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ALIMENTARY TRACT

Production of Viable *Giardia* Cysts In Vitro: Determination by Fluorogenic Dye Staining, Excystation, and Animal Infectivity in the Mouse and Mongolian Gerbil

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The purpose of this research was to document the formation of viable *Giardia* cysts in vitro. Viability staining, using fluorogenic dyes that required metabolic conversion for detection, and immunocytochemistry at the light microscopic level provided information on viability and for the identification of antigens that were similar to those found with cysts formed in vitro. Analysis of cysts formed in vivo and in vitro showed similar morphologic appearances by both light and electron microscopy. Cysts formed in vitro were capable of establishing infections in both mouse and gerbil models for giardiasis. Trophozoites obtained from mice experimentally infected with in vitro-formed cysts could be maintained in culture and induced a second time to form cysts in vitro. This model for the production of viable *Giardia* cysts in vitro should facilitate research on controlling the complete life cycle of *Giardia* outside an animal host.

Giardia is a parasitic protozoon capable of causing severe, chronic diarrhea in humans. The life cycle of this organism consists of two forms: trophozoite and cyst. Giardiasis, the intestinal disease resulting from *Giardia* infection, has a worldwide distribution; however, little is known about the formation of the diagnostically significant, environmentally resistant cyst form of the organism. The cyst is clinically significant for two reasons: (a) ingestion of *Giardia* cysts in food or water is a common mechanism for the transmission of giardiasis (1,2); and (b) the detection of fecal cyst shedding is used as a routine diagnostic indicator of infection (3).

In vitro excystation of *Giardia* cysts has been induced by acidic conditions that mimicked the gastric milieu (4,5). This yielded viable trophozoites that could be maintained in culture (4,5). Recent evidence has suggested that *Giardia* cyst antigens were formed in vitro (6); however, no description of viable *Giardia* cysts produced in vitro has been reported, even though axenic cultures of *Giardia* trophozoites were first developed almost a decade ago (7). The development of an in vitro model for encystation would enable the complete life cycle of this protozoon to be manipulated outside of an animal host.

The specific goals of this research were: (a) to show that cysts formed in vitro were viable as determined by incorporation of fluorogenic dyes (8), their response to in vitro excystation (7), and their ability to establish an infection in animal models susceptible to the species of *Giardia* under investigation (9–12); and (b) to demonstrate that cysts formed in culture were morphologically and immunocytochemically identical, at both the light and electron microscopic levels, to cysts isolated from infected human hosts.

Abbreviations used in this paper: **DIC, differential interference contrast; FDA, fluorescein diacetate; PI, propidium iodide.**

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Materials and Methods

Cell Culture

Axenic cultures of *Giardia* were maintained on bile-supplemented (2.5 mg/ml) TYI-S-33 medium (ATCC medium 1404), as previously described by Keister (13). The trophozoites were transferred twice weekly. *Giardia* encystation was stimulated by increasing the concentration of bile (bovine; Sigma Chemical Co., St. Louis, Mo.) in the TYI-S-33 culture medium from 2.5 to 5.0 mg/ml. Encystation was studied by varying the concentration of bile in the growth media from 1 to 20 mg/ml. Log phase cultures were harvested by cooling the culture tubes on ice for 10 min, followed by centrifugation at 1000 g for 10 min. Total cyst counts were made from the pelleted material using light microscopy.

Morphology

Light microscopy. Wet mounts for light microscopy were made on slides directly from the pellet obtained after centrifugation of the culture tubes. The slides were coverslipped and sealed with wax. Micrographs were taken with an Olympus BH-2 microscope (Olympus Optical Co., Ltd., Tokyo, Japan) equipped with differential interference contrast (DIC) and epiillumination optics, using Technical Pan film (Eastman Kodak Co., Rochester, N.Y.).

