *C. parvum* genotype II infected HCT-8 cells evenly (Fig. 1B), internalized genotype I parasites formed discreet clusters throughout the cell monolayers (Fig. 1A).

**Host cell actin cytoskeletal rearrangement is similar after invasion of HCT-8 cells by both genotype I and genotype II Cryptosporidium isolates.** The effects on the host cell actin cytoskeleton after invasion with both genotypes were compared by using confocal scanning microscopy to visualize colocalization of sporozoites with actin. Actin accumulation occurred in association with internalized sporozoites of both types (Fig. 2).

In addition, disruption of the host actin cytoskeleton with cytochalasin B or cytochalasin D significantly inhibited entry of both genotypes. Pretreatment of HCT-8 cells for 1 h with either microfilament inhibitor substantially reduced infection (Fig. 3A). The reason for the different efficiencies of the two inhibitors is unclear. However, it may reflect differences in the permeability of the oocyst wall to the drugs since invasion of the cells in the presence of either inhibitor markedly reduced infection regardless of the genotype or inhibitor type (Fig. 3B). Preexposure of oocysts of both genotypes to cytochalasin D and cytochalasin B did not affect subsequent invasiveness.

**Pretreatment of oocysts with microtubule inhibitors attenuates cattle but not human genotype invasion of HCT-8 cells.** In order to determine whether *C. parvum* genotype I also requires an intact microtubule cytoskeleton for efficient infection of HCT-8 cells, we examined the effect of the microtubule-depolymerizing agents, colchicine and vincristine, on infection. As described in previous studies (7), inhibition of host cell microtubules did not affect *C. parvum* invasion (data not shown). However, pretreatment of genotype II oocysts with either microtubule inhibitor for 1 h reduced infection of HCT-8 cells in a dose-dependent manner (Fig. 4A and B). Infection in the presence of either $10^{-3}$ M colchicine or $10^{-4}$ M vincristine throughout the infection period reduced the in-

**FIG. 1.** Comparison of the pattern of infection of *C. parvum* genotypes I and II in HCT-8 cells. Genotype I *C. parvum* invasion occurred in clusters (A), whereas genotype II *C. parvum* invasion was evenly distributed throughout the monolayer (B). Slides were examined by using a fluorescence microscope. Magnification, ×400.

**FIG. 2.** Confocal laser scanning micrographs of host-actin colocalization during invasion of genotype I (A) and genotype II (B) into HCT-8 cells. The green color shows the stained actin, and the red color indicates the intracellular stages of *C. parvum*. Colocalization of host actin and the parasite appears yellow (arrows). Slides were examined by using a laser scanning confocal microscope. Magnification, ×400.
Infection of genotype II oocysts from 88.4% ± 1.59% to 30.7% ± 1.14% and 7.7% ± 2.5%, respectively (Fig. 4C). In contrast, neither vincristine nor colchicine treatment of *C. parvum* genotype I oocysts affected the rate of infection of HCT-8 cells (Fig. 4A and B). These data suggest that either the mechanism of host cell entry utilized by *C. parvum* genotype I sporozoites is independent of the parasite’s microtubule structures or genotype I oocysts are impermeable to microtubule-depolymerizing drugs used.

**Culture of primary human cells and invasion by *C. parvum* genotypes I and II.** Isolated cells and crypts attached to the coverslips and spread within 24 to 48 h. The 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 labeling with cytokeratin.

*C. parvum* genotype II entered primary human cells with an efficiency comparable to the invasion of HCT-8 cells. However, *C. parvum* genotype I infected primary human intestinal cells significantly more efficiently than was the case for HCT-8 cells. In addition, the clustered pattern of infection observed for *C. parvum* genotype I entry into HCT-8 cells was not evident in primary cells, with infection being equally distributed throughout the cell monolayer (Fig. 5).

