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Effect of low pH on the morphology and viability of *Cryptosporidium andersoni* sporozoites and histopathology in the stomachs of infected mice

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ABSTRACT

The genus *Cryptosporidium* includes many common parasites infecting animals and humans, and is a major cause of diarrheal illness worldwide. The biology of gastric *Cryptosporidium* spp., including replication in the stomach, has not been well documented. This study evaluated the viability of *Cryptosporidium andersoni* sporozoites in gastric environments after excystation and examined the endogenous development and histopathological changes in the stomachs of infected mice, using a novel type of *C. andersoni*. Sporozoites were affected by low pH (61.6% viability after 3 h at pH 2.0). Electron microscopy revealed developmental parasites on the gastric foveolae but not on the surface of the gastric mucosa. Histopathological examinations at 1, 2, 4 and 12 weeks p.i. uncovered three different lesions. The gastric mucosa of foveolae filled with parasites was extended and the amount of neutral mucopolysaccharide at the mucosal surface was decreased with the first type of lesion. The gastric mucosa was atrophied, some gastric glands were disrupted and the amount of acid mucopolysaccharide at the mucosal surface was increased with the second type. Finally, the gastric mucosa was slightly extended and goblet cells were present in the gastric mucosa, indicating intestinal metaplasia, in the third type. No parasites were detected in these areas with increased acidic mucin and indications of metaplasia. The results suggest that *C. andersoni* parasites could not survive in acidic environments for a long period before invading host cells and preferentially develop in neutral sites of the gastric mucosa, resulting in histopathological changes and chronic shedding of oocysts.

1. Introduction

Parasites of the genus *Cryptosporidium* infect vertebrates and sometimes cause diarrheal illness. Cryptosporidiosis caused by some species has been reported following zoonotic transmission from infected animals and contamination of water supplies (O'Donoghue, 1995). Currently, 19 species of *Cryptosporidium* are recognized based on biological and molecular characterization (Fayer, 2010). Most of these, including *Cryptosporidium parvum* (with a wide range of hosts), infect epithelial cells of the small intestine, while *Cryptosporidium muris* (mainly infecting rodents) and *Cryptosporidium andersoni* (mainly infecting cattle) have been described as causative agents of gastric cryptosporidiosis in mammals (Tyzzer, 1907; Lindsay et al., 2000). Although the pathogenicity of these gastric *Cryptosporidium* spp. remains unclear, chronic *C. andersoni* infections in cattle have been reported to cause gastritis, reduced milk yields and poor weight gain, and therefore have potential economic impacts (Anderson, 1998).

Both gastric and small intestinal *Cryptosporidium* infections are initiated by the ingestion of oocysts containing four sporozoites. These infective sporozoites are released from oocysts in the gastrointestinal tract, and are thought to advance upon a target host cell for invasion using an active process termed "gliding motility" relative to organelles at the apical end (termed the apical complex) (Wetzel et al., 2005). The sporozoites then attach to and invade the membrane of a host epithelial cell, form a parasitophorous vacuole, and undergo sexual and asexual development. Although the cellular and molecular mechanisms of the infection process remain poorly understood, attachment and invasion are thought to be

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associated with apical organelles including rhoptries and micronemes, and cytoskeletal activity such as the actin polymerization of sporozoites and host cells at the attachment site (Forney et al., 1998; Blackman and Bannister, 2001; Elliott et al., 2001; Chen et al., 2003).

Compared with that of small intestinal *Cryptosporidium*, the biology of gastric *Cryptosporidium*, including attachment, invasion and successive replication in the stomach by sporozoites or merozoites, has not been well documented. In cattle, *C. andersoni* oocysts are thought to excyst and infect the microvillous border of epithelial cells in the abomasum (Lindsay et al., 2000). Histopathologically, inflammatory cells have frequently been observed in the lamina propria of the abomasum, with the oocysts defecated for 14–35 months (Masuno et al., 2006). How long excysted sporozoites of *C. andersoni* can survive when exposed to an acidic environment before invasion or how the histopathology in the infected stomach changes during endogenous development of the parasite remains unclear. We have recently reported a novel type identified genetically as *C. andersoni* and successfully infected laboratory mice (gastric gland) (Matsubayashi et al., 2004, 2005). Severe combined immunodeficiency (SCID) mice infected with *C. andersoni* novel type shed 10^6–10^7 oocysts per day for at least 91 days without showing clinical symptoms such as diarrhea (Matsubayashi et al., 2004, 2005). The present study used this model to evaluate the viability of *C. andersoni* sporozoites exposed to low pH, and examined endogenous development and histopathological changes in the infected mouse stomachs.

2. Materials and methods

2.1. Parasites and animals

Oocysts of the novel *C. andersoni* sp. were isolated from feces of cattle reared in Saga Prefecture, Japan and analyzed as described previously (Nagano et al., 2007; Matsubayashi et al., 2008a). Oocysts were maintained by passage in SCID mice (C.B-17/Icr-SCID Jcl, Clea Japan, Tokyo, Japan). Mice were housed individually in wire-bottomed cages placed on a tray containing 5 mm of water to keep the feces wet. All cages were kept in an environmentally controlled room maintained at a temperature of 25 °C. Mice were given sterilized water and a standard pellet diet (CE-2; Clea Japan). All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" for the Department of Parasitology, Graduate School of Medicine, Osaka City University, Japan.

