Excystation of In Vitro-Derived *Giardia lamblia* Cysts

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Received 16 April 1990/Accepted 10 August 1990

This is the first in-depth analysis of the excystation of *Giardia lamblia* cysts prepared in vitro. Its goals were both to achieve efficient excystation and to gain insights into this crucial but poorly understood process. To identify the critical elements of excystation, we tested the sequential low-pH induction and protease treatments which had been reported to be important for excystation of fecal cysts. The optimal pH for induction of excystation was 4.0. Emergence was greatly (~10-fold) stimulated by subsequent exposure of in vitro-derived cysts to chymotrypsin, trypsin, or human pancreatic fluid. The stimulatory activity of each was abolished by soybean trypsin inhibitor, demonstrating that the activity of pancreatic fluid was due to these proteases. Excystation of in vitro-derived cysts was ~10 to 20% of the walls of in vitro-derived cysts were partially digested by protease treatment, trophozoites emerged only from one pole, as observed with fecal cysts. The conditions of excystation also determined the efficiency of excystation. Specifically, excystation in the presence of lactate, a major metabolite of colonic bacteria, stimulated excystation approximately fourfold, although it did not increase the total numbers of cysts. These experiments have shown that excystation of in vitro-derived cysts reflects that of cysts purified from human feces in that it is dependent upon conditions which simulate the passage of cysts through the human stomach (low pH) and into the small intestine (pancreatic proteases).

Since excystation of ingested *Giardia lamblia* cysts is essential to the transmission of disease, it may be considered a virulence factor in human giardiasis. Nonetheless, this process is not well understood, largely because, until recently, the only source of *G. lamblia* cysts was the feces of infected humans or experimentally infected animals. Excystation of fecal cysts varied from <0.1 to >95% (17; unpublished data). Cysts prepared in vitro have the advantages of being free of fecal contaminants and of more consistent physiologic conditions for studies of excystation.

During natural infection, ingested cysts pass through the stomach of the host, where they are exposed to gastric acid (2). Subsequently, cysts enter the duodenum, where the gastric chyme is rapidly neutralized by influxes of bicarbonate (3). It is important that trophozoites not emerge from cysts in the stomach because they would be killed by the acid. Excystation is presumed to occur in the upper small intestine, but the exact site is not known. Descent past the entrance of the common bile duct would expose the cysts to a variety of degradative enzymes and bile salts, which have detergent activity (3, 10).

The pioneering studies of fecal cysts by Bingham et al. (1, 2) demonstrated that excystation was dependent upon “those conditions most closely approximating the organism’s *in vivo* environment” (2). Specifically, they showed the importance of exposing cysts to a low pH, which mimics passage through the stomach. Trophozoites emerged when the acid-treated cysts were transferred to a neutral medium, reproducing entry into the duodenum (2). Excystation methods published since then have incorporated other physiologic intestinal factors, including bicarbonate, trypsin (a pancreatic protease), thiol-reducing agents (16, 20), and bile salts (11).

In a previous study (7), we demonstrated the production of large numbers (~10^5/ml) of water-resistant cysts with type I morphology (smooth, oval, phase bright, with cyst organelles visible in relief by differential interference contrast microscopy). Some of the cysts (range, ~1 to 9.5% of total cysts) completed the life cycle by excysting in vitro, but most of the trophozoites emerged only partially from the cyst wall. Since ~90% of the type I cysts were viable (7), as determined by the fluorogenic dye method of Schupp and Erlandsen (19), we hypothesized that the efficiency of excystation could be increased. Therefore, the goals of the present study were to achieve high-efficiency excystation of in vitro-derived *G. lamblia* cysts and to gain an increased understanding of this important biologic process.

MATERIALS AND METHODS

Materials. Unless otherwise noted, all reagents were obtained from Sigma Chemical Co. (St. Louis, Mo.). Bicarbonate was purchased from Fisher Scientific, and crude trypsin, from U.S. Biochemicals, was the gift of Judith Sauch. Cysts were isolated from the feces of a patient with symptomatic giardiasis by the method of Douglas et al. (5).