Scanning electron microscopy. The specimens were prepared by washing the pelleted culture with 0.9% saline and centrifuging as described above. A drop of the washed culture contents was placed on glass chips coated with poly-l-lysine (Sigma) and allowed to adhere for 1 h. The cells were fixed for 4 h in 2.5% glutaraldehyde buffered with 0.1 M sodium cacodylate-HCl (pH 7.4). This was followed by postfixation for 1 h at 4°C in 1% osmium tetroxide diluted in the same buffer. The glass chips, with attached *Giardia* trophozoites and cysts, were dehydrated in an ascending ethanol series and critical-point dried (14). The glass chips were mounted on stubs with silver paint or copper tape and sputter-coated with gold palladium in a Denton DV 502 vacuum evaporator (Denton Vacuum, Inc., Cherry Hill, N.J.). The prepared specimens were examined in a Hitachi S450 scanning electron microscope operating at 20 kV.

Transmission electron microscopy. Cultures were fixed for transmission electron microscopy as described for scanning electron microscopy, except that a few drops of albumin were added to the cultured material between primary and postfixation steps. The albumin was fixed for 5 min in 2.5% glutaraldehyde. The fixed albumin block that contained the cell suspension was dehydrated in an ascending ethanol series and embedded in Epon (Shell Chemical Co., New York, N.Y.) as described by Luft (15). Sections were cut on a LKB Huxley ultramicrotome (LKB-Produkter AB, Bromma, Sweden), and then stained with uranyl acetate and Reynolds lead citrate (16) and examined with a JEOL 100 CX electron microscope (JEOL Ltd., Tokyo, Japan) operating at 80 kV.

Immunocytochemistry

Four of the antisera, directed against *Giardia* cysts, were obtained from Dr. J. Sauch and Dr. J. Riggs (17,18). The other antiserum used was produced in our laboratory by inoculation of guinea pigs with pooled *Giardia lamblia* cysts, according to the technique of Sauch (17). The primary antisera at a 1:20 dilution with phosphate-buffered saline were added directly to the washed pelleted contents of the culture tubes. Goat-antirabbit immunoglobulins conjugated with fluorescein or rhodamine were used as the secondary antisera. The primary antisera were incubated with the cells for 2 h, followed by three centrifugal washes in phosphate-buffered saline. The secondary antibody was applied to the pelleted contents at a dilution of 1:20 and incubated for 3 h, followed by three centrifugal washes as previously described. The washed pelleted contents were placed on a slide, coverslipped, and sealed with wax for subsequent immunofluorescent microscopy.

Cyst Viability

Fluorogenic dye staining. The cultured cysts were separated from the trophozoites either by incubation of 30–60 min in distilled water, or by differential buoyant density gradient centrifugation. For the density gradient technique, the pelleted culture contents were mixed with 10 ml of Percoll (Sigma) with a density of 1.090 g/ml. This was overlaid with 15 ml of Percoll with a density of 1.045 g/ml followed by 15 ml of Percoll with a density of 1.020 g/ml. The tube was topped with 10 ml of distilled water and centrifuged for 30 min at 1000 g. The fraction containing *Giardia* cysts devoid of trophozoites was removed from the 1.090–1.045 interface and stored in distilled water at 4°C until needed. The viability of the cyst fraction was determined using the fluorogenic dyes fluorescein diacetate (FDA) and propidium iodide (PI) (Sigma) as described by Schupp and Erlandsen (8).

Animal infectivity. Cysts, isolated as described above, were inoculated by gastric lavage into 8 Mongolian gerbils (Tumblebrook Farms, West Brookfield, Mass.) according to the technique of Belosevic et al. (9). Ten 5-day-old mice and six 15-day-old mice (Harland Sprague-Dawley, Indianapolis, Ind.) were also inoculated by gavage, according to the technique of Roberts-Thomson et al. (12). The feces of each animal were checked for the presence of *Giardia* for 5 days before the start of the experiment. Uninfected controls and inoculated animals were placed in the same cage to ensure that neither an endogenous infection nor intrasibling transmission were sources of infection. The presence of cysts in the feces or trophozoites in the small intestine was used as an indicator of an established infection.

