IN VITRO ENCYSTATION OF GIARDIA LAMBLIA: LARGE-SCALE PRODUCTION OF IN VITRO CYSTS AND STRAIN AND CLONE DIFFERENCES IN ENCYSTATION EFFICIENCY

Anne V. Kane, Honorine D. Ward*, Gerald T. Keusch*, and Miercio E. A. Pereira*
Intestinal Microbiology Laboratory, Center for Gastroenterology Research on Absorptive and Secretory Processes, New England Medical Center, Box 842, 750 Washington Street, Boston, Massachusetts 02111

ABSTRACT: A method for obtaining large numbers of Giardia lamblia cysts in vitro was developed based on modification of earlier methods of in vitro encystation. Maximal numbers of cysts were obtained by growing trophozoites to confluence in TYI-S-33 growth medium containing 0.5 mg/ml of bovine bile, followed by incubation in medium containing 10 mg/ml of bovine bile, at pH 7.8 for 96 hr at 37 C. Up to $4 \times 10^5$ cysts were obtained per milliliter of encystation medium. Cysts thus obtained were similar in structure to those in vivo, were resistant to hypotonic lysis, and reacted with a cyst-specific monoclonal antibody. Further modification of this method by returning the trophozoites to growth medium after 24 hr of exposure to encystation medium resulted in production of cysts that were shown to be viable by fluorogenic dye staining and ability to excyst. This method was scaled up using roller bottles, which resulted in production of up to $1.6 \times 10^8$ cysts per roller bottle. In addition, of 4 strains tested, the LT strain yielded the highest number of cysts. Of 4 clones of the WB strain, clone A consistently produced the largest number of cysts.

Giardia lamblia is a significant cause of diarrheal disease worldwide (Dupont and Sullivan, 1986). Infection is initiated by ingestion of cysts, followed by excystation and release of the trophozoite. Colonization occurs in the duodenum where some trophozoites undergo encystation and are excreted into the external environment to complete the life cycle. Study of the processes of excystation and encystation is essential not only from the point of view of determining the developmental biology of the parasite but also for designing strategies to prevent differentiation of one form of the parasite to the other. The ability to induce these developmental changes in vitro is critical to that effort.

Recently, Gillin et al. (1987) induced encystation in G. lamblia strain WB by replacing bovine bile in the growth medium with various primary and secondary bile salts with or without oleic acid. Schupp et al. (1988) reported the production of cysts from 6 isolates of G. lamblia in vitro simply by increasing the concentration of bovine bile in the TYI-S-33 medium to 5 mg/ml. In addition to appearing structurally similar to in vivo cysts by light and electron microscopy and by reaction with a cyst-specific antibody, these cysts were found to be viable by fluorogenic dye staining, animal infectivity, and ability to excyst. These same criteria for viability of in vitro-derived cysts subsequently were upheld by studies (Gillin et al., 1988, 1989) that employed several encystation media to examine the relationship between bile composition and the quality and quantity of resulting cysts. The optimal conditions as determined by these studies were growth in TYI-S-33 medium without bile with subsequent encystation in the same medium at pH 7.8, containing porcine (0.25 mg/ml) bile plus lactic acid (5 mM). The maximum cyst yield attained by Gillin et al. (1987, 1988, 1989) or by Schupp et al. (1988) was $\sim 1 \times 10^5$/ml medium, with a viability of $\sim 50\%$.

Studies directed at the biochemical and molecular mechanisms underlying the process of encystation require large quantities of cysts for carbohydrate and protein analysis as well as for DNA and RNA extraction. The first aim of the present study was to determine optimal conditions for the large-scale production of in vitro cysts. Because recent reports have suggested marked variation in structural and functional parameters of different strains, as well as among different clones of the same strain (Nash, 1989), a second aim was to determine the relative encystation efficiencies of various strains and clones of the parasite.