Human pancreatic fluid. An adult male volunteer was intubated orally with a duodenal tube (AN 20; A. W. Anderson, Santa Monica, Calif.). The tube was allowed to pass through the stomach and into the duodenum under fluoroscopic guidance to an area between the ampulla of Vater (common bile duct) and the beginning of the jejunum. Before samples were collected, all residual pancreatic and duodenal fluids were withdrawn through the duodenal tube and discarded. Basal pancreatic secretions were collected for 15 min. Pancreatic secretions were then stimulated by intravenous injection of 75 U of secretin (Ferring Laboratories, Sufferin, N.Y.). Two sequential 15-min stimulated pancreatic fluid samples were then collected.

Parasite cultivation. *G. lamblia* WB (ATCC 30957) was routinely cultivated in Diamond TYI-S-33 medium (4) with 10% adult bovine serum (Irvine Scientific) and bovine bile (13) but without added iron, vitamins, or antibiotics as previously described (7), with subculturing twice weekly. The same lots of Biosate (BBL) and serum were used.

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throughout these experiments, since any change which affected growth tended to decrease encystation.

**Encystation.** (i) **Pre-encystation cultures.** The condition of the pre-encystation trophozoite monolayer appeared to play an important role in the efficiency of in vitro encystation (7). Cultures grown to the late log phase in growth medium were chilled, inverted 12 times, and counted in a hemacytometer chamber. Trophozoites (5,000 per ml, final concentration) were added to chilled pre-encystation medium. The pre-encystation medium was freshly prepared TYI-S-33 growth medium (pH 7.1) containing the antibiotics piperacillin (500 \( \mu \)g/ml; Lederle Laboratories) and amikacin (125 \( \mu \)g/ml; Bristol Laboratories) but no bovine bile. Pre-encystation cultures (in 8-ml borosilicate glass screw-capped tubes [13 by 100 mm]) were grown for 3 days at 37.5\(^\circ\)C and at 5\(^\circ\) from horizontal. Cultures were still in the log phase, and monolayers were \( \sim 50\) to 80\% confluent. The tubes were inverted eight times, and the unattached trophozoites and medium were discarded. The attached trophozoite monolayers were refed with fresh encystation medium (see below).

(ii) **Encystation cultures.** Unless otherwise specified, the encystation medium consisted of pre-encystation medium adjusted to pH 7.8 with 1 M NaOH and supplemented with porcine bile (0.25 mg/ml, final concentration) and lactic acid (hemi-calcium salt, 5 mM, final concentration) (7). The medium, bile (10-mg/ml stock solution), and lactic acid (100 mM stock solution) were all freshly prepared and filter sterilized separately. Encysting trophozoites were incubated for 66 h, since initial experiments showed that this length of incubation was optimal for the biologic activity of cysts (unpublished data). Parasites were harvested and water-washed in 15 ml of double-distilled water, and incubated for 30 to 45 min at room temperature in 15 ml of double-distilled water. Trophozoites and cysts with incomplete walls were decanted into 15-ml conical centrifuge tubes, pelleted, washed in 15 ml of double-distilled water, and incubated for 30 to 45 min at room temperature in 15 ml of double-distilled water. Trophozoites and cysts with incomplete walls were lysed by this treatment. Type I and type II water-resistant cysts were collected by low-speed centrifugation (5 min, 135 \( \times \) g, 4 to 10\(^2\)C), suspended in the volume (-200 \( \mu \)l) of water remaining in the tube, and enumerated in a hemacytometer chamber by differential interference contrast microscopy (7). As defined previously (7), type I cysts have a smooth, oval shape, are somewhat refractile, and have cyst organelles appearing in relief. Cysts not fulfilling these criteria are referred to as type II.