Excystation. The excystation procedure was performed using a modification of the technique of Bingham and Meyer (4). This method used the aseptic dilution of 6 ml of 100 mM cysteine-ascorbic acid solution in Eagle's minimal essential medium to a total of 50 ml in Hanks' balanced salt solution. Culture tubes (13 × 100 mm) containing 6 ml of the diluted cysteine-ascorbic acid solution were incubated in a water bath at 37°C. Each tube

was inoculated with 7.5×10^5 in vitro-produced *Giardia* cysts in 0.1 ml of distilled water. The tubes were then stoppered and incubated for 15 min at 37°C. The pH was adjusted to neutrality by the dropwise addition of 1 N $NaHCO_3$ after the incubation. The cysts were immediately centrifuged at 1000 g for 10 min at 4°C. The supernatant was removed, and the tubes were refilled with fresh TYI-S-33 medium. The tubes were then placed in a 37°C incubator and examined periodically for 48 h for signs of excystation and the presence of trophozoites.

Results

Encystation

Six different isolates of *Giardia* including the WB strain isolated from humans (19) (American Type Culture Collection #30957, Bethesda, Md.), four beaver isolates (IP-0482:1 and IP-0583:1 from Dr. L. Diamond; PB-1 and B-5 from Dr. H. Stibbs), and one muskrat isolate (MR4 from Dr. H. Stibbs and Dr. P. Wallis) were grown axenically and shown to be capable of forming viable cysts in vitro. Trophozoites grown in TYI-S-33 medium that had 2.5 mg/ml or less bile did not form cysts. However, the trophozoites exhibited typical growth and reproduction (Figure 1). When the culture media were replaced with fresh TYI-S-33 media that had a 5-mg/ml bile content, encystation occurred at rates ranging from 5% to 50%, depending on the isolate being grown.

The response of one isolate (MR4) to varying concentrations of bile in the growth medium is illustrated in Figure 1. In a representative experiment using five different concentrations of bile (1, 2.5, 5, 10, or 20 mg/ml) added to separate tubes containing the standard TYI-S-33 media, the percentage of encystation increased with increasing bile concentrations. No cysts were detected in the tubes containing either 1 or 2.5 mg/ml of bile; however, at a bile content of 5 mg/ml, an encystation rate of 32% was observed by DIC microscopy (n = 5). Increasing the bile concentration to 10 or 20 mg/ml produced an increase to 35% encystation (n = 5) or a reduction to 11% encystation (n = 5), respectively. When organisms from these experimental tubes were transferred for a second passage into their respective media with the same concentration of added bile, the encystation rate was always less than the level attained during the first passage. Continued subculture resulted in a further decrease in the encystation rate (not illustrated).

Morphologic Studies

Examination of the six *Giardia* isolates with DIC microscopy revealed cyst profiles that were morphologically identical to control cysts isolated from patients with symptomatic giardiasis. Figure 2*a*

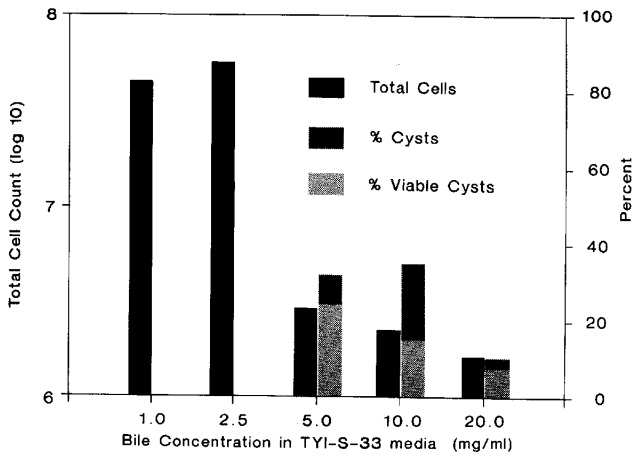

Figure 1. Giardia encystation.

