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Effects of host temperature, and gastric and duodenal environments on microsporidia spore germination and infectivity of intestinal epithelial cells.

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Abstract

Approximately 14 of the more than 1,000 species of microsporidia infect humans, only 2 of which, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, cause intestinal microsporidiosis. Clinical isolates of three microsporidia species, *E. intestinalis*, *Encephalitozoon hellem*, and the insect parasite, *Anncaliia* (*Brachiola*, *Nosema*) *algerae* were used in a spore germination assay, and enterocyte attachment and infection assays to model the potential roles of gastric and duodenal environments, and host temperature in determining why only one of these microsporidia species causes intestinal microsporidiosis. Enterocyte infection with *A. algerae* spores was 10% that of the *Encephalitozoon* species, a difference not attributable to differences in spore attachment to host cells. Prior spore treatment with pepsin in HCl, pancreatic enzymes or ox bile did not inhibit germination or enterocyte infection by the three microsporidia species. While the *Encephalitozoon* species differentiated to mature spores within 3 days, the time taken for many enterocytes to turn over, *A. algerae* took 3 - 5 days to produce mature spores, near the upper limit for enterocyte turnover in vivo. Thus host temperature may contribute to *A. algerae* not causing human intestinal microsporidiosis, but none of the factors tested account for the inability of *E. hellem* to cause such an infection.

INTRODUCTION

Approximately 14 species of microsporidia infect humans (Didier, 2005). Microsporidia spores from several of these species are ubiquitous in the environment and have been found in potential drinking water and food sources (Cotte et al, 1999; Dowd et al, 1998; Graczyk et al, 2004; Kwakye-Nuako et al, 2007; Graczyk et al, 2008). However, only two species, *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, appear to infect the gastrointestinal tract causing significant diarrhea and wasting, particularly in AIDS patients (Kotler and Orenstein, 1998). Franzen and colleagues (2005) found that spores from several species could infect human intestinal epithelial cells in culture, although only one of the tested species causes intestinal disease, and they concluded that the route of administration was a major factor in determining host cell organ specificity.

There are many examples of spores of various microsporidia species readily infecting human and other mammalian cell lines (e.g. Visvesvara et al, 1999; Mathis et al 2005). The present study used three microsporidia species isolated from human infections. The species used were the second most common cause of intestinal disease, *E. intestinalis* (Kotler and Orenstein, 1998), *Encephalitozoon hellem*, a cause of ocular and disseminated infections, not generally considered a cause of intestinal disease (Didier, 2005), and *Anncaliia* (*Brachiola*, *Nosema*)

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algerae, an insect parasite, (Undeen, 1975), that has caused several human infections (Moura et al, 1999; Coyle et al, 2004; Visvesvara et al, 2005). In preliminary studies we found that all three microsporidia species readily infect human Caco2 cells in culture, yet clinically only *E. intestinalis* appears to cause intestinal microsporidiosis.

Infection by obligate intracellular microsporidia parasites appears to occur in one of two ways. The spore may deploy a tube, impaling a nearby target cell, and then inject its content of infectious sporoplasm, into the host cell. Deployment of the tube is known as germination. Alternatively a spore may be phagocytized by a host cell and intracellular germination follows to cause infection (Franzen, 2004). While phagocytosis is probably favored in phagocytic and perhaps poorly differentiated cells (e.g. Couzinet et al, 2000), impalement by a deployed polar tube seems more likely as the method of infection in differentiated intestinal epithelial cells (Leitch et al, 2005). Spore adherence to target cell glycosaminoglycans also correlates with infection (Hayman et al, 2005; Southern et al, 2007). For these reasons we used a spore germination assay, and a host cell attachment assay and an infection assay using differentiated human intestinal epithelial cells to model the role of host factors in intestinal microsporidiosis.

MATERIAL AND METHODS

Microsporidia culture and spore isolation

Isolates of *E. intestinalis* and *E. hellem* were obtained from American Type Culture Collection (ATCC 50651 and 50451 respectively), Manassas, VA, while an isolate of *A. algerae* (CDC:V422) was obtained from the Centers for Disease Control and Prevention, Atlanta, GA. The microsporidia cultures were maintained in green monkey, Vero, E6 cells in a 37°C CO₂ (5%) incubator as described elsewhere (Visvesvara et al, 1999), except in the case of the *A. algerae* cultures which were maintained at 33°C. The *Anncalia* cultures were maintained at the lower temperature to provide optimal spore yield and because we have found that this human isolate of *A. algerae* cultured at 30°C readily germinates in vitro and infected mammalian cells maintained at 37°C (Kucherova et al, 2004). Spores were purified as described previously (Leitch et al, 2005), maintained in water at 4°C and used within 2 weeks of isolation.