**Encystation.** Unless otherwise specified, encystation was carried out on the same day that in vitro-derived cysts were harvested. Our standard encystation procedure incorporates variations of the two-step method of Rice and Schaefer for fecal cysts (16), based on the results of the experiments presented here. The variables tested are described in each experiment. A low-pH induction solution was prepared fresh by mixing 6.8 ml of Hank's balanced salt solution containing l-cystine hydrochloride (57 mM) and reduced glutathione (32.5 mM) with 6.8 ml 0.1 M NaHCO\(_3\) and 11.3 ml of double-distilled water. The pH of the solution was adjusted to 4.0 with 0.01 N HCl. In the first, or induction step, 50 \( \mu \)l of cyst suspension (\( \sim 10^6 \) to \( 5 \times 10^6 \) cysts) and 0.55 ml of the low-pH induction solution were mixed in a 1.5-ml Eppendorf tube. The tube was capped, vortexed, and incubated for 30 min in a 37.5\(^\circ\)C water bath. The cysts were sedimented for 3 min at 135 \( \times \) g and room temperature, and the supernatant was aspirated and discarded.

In the second, or encystation, step, 1 ml of a prewarmed mixture containing 1 mg of chymotrypsin (from bovine pancreas, 3X crystallized, 50 U/mg of protein) in Tyrode salt solution (16), with the pH raised to 8.0 with freshly prepared 7.5\% sodium bicarbonate, was added to the cyst pellet. The tube was capped, vortexed, and incubated for 30 min in a 37.5\(^\circ\)C water bath. The cysts were sedimented for 3 min at 135 \( \times \) g and room temperature, and the supernatant was removed to avoid prolonged exposure to chymotrypsin, which was detrimental to the emerging trophozoites.

After the encystation step, 100 \( \mu \)l of prewarmed TYI-S-33 growth medium was added to the pellet, which was suspended with a Pipetman, to promote trophozoite motility and viability. Twenty microliters of each sample was loaded into a hemacytometer chamber, which was placed in a 37\(^\circ\)C humidified chamber. Partially and totally excysted trophozoites and intact cysts were enumerated by differential interference contrast microscopy with a 20X objective. Early experiments showed that the percent encystation did not change significantly during the time required to count all the samples (~4 h).

The formula of Bingham et al. (1) was used to determine percent encystation. The original formula was as follows: percent encystation = \([\{(TET/2) + PET\}/(TET/2) + PET + IC\}] \times 100\), where TET is the number of totally excysted trophozoites, PET is the number of partially excysted trophozoites, and IC is the number of intact cysts. PET are motile and active but not completely emerged from the cyst wall. This formula included a twofold factor based on the observation that TET from fecal cysts divided immediately upon emergence, producing two trophozoites. However, on the basis of morphologic observations, the division of strain WB trophozoites emerging from cysts prepared in vitro was delayed by several hours. Therefore, for in vitro cysts, we did not divide TET by two, unless the trophozoites had already divided, as determined microscopically.

Each figure shown is from a single experiment representative of two to six repetitions with different cyst preparations. Each determination represents an average of four fields with \( \sim 200 \) cells per field. The results of each repetition were consistent with those shown in the figures. Significance was determined by Student's t test.

**RESULTS**

**Roles of acid and protease treatments in encystation.** In an initial experiment, we assessed two published methods of encystation. With the method of Rice and Schaefer (16), we observed 13.5\% \( \pm \) 0.8\% encystation. Moreover, \( >97\% \) of the excysted trophozoites emerged completely from the cyst wall. In contrast, with the method of Schupp et al. (20), which does not include protease treatment, we observed only 4.3\% \( \pm \) 0.8\% encystation. Moreover, most (90.1\%) of the trophozoites were only partially excysted, meaning that the trophozoites did not emerge completely from the cyst wall or remained partially adherent to the cyst shell (\( P < 0.0005 \)). Therefore, we investigated the two major parameters of the method of Rice and Schaefer: pH in the first, or induction, step and treatment of cysts with protease in the second, or emergence, step.

The studies of Bingham et al. (1, 2) demonstrated the importance of the exposure of fecal cysts to a low pH for subsequent encystation. Therefore, we assessed the effect of exposure to an acidic pH on encystation of in vitro-prepared cysts (Fig. 1). The pH curve was quite broad, with 10 to \( >35\% \) encystation after exposure to pH 2 to 8, with an optimum at pH 4.0, which was used in subsequent experi-
ments. In other experiments, excystation did not differ greatly at pHs between 2 and 4 but always decreased gradually with increasing pH above pH 4. Although the proportion of totally excysted trophozoites decreased slightly with increased pH, it remained >80%.