illustrates MR4 organisms from an axenic culture that had been stimulated to undergo encystation with TYI-S-33 medium containing 5 mg/ml of bile. Characteristic flagellated, pear-shaped *Giardia* trophozoites were seen attached to the substratum and were intermixed with the cysts that had been produced in vitro. The cysts were typically round to oval in shape and were ~7.9 ± 0.8 μm in length and 4.8 ± 0.3 μm in width (n = 15). The cysts formed in vitro were of two different morphologic types, based on cytoplasmic appearance. One phenotype, which had a granular-appearing cytoplasm, was shown to be nonviable using morphologic criteria (20) as well as by fluorogenic dye staining patterns (8; discussed below). These nonviable cysts had an intact cyst wall, but the cytoplasmic contents often appeared shrunken and the peritrophic space was enlarged. The other observed phenotype, from in vitro-derived cysts, was characterized by a clearly defined cyst wall, peritrophic space, profiles of cytoplasmic organelles including flagellar axonemes and elements of the adhesive disk, and the presence of two to four nuclei (see *C* in Figure 2*a*). This latter type of *Giardia* cyst, formed in vitro, was considered to be viable because (a) the cyst morphology was comparable to *Giardia* cysts that had been isolated from a symptomatic patient with giardiasis (Figure 2*b*); and (b) it possessed the same morphologic characteristics (the hyaline appearance of the cytoplasm, clearly defined cyst wall, peritrophic space) described for viable *G. muris* cysts (20).

The ultrastructural appearance of both the *Giardia* trophozoites and cysts from culture are seen by scanning electron microscopy in Figure 2*c*. Trophozoites are typified by an adhesive disk on the ventral surface, a peripheral ventrolateral flange, four pairs of flagella, and a bilaterally symmetrical shape. The cysts are round to oval in shape, with a finely textured cyst wall that is smooth in appearance.