*C. parvum* genotype II but not genotype I infects primary bovine intestinal cells. In order to explore the possibility that the restriction of *C. parvum* genotype I to human hosts reflects species-specific tissue tropism for this genotype, we isolated and infected in vitro bovine intestinal cells. The viability of isolated bovine intestinal cells was monitored by the trypan blue exclusion assay. Microscopically, after 24 to 48 h, at which time they were used for infection studies, these cells appeared healthy and viable. *C. parvum* genotype II infected bovine cells with an efficiency comparable to infection of primary human cells and HCT-8 cells (92.3% ± 1.34%). However, genotype I (*C. parvum* 5942) organisms did not infect primary bovine cells. No parasites were seen in association with the bovine cells either adherent (after 1 h without washing) or after a 22-h invasion assay (Fig. 6). In order to corroborate this finding, we used a second *C. parvum* isolate. As observed with isolate 5942, TU502 efficiently invaded primary human cells but did not infect primary bovine intestinal cells (data not shown).

**DISCUSSION**

Studies of the pathogenesis of *C. parvum* in vitro to date have relied on the use of *C. parvum* genotype II isolates or human-derived isolates of unknown genotype (5–8, 12, 23).
Given that *C. parvum* genotype I is the predominant genotype in the majority of studies of sporadic and outbreak cases of human cryptosporidiosis (15, 16, 24, 25), it is essential to characterize and compare its pathogenesis with that of *C. parvum* genotype II.

In the present study a combination of established in vitro tissue culture and an ex vivo primary intestinal cell model of *Cryptosporidium* infection was used to compare infection by *C. parvum* genotype I and genotype II. The data presented here indicate that *C. parvum* genotype I differs from genotype II with regard to the efficiency and pattern of infection and susceptibility to microtubule depolymerizing drugs. In addition, we have shown that *C. parvum* genotype I isolates are unable to infect bovine intestinal cells ex vivo, indicating that host cell tropism underlies species restriction among human and bovine genotypes of *C. parvum*.

Primary intestinal cells have a number of advantages over immortalized tissue culture cell lines for the study of host-parasite interactions in vitro. As a biologically meaningful surrogate model of direct challenge studies in vivo, primary cells have provided important novel insights into other gastrointestinal pathogens, including *Campylobacter* and *Helicobacter* spp. (9, 26). Comparison of *Cryptosporidium* infection of primary and conventional cell lines in the present study demonstrates the potential usefulness of primary cells for the study of *C. parvum* infection. For example, our results indicate that the apparent efficiency of *C. parvum* infection in vitro may be critically dependent on the infection model used. A previous study suggested that genotype I appeared to be more aggressive in its growth in HCT-8 cells than genotype II (19). However, in the present study, entry of a *C. parvum* genotype I isolate into human-derived HCT-8 cells was less efficient than

![FIG. 5. Lectin VVL staining of primary intestinal human epithelial cells infected with *C. parvum* genotype I (A) and genotype II (B). Epithelial cells were infected with $2 \times 10^5$ oocysts/ml of genotype I and genotype II. *C. parvum* and stained with lectin VVL. Background staining by the lectin VVL also revealed cell morphology. The efficiency and pattern of infection was similar for both *C. parvum* genotypes (96.2% ± 0.36% for genotype I and 97.4% ± 0.1% for genotype II). Slides were examined by using a fluorescence microscope. Magnification, ×400.](image1)

![FIG. 6. Infection of primary bovine intestinal cells with genotype I *C. parvum* isolate 5942 (A) and genotype II *C. parvum* (B). Whereas genotype II *C. parvum* efficiently invaded bovine cells, genotype I did not. Slides were examined by using a fluorescence microscope. Magnification, ×400.](image2)
that of a genotype II isolate, and the genotype I isolate showed a distinct, focal pattern of infection in the monolayer. The reason for this difference in pattern of infection of HCT-8 cells by genotype I and genotype II C. parvum is uncertain, but it may reflect different adhesion-receptor interactions subverting invasion by the two genotypes of these immortalized cells. On the other hand, in a primary human cell model the two isolates were indistinguishable in terms of the pattern and efficiency of infection. More importantly, our primary cell model clearly shows that bovine cells are resistant to infection by C. parvum genotype I.