Following exposure to gastric acid, ingested cysts pass into the small intestine, where they are exposed to pancreatic proteases at a slightly alkaline pH. This process was modeled by Rice and Schaefer (16), who treated fecal cysts with a crude preparation of trypsin. To elucidate the role of proteases in excystation, we exposed in vitro-derived cysts to crude and purified trypsin preparations and to purified chymotrypsin. The highest level of excystation (19.2% ± 7.7%) was observed following exposure of in vitro-derived cysts to purified chymotrypsin, as compared with 15.7% ± 5.1% with crude trypsin at 5 mg/ml or 6.8% ± 1.6% with purified trypsin at 1 or 5 mg/ml (data not shown). Since chymotrypsin was effective at lower concentrations, we used it in all subsequent experiments.

We next compared the effects of a low pH and proteolytic treatment on the excystation of cysts isolated (5) from the feces of a patient with symptomatic giardiasis (Fig. 2). Excystation was maximal after exposure to pH 2.0 and almost absent after exposure to pH 6.0. Moreover, chymotrypsin stimulated the excystation of fecal cysts by approximately twofold. The maximal excystation (~38%) of fecal cysts in this experiment was directly comparable to that of the in vitro-derived cysts in Fig. 1, since these experiments were performed at the same time.

Since trypsin and chymotrypsin have different substrate specificities, we next tested whether other endoproteases would also stimulate excystation of in vitro-derived cysts. Four relatively nonspecific proteases, proteinase K, subtilisin, thermolysin, and elastase, stimulated excystation as efficiently (14.0% ± 3.3% to 19.7% ± 1.6%) as did chymotrypsin (16.5% ± 1.2%; P > 0.05), but neither the exoproteases carboxypeptidase A and leucine aminopeptidase nor pepsin (incorporated into the low-pH induction step) stimulated excystation (data not shown).

**Stimulation of excystation of in vitro-derived cysts by pancreatic fluid.** Since trypsin and chymotrypsin are secreted into the small intestine by the pancreas, we tested the effects of pancreatic secretions from a healthy human volunteer on excystation. Pancreatic fluids collected from the same subject before and after stimulation with secretin (3) greatly increased excystation (P < 0.002) (Fig. 3). Stimulated pancreatic secretions were more effective at lower concentrations and consistently yielded higher levels of excystation than unstimulated pancreatic secretions. Both stimulated pancreatic fluid and chymotrypsin increased excystation over a >100-fold concentration range. Concentrations of stimulated pancreatic fluid above 2% or of chymotrypsin above 1 mg/ml led to decreased excystation (data not shown).

To determine whether the stimulation of excystation by pancreatic fluid was due to chymotrypsin and/or trypsin activity, we assessed the effect of soybean trypsin inhibitor, an inhibitor of both proteases (Fig. 4). Stimulation of excystation by both chymotrypsin and pancreatic fluid was virtually eliminated by soybean trypsin inhibitor (P < 0.01).

**Effect of cyst storage on biological activity.** Since Bingham et al. (1) showed that fecal cysts were able to excyst after >30 days of storage in distilled water at 8°C, we monitored the biologic activity of two preparations of in vitro-derived cysts from the day they were harvested (day 1 in Fig. 5). Biologic activity was detected for up to 26 days of incubation at 4°C, after which excystation dropped below 0.1%. Although the initial percent excystation of the two cyst preparations differed by a factor of approximately three, it was highest on the day of harvest and declined with incubation at 4°C.

**Effect of excystation conditions on the biologic activity of cysts.** Using the optimized excystation procedure, we tested the hypothesis that the conditions of excystation determine cyst biologic activity and the idea that metabolites of colonic bacterial flora may be important for this process. To do this, we assessed the effects of organic acids produced by the colonic bacterial flora (3) on both the numbers of cysts and the efficiency of excystation. None of the organic acids tested increased the numbers of total cysts as compared with porcine bile alone (at pH 7.8), although lactic acid led to slightly increased numbers of type I cysts (Fig. 6A) (7). In contrast, the percent excystation was increased more than fourfold by the inclusion of 5 mM lactic acid in the excystation medium (P = 0.001) (Fig. 6B).
DISCUSSION

Taken together, data from our laboratory (6–9, 24) and other laboratories (e.g., 1, 2, 11, 13, 16, 23) show that conditions encountered by *G. lamblia* during each step of its natural life cycle are crucial to reproducing that step in vitro. By varying the conditions of encystation (7) and excystation, we have consistently obtained in vitro-derived cysts of *G. lamblia* with levels of biologic activity comparable to those of fecal cysts.