Germination assay

In all germination assays purified spores samples were fixed for the measurement of baseline (0 time) germination, while other samples from the same stock of spores were incubated in a test solution for 15 min at 37°C, washed by centrifugation where indicated, incubated in a germination buffer at 37°C for an additional 30 min, and then fixed with 5% neutral formalin. Fixed spore samples were then placed on chambered coverslip slides, the spores allowed to settle overnight, and the percentage of germinated spores determined by phase contrast microscopy as described previously (Leitch et al, 1993). The germination solution used for all three microsporidia species was 140 mM NaCl, buffered to pH 7.5 with 20 mM Hepes to which was added H₂O₂ to a final concentration of 5%. In one *A. algerae* experiment a second germination solution was also used consisting of 140 mM NaCl, buffered to pH 9.5 with 20 mM glycine (Frixione et al, 1997)

The test solutions used were PBS (control), 100 mM HCl, HCl containing 1 mg/ml hog pepsin (EC3 4.23.1), PBS containing 1 mg/ml pancreatin (EC 232.468.9), or PBS containing 1 or 10 mg/ml ox bile, all obtained from Sigma Chemical Co. (St. Louis, MO) Following 15 minutes exposure at 37°C, the control and treated spore samples were washed twice at room temperature in PBS by centrifugation. The same washing conditions were used with all samples to control for any effect of washing and/or centrifugation on germination. The samples were then incubated in the germination solution for 30 min at 37°C prior to fixation.

Infection assay

The Caco2 clone, C2Bbe1 (ATCC CRL-2102), was used in all infection assays. Cells were cultured on collagen coated chamber slides or 1 cm² transwells and maintained at 37°C in DMEM medium supplemented with 10µg/ml human transferrin and 10% fetal bovine serum. At 7 days post-confluence, by which time cells were significantly differentiated, monolayers were infected with 4×10^5 spores per well of the 8 well-chamber slides or 10^6 spores per transwell. The slides were used for the estimation of infection using a calcofluor staining method (Didier et al, 1995), while the transwells were used for transmission electron microscopy. Spores were used without pretreatment or following pretreatments that mimicked gastric and duodenal environments as above before being used in the infection assays. Twenty-four hours post infection (PI) the monolayers were washed twice with Opti-MEM containing 1mg/ml chondroitin sulfate to remove spores that had attached to the monolayer but had not yet germinated and infected the host cells (Hayman et al, 2005; Leitch et al, 2005). This first wash was allowed to remain in the wells for 30 minutes and a second wash for 10 minutes. The infected wells were then refilled with medium and 48 hours later (3 days PI) the medium removed and the cells prepared for calcofluor staining.

Calcofluor staining

Cells fixed with 3% paraformaldehyde for 10 minutes were washed 3 X with PBS, exposed to 0.1% triton X100 in PBS for 2 minutes, and washed again 3 X with PBS. Calcofluor (Fluorescent Brightner 28, Sigma Chemical Co, St. Louis, MO) was then used to identify parasite spores (Didier et al, 1995). Calcofluor (50 µl, 1mg/ml in water) was added to each well for 2 minutes, and the wells were again washed 2 X with PBS before adding 0.1% Evans blue in PBS. After 2 additional washes with PBS, 50 µl propidium iodide (1mg/ml water) was added to each well. The propidium iodide staining was used to localize host cell and intracellular parasite nuclei. The wells were then washed 2 X with PBS, allowed to air dry, and coverslips were mounted with Fluoromount-G (Southern Biotech, Birmingham, AL). An Olympus BX41TF microscope with Microfire Digital camera was used to capture images using Picture Frame software, and the number of infected cells counted in randomly chosen 2,300µm² fields. Because of chamber wall edge effects, the areas adjacent to the site of the chamber slide scaffolding were avoided.

Electron microscopy

Transwells were fixed by emersion of both apical and basolateral surfaces in 4°C, 2.5% glutaraldehyde, buffered to pH 7.4 with 0.1M cacodylate buffer. Preparations were processed as described previously (Visvesvara et al, 1991). All sections were cut at right angles to the transwell surface.