In order to avoid the confounding effect of variation in isolate pathogenicity (30, 31, 42) on the interpretation of our results, we used two geographically distinct isolates of C. parvum genotype I for studies on primary cells. Neither isolate could enter primary intestinal bovine cells. Both entered primary human small bowel cells with similar efficiency. That the observed tropism was not simply a result of host cell adaptation is underlined by the fact that genotype I isolate, TU502, was extensively passed through gnotobiotic neonatal pigs (a species successfully infected with C. parvum genotype I experimentally) before use.

The results of previous studies of the effects of microtubule inhibitors on Cryptosporidium infectivity have been contradictory (7, 48). Our data support existing reports indicating that the host microtubule cytoskeleton is not involved in C. parvum infection (7). In addition, our results are in agreement with those of Wiest et al. (48), who showed that pretreatment of C. parvum oocysts with colchicine and vincristine attenuated infection in a dose-dependent manner. However, in the present study the infectivity in vitro of a C. parvum genotype I isolate was not affected by preincubation with colchicine or vincristine. It is possible that genotype I does not require its microtubule cytoskeleton for cell invasion. However, an alternative explanation could be a variation in oocyst permeability of the two C. parvum genotypes. Whether isolates of C. parvum differ in their requirement for microtubule function during the infection process or vary in their sensitivity to the effects of inhibiting agents requires further study. However, these data suggest that the parasite’s microtubule structure is unlikely to be an attractive target for developing novel human anticytosporidial therapies.

It is possible that C. parvum genotype II uses a pathway distinct from that of C. parvum genotype I for infection of both human and bovine intestinal cells. Alternatively, C. parvum genotype II may have evolved a specialized mechanism of host-parasite interaction specific for infecting bovine intestinal cells that is not shared by C. parvum genotype I. Other apicomplexan invasion mechanisms have been shown to have a high degree of redundancy (29, 32). It is possible that genotype I has become specifically adapted for human cells and therefore has less redundancy in invasion pathways than genotype II. Our studies with microfilament inhibition and confocal microscopy showed that, like C. parvum genotype II (6, 7), the human genotype requires the participation of the host cell actin cytoskeleton for entry into cells. This, together with the finding that C. parvum genotype I did not attach to bovine intestinal cells, strongly suggests that the species tropism we observed occurred at the level of receptor-ligand interaction during the initial stages of infection. Exploration of potential sporozoite lectin-carbohydrate interactions (7) that subserve these distinct attachment specificities will be an important area for future investigation.

C. parvum genotype I isolates have only rarely been described in nonhuman primates, sheep, and dugongs (14, 27, 39, 46). Under experimental conditions genotype I isolates can infect gnotobiotic piglets, and mixed (genotype I and II) infections have been described following propagation studies in calves (41). However, from an epidemiological perspective this subtype of C. parvum is considered to infect humans almost exclusively (45). Under both clinical and experimental conditions C. parvum genotype I and II isolates maintain a separate reproductive cycle, indicating a lack of genetic recombination between them (45). Based on these and other molecular genetic and biological data (28), there now exists compelling evidence that C. parvum genotype I is a separate species from C. parvum genotype II. Our results showing species-restricted host cell tropism adds further support to the proposal that C. parvum genotype I should be redesignated as a novel species, Cryptosporidium hominis (28, 45).

In conclusion, the data presented here show that the pathogenesis of C. parvum genotype I differs from that of genotype II. C. parvum genotype I was less infective in HCT-8 cells, and its infectivity was not affected by the addition of microtubule depolymerizing drugs. Furthermore, C. parvum genotype I was not able to infect primary bovine intestinal cells, indicating that the host species restriction of this genotype reflects tissue tropism in vivo. It will be important now to investigate and compare with C. parvum genotype II the relative effects of C. parvum genotype I on signal transduction events (6, 7, 11, 12), cell death (5, 8), and the inflammatory response (20) in existing immortalized cell models and primary intestinal cells.

ACKNOWLEDGMENTS

This study was supported by the Irish Health Research Board and the Children’s Research and Medical Foundation.

We thank Kristin Elwin and Anne Thomas for maintenance and genotyping of the National Collection of Oocysts at the Cryptosporidium Reference Laboratory. We are grateful to Saul Tzipori, Department of Geographical Medicine, Tufts University, for providing TU502 C. parvum isolates.

REFERENCES