Our experiments, based on earlier work with fecal cysts (2, 16), have permitted us to identify two parameters necessary for the excystation of in vitro-derived cysts. The first, exposure of in vitro-derived cysts to a low pH, which mimics the passage of ingested cysts through the stomach, was shown by Bingham et al. (1, 2) to be crucial for the excystation of fecal cysts. The second, exposure of in vitro-derived cysts to trypsin, as reported by Rice and Schaefer (16) for fecal cysts, or to chymotrypsin, mimics the passage of cysts into the lower duodenum, where they are bathed in pancreatic secretions containing these proteases (3).

At the optimal pH for each, we observed equal excystation of fecal and in vitro-derived cysts. The optimal pH (2.0) for triggering the excystation of fecal cysts was slightly lower than that for triggering the excystation of in vitro-derived cysts (4.0). A second difference was that excystation of in vitro-derived cysts was totally dependent upon exposure to proteases, whereas excystation of fecal cysts was depressed only by 50 to 60% in the absence of proteases.

Differences between the excystation of in vitro-derived and fecal cysts may simply be related to differences between strains. The WB strain, which typifies the most common group of *G. lamblia* isolates (14), was isolated in 1979 from a patient infected in Afghanistan, whereas the fecal cysts used in this study were isolated from a patient infected in Kenya. Alternatively, the physical state of the wall of in vitro-

![FIG. 3. Stimulation of the excystation of in vitro-derived cysts by pancreatic fluid. Unstimulated or stimulated pancreatic fluid or chymotrypsin at the concentrations shown was substituted for the standard 1-mg/ml concentration of chymotrypsin in the second step of excystation. *, Significant increase in percent excystation ($P \leq 0.002$), compared with the control with no pancreatic fluid or chymotrypsin.](http://iai.asm.org/)

![FIG. 4. Inhibition by soybean trypsin inhibitor of stimulation of the excystation of in vitro-derived cysts by pancreatic fluid or chymotrypsin. Chymotrypsin or pancreatic fluid was preincubated with soybean trypsin inhibitor at the indicated concentrations for 15 min at 37°C prior to being used in the second step of excystation. *, Significant inhibition of excystation by soybean trypsin inhibitor ($P < 0.001$). **, Significant inhibition ($P = 0.006$); stim., stimulated; unstim., unstimulated.](http://iai.asm.org/)
derived cysts may differ in subtle ways from that of the wall of fecal cysts. This difference may be due to incubation of the latter within the fecal mass both before and after passage. For example, the cyst wall may be acted upon by bacterial or host enzymes or metabolites.

Exposure of cysts to an acidic pH has been a hallmark of published excystation procedures (1, 2, 11, 17, 18). The pH curve for excystation of in vitro-derived cysts was broad. The percent excystation was maximal at pH 2 to 4 but always declined steadily at pHs above 5. Even so, substantial excystation (25 to 35% of maximal) was observed following exposure to pH 7 to 8. This result may have been due to prior exposure of cysts during harvesting, incubating, and washing in double-distilled water at a pH of ~5.7. Such exposure may trigger low levels of excystation which can be subsequently increased by exposure to a lower pH. This process may explain the incidence of giardiasis in patients with reduced gastric acidity.

In the present study, excystation of in vitro-derived cysts was >90% dependent on exposure to proteases in the second step. The stimulatory activity of human pancreatic fluid was due to trypsin and chymotrypsin, since it was virtually abolished by soybean trypsin inhibitor, an inhibitor of both proteases. This observation shows that other components in or collected with the pancreatic secretions, such as bile salts or other enzymes, are not required, although they may have a contributory role (11). The requirement for proteases was not specific, since all endoproteases tested were effective. The idea that proteins are important components of the cyst wall is consistent with our earlier observation that cyst antigens separated by polyacrylamide gel electrophoresis and transferred to nitrocellulose were digested by trypsin (15).