Spore attachment

Spore attachment was assessed in differentiated C2Bbe1 cells by exposing 7 day post-confluent cultures to 4×10^5 spores per chamber of a chamber slide for a period of 4 hours (Leitch et al, 2005). The monolayers were then fixed with 3% paraformaldehyde. The cells were not subsequently permeabilized and spores attached to the apical cell surface were visualized using calcofluor staining.

Statistical evaluation

In germination assays treatment effects were calculated using the percent germination in a sample after correcting for any pretreatment germination (Leitch et al, 1993), comparing pairs of treated and control samples, both from the same stock of purified spores. A minimum of 4 replicate experiments were performed for each treatment. Differences between control and treatment means were evaluated using paired Student t tests.

In the case of infection assays an average of 15 fields were evaluated for the number of infected cells and the significance of differences between means determined by one-way ANOVA of repeated measures using aggregate data sets. Post hoc analyses were performed using Tukey's tests, and p values of <0.05 were considered significant.

RESULTS

Spore attachment and infection

Figure 1 illustrates the relationship between spore attachment and infection of C2Bbe1 cells with the three microsporidia species under study. Infection was significantly less (~10%) in the case of *A. algerae* 3 days PI when compared with either of the *Encephalitozoon* species. However, this effect did not appear to be due to a difference in spore adherence as there was no significant difference between the three species in their spore attachment to the target cells measured at 4 h PI.

Gastric and duodenal environment effects

Exposure to HCl, with or without pepsin, a mixture of pancreatic enzymes, or ox bile did not inhibit H₂O₂-stimulated spore germination in any of the three parasite species used here (Table 1). Similarly, prior exposure to pepsin in 100 mM HCl, pancreatin or ox bile did not reduce infection of differentiated C2Bbe1 cells (Figure 2).

Effect of temperature on microsporidia infection of enterocytes

Transmission electron micrographs of infected, differentiated C2Bbe1 cells grown on transwells are shown in figure 3. *Encephalitozoon*-infected cells were fixed 3 days PI and *A. algerae*-infected cells were fixed at 3 and 5 days PI. When the cultures were maintained at 37°C mature spores were frequently evident within parasitophorous vacuoles in both *E. intestinalis* and *E. hellem* infected cells (Figure 3 A and B). However we were unable to detect mature *A. algerae* spores in 3 day 37°C cultures, while by 5 days cells were observed containing multiple empty (germinated) spores (Figure 3 C). Such infected host cells were more vacuolated than were *Encephalitozoon*-infected cells. When *A. algerae*-infected cells were maintained at 33°C for 3 days sporogonial stages were evident and there were examples of intracellular spore germination indicating that differentiation occurred more rapidly with this species at the lower temperature (Figure 3D and insert).

A similar infection experiment was conducted using C2Bbe1 cultures on chamber slides employing a calcofluor stain that identifies the chitinous spore coat of sporogonial stages (Didier et al, 1995). With the calcofluor staining method it was possible to distinguish and count the larger individual intracellular *A. algerae* spores as these are separate within the host cell cytoplasm and not aggregated within parasitophorous vacuoles, as is the case with the *Encephalitozoon* microsporidia. Figure 4 illustrates both the number of *A. algerae*-infected cells per field and the number of spores per field when C2Bbe1 cells were maintained at 33°C and at 37°C for 3 and 5 days PI. There was a significant increase in both parameters as the incubation continued from 3 to 5 days, and at each period both the number of infected cells and the number of spores were significantly greater in the 33°C cultures than in the 37°C cultures. The spore count included all parasite stages that stained positive with calcofluor and is thus probably an overestimation of mature, infectious spores. On the other hand the low incidence of *A. algerae* mature spores in 37°C cultures makes it likely that they might be missed with the sampling constraints of transmission electron microscopy. Nevertheless the calcofluor and transmission electron microscopic data indicate that at mammalian body temperature there is a lower likelihood of *A. algerae* completing its life cycle to infective mature spores within the life span of the target enterocytes than is the case with either of the *Encephalitozoon* species.

Effect of temperature on *A. algerae* spore germination

Anncaliia algerae infection could also have been reduced in the 37°C cultures by a delay or reduction of spore germination. Figure 5 shows that this possibility is unlikely as in vitro spore germination was stable over the temperature range employed in the infection assays, using two different germination media.