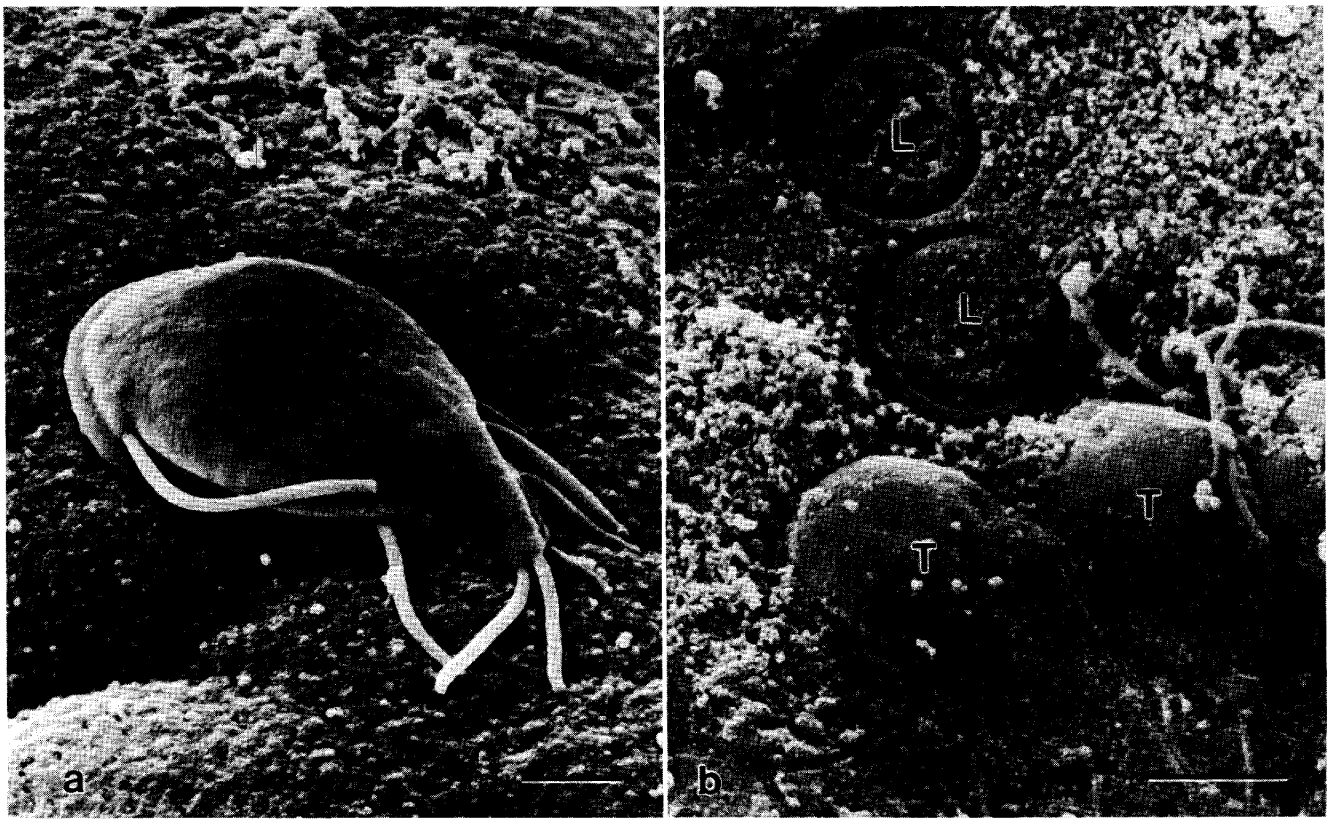

Figure 6. Scanning electron micrographs of *Giardia* trophozoites attached to the surface of intestinal epithelial cells of a Mongolian gerbil infected with *Giardia* cysts formed in vitro. *a*. A bilaterally symmetrical trophozoite is seen adhering to the microvillous border of intestinal epithelial cells. *b*. Lesions (*L*) within the microvillous border were caused by the attachment of the *Giardia* trophozoite via the ventral adhesive disk. Two trophozoites (*T*) can also be seen firmly attached to the epithelial cell surface. Magnification bar = 5 μm.

criteria for cyst morphology based on cysts produced in vivo (23,24). In addition to morphology, this study used three separate physiologic methods (fluorogenic dye uptake, excystation, and animal infectivity) to show that cysts formed in vitro are viable. Furthermore, the cysts are shown, by light microscopy, to be immunoreactive with antisera that recognize *Giardia* cysts produced in vivo (17,18).

Methods used to determine *Giardia* cyst viability included the incorporation of fluorogenic dyes (8) and the ability of cysts to undergo excystation (4). Viability, as determined by fluorogenic dye uptake, has been correlated with animal infectivity and cyst morphology (8,20), whereas excystation has been used as a measure of viability in studies on inactivation of *Giardia* cysts with chlorine (25), iodine (26), ozone (27), and heat (28). *Giardia* cysts formed in vitro were shown to have a viability of up to 50% by incorporation of the fluorogenic dyes FDA and PI, and an excystation rate of <1%. These values for cysts formed in culture were much less than that reported for cyst viability as determined by dye uptake (8) in freshly isolated *G. muris* cysts (85–90% ± 3%) or for excystation of either *G. muris* (29) or *G. lamblia* cysts (4,5). However, even though the excystation level of cysts formed in vitro was extremely low, it should be noted that Kasprzak and Majewska (30) have reported that human strains of *Giardia* can be established in culture from cysts having an apparent excystation level of 0%. These lower values of viability for *Giardia* cysts formed in vitro may have reflected the deleterious effects of culture conditions (elevated temperatures, proteases from trophozoite lysis, oxidized fatty acids) and the prolonged period of time before sampling (3 days after bile stimulation). Previous studies have shown that storage of isolated *Giardia* cysts at 37°C for <24 h greatly reduced their viability (as judged by the criterion of excystation) and that cysts never survived longer than 4 days at 37°C (4,28,31,32).

Viability of *Giardia* cysts formed in vitro was also demonstrated using the neonatal mouse model (10) and the Mongolian gerbil model of Belosevic et al. (9). Infection of mice (30%) and gerbils (37%) was demonstrated by the presence of intestinal trophozoites at necropsy, although fecal cysts were never seen. The presence of intestinal trophozoites and the absence of fecal cysts in animal model infections with *Giardia* has been noted by others (31).

Ultrastructural analysis of the microvillous border

in infected gerbils revealed distinct morphologic lesions similar to those reported for intestinal giardiasis caused by *G. muris* in the mouse (21) and rat (22).

This interaction with the intestinal mucosa constitutes direct evidence for the establishment of a continuing infection within this host as opposed to the notion that trophozoites are merely transients. Further studies are needed to (a) delineate the duration of infections in animals inoculated with in vitro-produced cysts; (b) document the presence of cysts in feces; and (c) determine whether or not changes occur in the physiologic function of intestinal epithelial cells, such as enzymatic content or cell turnover.