Compared with the walls of fecal cysts, the walls of in vitro-derived cysts appeared by light microscopy to be more transparent after protease treatment. However, trophozoites always emerged from a pole, as reported earlier for fecal cysts (2), suggesting the presence of a protease-sensitive component or area at the pole of the cyst, possibly produced or unmasked by prior exposure of the cyst to acid and/or thiol-reducing agents. In contrast, treatment of cysts with purified chitinase did not stimulate excystation (data not shown), suggesting that chitin, which was previously reported to be a cyst wall constituent by one group (22) but not by another (12), may not be located at the site of trophozoite emergence.

Excystation is the most stringent criterion of cyst biologic
activity and is extremely sensitive to the conditions of encystation. For example, although encystation in the presence of lactic acid led to relatively small (∼20 to 100%) (Fig. 6) (7) increases in type I cysts but not in total cysts, it stimulated encystation strongly (more than fourfold). The idea that lactic acid may have a role in the terminal stages of encystation is consistent with the fact that it is a major metabolite of bacteria in the large intestine. Moreover, the addition of lactic acid to cultures after 24 h in encystation medium did not decrease encystation (data not shown).

Our earlier study (7) showed that viability, defined by the fluorogenic dye assay of Schupp and Erlandsen (19), was a less stringent criterion for cyst quality, since viable cysts were not necessarily able to excyst (20). Similarly, fecal cysts retained the ability to exclude eosin (viability) after they lost the ability to excyst (1).

In the present study, encystation of different preparations of in vitro-derived cysts using the same encystation and excystation procedures varied from ∼8 to 38%. This value is well within the broad range (1 to 95%) reported for cysts isolated from human patients (17). Nonetheless, the apparent efficiency of encystation of in vitro-derived cysts may be low because the calculation is based on total numbers of water-resistant cysts, a heterogeneous population of which only ∼10 to 30% has type I morphology. While ∼90% of type I cysts are viable (7) and therefore are theoretically capable of excystation, the remaining type II cysts include cysts with a shrunken cytoplasm as well as cysts which appear morphologically less mature. Only ∼33% of type II cysts were found viable (unpublished data) by the fluorogenic dye method (19). We have not been able to determine whether type I or type II cysts or both excyst, because the excystation procedure obscures cyst morphology. However, on the basis of morphology and viability, type I cysts may be more competent at excystation. The variability in cyst morphology is not an artifact of encystation in vitro, since both type I and type II cysts are isolated from feces (7, 19). Moreover, the numbers of cysts passed by infected humans fluctuate greatly. We have observed cyst numbers from ∼10⁶/g of stool to barely detectable in the same untreated patient (unpublished data; 21), suggesting that the formation and/or shedding of cysts in vivo is highly sensitive to variations in conditions which have not yet been identified. Our in vitro encystation conditions yield more consistent numbers of biologically active cysts.

Our experiments have shown that a low pH and pancreatic proteases which are important for the excystation of fecal cysts also induce high-efficiency excystation of in vitro-derived cysts. These observations again underscore the value of mimicking human gastrointestinal tract conditions for completing the life cycle of G. lamblia in vitro. The availability of all stages of the life cycle of G. lamblia will permit new cellular, molecular, and immunologic studies of this important pathogen.

ACKNOWLEDGMENTS

We are grateful to our colleagues at the Environmental Protection Agency for suggesting the method of Rice and Schaefer, to D. Reiner for stimulating discussions, to Dan Hogan (Division of Gastroenterology, University of California, San Diego) for the pancreatic fluid, to C. Davis and J. Sauch for critiquing the manuscript, to W. Strum (Scrpps Clinic) for patients, and to S. McFarlin for preparing the manuscript. This study was supported by U.S. Environmental Protection Agency cooperative agreement CR 814373 and by Public Health Service grants AM 35108, AI 24285, and AI 19863 from the National Institutes of Health.

LITERATURE CITED