DISCUSSION

Microsporidia spores have been recovered from surface water and drinking water sources (Dowd et al, 1998; Cotte et al, 1999). Fecal-oral transmission and person-person transmission have been implicated in microsporidiosis and the gastrointestinal tract and lungs would seem the most probable sites for infection (Mathis et al, 2005). The present study was designed to determine what host gastrointestinal factors contribute to determining why the three microsporidia species under study can all infect enterocytes in culture, but have such different organ specificity in vivo.

In humans, intestinal microsporidiosis is generally limited to two species, *Enterocytozoon bieneusi* and *E. intestinalis* (Kotler and Orenstein, 1998; Didier, 2005). *Encephalitozoon hellem* causes enteritis in birds (Mathis et al, 2005), but is not generally associated with human intestinal microsporidiosis, with the possible exception of two diarrhea cases in related individuals who were HIV negative and were co-infected with *Enterocytozoon bieneusi* (Muller et al, 2001).

Brachiola (Nosema) algerae has recently been reassigned to the genus *Anncaliia* (Franzen et al, 2006). This species is probably ubiquitous in the environment (Avery and Undeen, 1987) and has been detected in three clinical cases to date (Coyle et al, 2004; Visvesvara et al, 2005), none involving the gastrointestinal tract. It was previously thought that this insect parasite could not infect mammals because of their higher body temperature. In addition to the clinical cases cited above there have been multiple reports of infection of mammalian cell lines and mammalian animal models by this species (Undeen, 1975; Undeen and Alger, 1976; Trammer et al, 1997; Moura et al, 1999; Lowman et al, 2000; Koudela et al, 2001). However spores from clinical isolates were significantly more infectious in mammalian cells cultured at 37°C than were spores from insect isolates (Kucerova et al, 2004).

In this study *A. algerae* spores infected significantly fewer cultured differentiated intestinal epithelial cells than spores of either of the *Encephalitozoon* species tested (Figure 1A). Microsporidia spores have been shown to express a protein, EnP1, on the spore coat, particularly in the area of the anchoring disc (Southern et al, 2007), that mediates spore attachment to target cell glycosaminoglycans (Hayman et al, 2005), and there appears to be a direct correlation between such spore attachment and infection. Thus a lower incidence of spore attachment is a potential explanation for the lower infection rate seen with *A. algerae* spores. However, as seen in Figure 1B there were no differences in spore attachment to C2Bbe1 cells among the three species tested.

When we modeled the effects of the gastric and duodenal environments on microsporidia spore germination and enterocyte infection we found no major antimicrosporidial effects with any of the three parasite species (Table 1, Figure 2). In fact there were modest but significant increases in *E. intestinalis* and *A. algerae* spore germination following pancreatin pretreatment, and a trend to increased enterocyte infection with all three species following spore exposure to pepsin, bile or pancreatin.

For a microsporidia parasite to successfully maintain an infection of the intestinal epithelium it must be able to complete its life cycle to the point of producing infectious mature spores

before the infected epithelial cell turns over, a period of approximately 3-6 days in the intestine depending on species, location, age, nutritional status and luminal flora (e.g. Lipkin, 1987; Jones and Gore, 1997). Alternatively the host could continuously consume spores. In an in vitro murine model *E. intestinalis* has been shown to be able to complete its life cycle well within this time frame (Wasson and Barry, 2003). Using transmission electron microscopy of transwell-cultured C2Bbe1 cells maintained at 37°C we were frequently able to detect all parasite stages, including mature spores, in both *E. intestinalis*- and *E. hellem*-infected cells 3 days after spore inoculation (Figure 3). While we were unable to find mature *A. algerae* spores, by 5 days PI there was evidence that intracellular spores had germinated. This contrasts with 33°C *A. algerae*-infected cells in which empty and germinating intracellular spores were seen by 3 days PI.