The morphologic appearance of viable *Giardia* cysts formed in vitro was indistinguishable by light and electron microscopy from that of in vivo-produced *Giardia* cysts of murine or human origin (21,24,33,34). The two phenotypes of *Giardia* formed in vitro appeared similar to the morphologic patterns observed by DIC microscopy of in vivo-produced viable and nonviable *G. muris* cysts (20). Viable cysts could be distinguished by the presence of a distinct cyst wall, a peritrophic space between cyst wall and cytoplasm, and the hyaline appearance of the cytoplasm by DIC microscopy. On the other hand, nonviable cysts formed in vitro were characterized by shrunken, granular cytoplasm and a deterioration of cellular organization. At the ultrastructural level the nonviable cysts from culture were similar to isolated nonviable *G. muris* cysts (35), but these cysts often showed signs of greater deterioration and contained only cytoplasmic remnants.

Previous studies have provided light microscopic descriptions of *Giardia* trophozoites undergoing encystation (36,37). In these, mucosal smears from the colon of infected animals were examined and forms that were rounded and possessed a different refractive index from the trophozoites were considered cysts. More recently, axenic cultures of trophozoites of the WB strain have been shown to form oval, cystlike forms that were immunoreactive with cyst-specific antisera (6). In that same report, cystlike structures were found to be resistant to lysis in distilled water and had a size and shape characteristic of *Giardia* cysts when viewed with phase microscopy. No information on viability was included (6).

Our results have demonstrated that the life cycle of *Giardia* can be repeated outside of the host. Three continuous trophozoite-cyst transformations were documented at the following times: (a) when the trophozoites formed cysts in culture; (b) when the in vitro-formed cysts were inoculated into the animals and yielded trophozoites; and (c) when the intestinal trophozoites isolated at necropsy from these animals were recultured axenically and induced to undergo encystation with bile-enriched (5 mg/ml) TYI-S-33 medium. The encystation level for these reaxenized trophozoites was the same as for the preinoculum group (20%).

The addition of bovine bile to the TYI-S-33 culture medium induced encystation in axenic cultures of *Giardia*. Farthing et al. (38,39) have previously shown that the addition of bile can directly influence the generation time of *G. lamblia* trophozoites in culture. They found that concentrations of 0.1–0.8 mg/ml were most effective in stimulating growth and that concentrations exceeding 2 mg/ml retarded growth. In our work, bovine bile concentrations of 5.0 mg/ml or higher stimulated encystation (Figure 1). It should be noted that the total numbers of cells (trophozoites) plus cysts were decreased at bile concentrations of 5 and 10 mg/ml, whereas the lowest number of cells plus trophozoites was observed at the highest bile concentration (20 mg/ml). Based on these limited data, it might be suggested that a relationship existed between inhibition of trophozoite multiplication by high bile concentrations and the induction of in vitro encystation. However, further experiments will be required to clarify the relationship, if any, between trophozoite multiplication and cyst formation.

This encystation model should facilitate study of the molecular biology of *Giardia* by providing an aseptic, constant source of *Giardia* cysts for such areas to be resolved as biochemical analysis of the cyst wall, mode of cyst wall production, and development of strategies to block wall formation (6,40). Based on studies using immunofluorescent lectins and enzymatic treatment, Ward et al. (40) have proposed that *N*-acetylglucosamine, present as polymers (chitin), may be a major structural component of the cyst wall in *Giardia*. These histochemical observations on the cyst wall have not yet been confirmed by biochemical analysis, but Gillin et al. (6) have reported the detection of chitin synthetase during in vitro encystation. Cyst walls formed in vitro have a morphologic appearance identical to in vivo cysts and also possessed similar antigenic reactivity; therefore, we might assume that their biochemical composition may be similar or even identical. However, no studies have been performed on the chemical composition of in vitro-formed cysts nor have they been tested for resistance to physical or chemical agents known to inactivate cysts formed in vivo. In addition, it should now be possible to incorporate a marker into cysts for cross-species transmission studies. Continued work with this in vitro model should provide insights into the mechanism by which bile stimulates encystation.

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