Oocysts were purified from feces using the sugar flotation method (Matsubayashi et al., 2005), resuspended in PBS (pH 7.2) and stored at 4 ℃ until 1 month before use. To prepare *C. andersoni* sporozoites, four million oocysts were excysted in RPMI 1640 medium containing 10% FCS (Invitrogen, CA, USA) for 20 min at 37 ℃, then filtered with a Disposable Syringe Filter (5-μm pore size; IWAKI, Chiba, Japan) to remove unexcysted oocysts and excysted oocyst walls.

2.2. Morphological observations

Sporozoites were incubated in RPMI 1640 medium containing 10% FCS (Invitrogen) at 37 ℃ for 0, 1, 2 or 3 h, then examined for morphological changes under light microscopy, as a previous study showed that *C. parvum* sporozoites were shortened in culture media, depending on the length of time cultured (Matsubayashi et al., 2010). Briefly, 300 sporozoites were counted on each slide and classified into four types based on morphology (1,>7.51 μm in length; 2, 7.50–5.01 μm in length; 3, 5.00–2.51 μm in length; and 4,<2.50 μm in length). These experiments were repeated five times.

2.3. Assay of sporozoite viability

The viability of *C. andersoni* sporozoites was estimated using LIVE/DEAD® Reduced Biohazard Viability/Cytotoxicity Kits (L-7013; Invitrogen) in accordance with the manufacturer's instructions with minor modifications (Matsubayashi et al., 2010). Namely, sporozoites were incubated in RPMI 1640 medium containing 10% FCS or a physiological saline solution of pH 7.0, 4.0, 3.0 or 2.0, at 37 ℃ for 0, 1, 2 or 3 h. After each incubation period, an aliquot was centrifuged at 8300 *g* and 4 ℃ for 3 min, and 200 μl of a dye working solution prepared according to the manufacturer's instructions were added. Pellets were resuspended in 100 μl of HEPES Balanced Salt Solution (HBSS) and 100 μl of 4% glutaraldehyde (GA) were added for fixation. Specimens were observed by differential interference contrast and fluorescence microscopy (Nikon, Tokyo, Japan). One hundred sporozoites were examined on each microscope slide. The green fluorescent dye stained all sporozoites (fluorescein isothiocyanate filter cube; Ex = 480/40, Em = 535/50) while the red fluorescent dye stained only dead sporozoites (tetramethyl rhodamine isothiocyanate filter cube; Ex = 545/30, Em = 620/60). At the same time, the morphological determinations described above were performed. These experiments were repeated three times.

2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

To observe the infected stomach using SEM, 5-week-old female SCID mice in groups of three were inoculated with 1×10^6 oocysts in 0.1 ml of distilled water by gastric intubation. At 56 weeks p.i. as a chronic infection, the glandular stomach was removed from euthanized mice and fixed in 2% GA and 2% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB) (pH 7.4) at room temperature for 2 h, additionally fixed with 2% GA at 4 ℃ for 1 week and 2% OsO_4 in 0.1 M PB at 4 °C for 2–3 h, and washed in 0.1 M PB. Samples were dehydrated in ethanol, cooled in liquid nitrogen and broken apart. These specimens were replaced in isoamyl acetate, critical point dried, coated with a layer of sublimated OsO_4 using an osmium plasma coater (OPC80N; Filgen, Nagoya, Japan), and examined under SEM (S-4700; Hitachi, Tokyo, Japan).

The infected stomach was examined by TEM as reported previously (Matsubayashi et al., 2008b). Briefly, after fixation the specimen was postfixed with 2% OsO_4 in 0.1 M PB at 4 °C for 2–3 h, dehydrated in an ethanol series, replaced in propyleneoxide and embedded in epoxy resin (Epon 812; TAAB, London, UK). Ultra-thin sections were cut with a diamond knife, stained with 2% uranyl acetate (Cerac, WI, USA) in distilled water for 15 min following by a lead staining solution (Hanaichi et al., 1986) for 5 min and examined under an electron microscope (H-7500; Hitachi).

2.5. Histopathological examination and staining of gastric mucin

For histopathological examination, 5-week-old female SCID mice in groups of three were inoculated with 1×10^6 oocysts. Two mice were inoculated with distilled water as a control. Animals were kept as described above. At 3 days before necropsy, the number of oocysts per day was estimated as previously reported (Matsubayashi et al., 2005). At 1 week (beginning of oocyst shedding), 2 and 4 weeks (period of increasing numbers of shed oocysts) and 12 weeks (chronic infection) p.i. according to a previous study (Matsubayashi et al., 2005), the glandular stomach was removed from euthanized mice, fixed in 10% buffered formalin at room temperature for 4–7 days, and embedded in paraffin. Each section was cut at a thickness of 4 μm, stained with H & E or Alcian blue (AB)-periodic acid Schiff (PAS) and examined using light microscopy. At the same time, serial sections of each sample were