When a calcofluor stain was used to monitor infection both the number of infected cells and the number of spores increased from 3 to 5 days PI and were always greater in 33°C cultures than 37°C cultures. The calcofluor stain did not permit distinguishing between mature spores and other sporogonial stages that were establishing a chitinous spore coat (Didier et al, 1995), but these experiments were not subject to the sampling limitations of the transmission electron microscopy. The fact that the number of *A. algerae*-infected cells increased with time can be explained by the fact that the calcofluor assay only detected sporogony stages, thus underestimating the number of infected cells but overestimating the number of mature spores. Taken together with the electron microscopy data the calcofluor experiments suggest that *A. algerae* differentiation to mature spores is temperature dependent and, while the life cycle may be completed by 3 – 5 days PI in 37°C cultures, host temperature would be expected to limit the ability of this species to cause intestinal microsporidiosis. This is consistent with other reports (Lowman et al, 2000; Koudela et al, 2001; Kucheroova et al, 2004) suggesting that mammalian body temperature slows differentiation in this parasite species. There is no reason to believe that *A. algerae* spore germination was less in the 37°C cultures than in the 33°C cultures as in vitro germination was stable over a wide temperature range (Figure 5).

This study suggests that gastric, pancreatic and hepatic secretions do not play a major role in determining if an ingested microsporidia spore will infect the intestinal epithelium. The lower infection rate of *A. algerae* spores could not be attributed to a lower attachment of spores to host cells in culture. However, the lower probability of this parasite completing its life cycle before target enterocytes turn over may contribute to the fact that this species has not been implicated in intestinal microsporidiosis. *Encephalitozoon intestinalis* and *E. hellem* were both able to complete their life cycles in enterocytes within 3 days, suggesting that other factors are responsible for the fact that only the former *Encephalitozoon* species is important in infecting the human gastrointestinal tract.

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Figure 1.

A. Number of infected C2Bbe1 cells per field 3 days PI with spores of *E. intestinalis*, *E. hellem* or *A. algerae*. B. Attachment of *E. intestinalis*, *E. hellem* and *A. algerae* spores to differentiated C2Bbe1 cells 4 hours after inoculation. Means $\pm$ SEM. (N=4). * Significantly different from the other two parasite species, $p < 0.05$.

Figure 2.

Number of infected differentiated C2Bbe1 cells per field 3 days after inoculation with *E. intestinalis*, *E. hellem* or *A. algerae* spores that had been exposed to PBS (control), 1 mg/ml pepsin in 100 mM HCl, 1 mg/ml pancreatin in PBS, or 10 mg/ml ox bile in PBS. Means $\pm$ SEM. (N=3)

Figure 3.

A, B. Transmission electron microscopic appearance of C2Bbe1 cells cultured on transwells at 37°C, 3 days PI. A. Several parasitophorous vacuoles containing *E. intestinalis* developing stages and mature spores (S). Brush border (BB). B. Large parasitophorous vacuole containing *E. hellem* developing stages and mature spores. C. Empty *A. algerae* spores in cells 5 days PI, cultured at 37°C. Arrow indicates a site near polar tube extrusion. D. *A. algerae* developing stages and mature spores in 33°C culture, 3 days PI. Germinating intracellular spore (inset). Bars, 2μm.

Figure 4. Number of infected cells (open bars) and number of spores (closed bars) detected in fields of C2Bbe1 cell cultures 3 and 5 days after inoculation with *A. algerae* spores. Cultures were maintained at 33°C or 37°C. Means $\pm$ SEM. (N=4).

Figure 5. Effect of incubation temperature on the germination of *A. algerae* spores. Spores were stimulated to germinate using a H₂O₂ germinating solution or a pH 9.5 germinating solution. Means $\pm$ SEM. (N = 4).

Table 1

Effects of prior exposure to HCl in the presence or absence of pepsin, pancreatic enzymes in PBS, or bile in PBS on microsporidia spore germination.

	<i>E. intestinalis</i>	<i>E. hellem</i>	<i>B. algerae</i>
HCl, 100m M	-1.3 ± 2.9^a	3.1 ± 3.3	1.1 ± 2.9
HCl/pepsin, 1 mg/ml	-2.0 ± 6.8	0.3 ± 0.7	4.1 ± 5.8
Pancreatin, 1 mg/ml	6.7 ± 0.8^b	10.6 ± 5.4	16.2 ± 2.8^b
Ox bile, 1 mg/ml	6.0 ± 5.5	0.8 ± 2.1	-1.3 ± 1.6
Ox bile, 10 mg/ml	10.2 ± 10.7	7.0 ± 4.9	2.2 ± 4.7

Results are expressed as percentage difference from the appropriate control replicate experiments.

^a Means $\pm$ SEM or 4 replicate experiments.

^b Significantly different from control, $p < 0.05$.