MATERIALS AND METHODS

Trophozoite cultivation

Trophozoites of the Portland 1 (Meyer, 1976), WB (Smith, Gillin, Spira, and Nash, 1982), LT (Smith,
Gillin, Kaushal, and Nash, 1982), and cat (Meyer, 1970) strains of *G. lamblia* as well as clones of the WB strain derived by limiting dilution in 2.5% soft agarose (Gillin and Diamond, 1980) were maintained in axenic culture by subculture twice weekly in growth medium (filtered sterilized TY1-S-33 medium containing 10% bovine serum [Biofluids, Rockville, Maryland], 0.5 mg/ml of bacteriological grade bovine bile [Sigma Chemical Co., St. Louis, Missouri, catalogue no. B8381], 100 U/ml of penicillin, and 0.1 mg/ml of streptomycin and adjusted to pH 7.1 [Keister, 1983]). Cultures were grown to confluence (usually 66–72 hr) at 37 °C in 15 × 125-mm (14-ml) borosilicate glass tubes.

**In vitro encystation**

After trophozoites were grown to confluence, the tubes were inverted several times, and the spent growth medium together with unattached trophozoites was poured off. The tubes then were filled with encystation medium, which consisted of growth medium adjusted to pH 7.8, containing bacteriological grade bovine bile 10 mg/ml (unless otherwise noted, as in experiments outlined below). Tubes then were returned to 37 °C. In initial experiments, trophozoites were incubated in encystation medium until the time of harvest (96 hr). For the later studies, cultures were chilled after 24 hr in encystation medium, pelleted by centrifugation at 500 g for 10 min, resuspended in growth medium, and incubated at 37 °C until harvest. Cultures were harvested by chilling the tubes, followed by centrifugation at 500 g for 10 min. Parasites were washed twice in 20 mM sodium phosphate, pH 7.1, containing 150 mM sodium chloride and counted by phase microscopy using a hemocytometer. To obtain cysts alone, trophozoites were removed by hypotonic lysis in distilled water for 1–24 hr at 4 °C, followed by successive washing in distilled water to remove debris. Cysts resistant to hypotonic lysis then were enumerated.

To ascertain the optimal pH of the encystation medium, trophozoites (strain WB) were encysted for 96 hr as described above using growth medium supplemented with 5 mg/ml bovine bile and adjusted with 1 N NaOH to various pHs ranging from 7.1 to 8.2. To determine optimal bile concentration, parasites were encysted for 96 hr in growth medium adjusted to pH 7.8 containing various concentrations of bovine bile (2.5, 5.0, 7.5, 10.0, and 12.5 mg/ml). To determine the time course of appearance of cysts, cultures were harvested 24, 48, 72, 96, and 120 hr after initiation of encystation.

Once the optimum conditions for production of cysts resistant to hypotonic lysis were established, factors affecting the viability of these cysts were examined. To determine the time of exposure to encystation medium required to initiate encystation, confluent cultures of attached trophozoites (strain WB clone A) were incubated in encystation medium. At various times (1, 2, 4, 8, and 24 hr) after addition of encystation medium, tubes were chilled and encysting parasites pelleted by centrifugation at 500 g for 10 min. Encystation medium then was aspirated and replaced with growth medium, and cultures were returned to 37 °C. Forty-eight hours after initial exposure to encystation medium, the parasites were harvested. Cysts were counted and assessed for viability by fluorogenic dye uptake/exclusion as described below.

To determine the optimal time of harvest for maximum viability of cysts, confluent cultures of trophozoites (strain LT) were incubated in encystation medium for 24 hr, then returned to growth medium at 37 °C as above. Cultures were harvested 48, 72, and 96 hr after initial exposure to encystation medium, and the cysts were counted and stained for viability.

**Staining of in vitro cysts by GCSA-1**

In vitro cysts were fixed in methanol and stained with GCSA-1, a cyst-specific monoclonal antibody using an immunofluorescence assay as described (Ward et al., 1990).

**Fluorogenic dye staining for viability**

Viability was assessed using uptake of fluorescein diacetate (FDA) and exclusion of propidium iodide as described by Schupp and Erlandsen (1987). Stained preparations were examined with a Nikon fluorescence microscope at excitation wavelengths of 510–560 nm and 450–490 nm. The percentage of cysts staining positive by FDA was determined.

**Excystation**

Excystation was performed using a modification of the methods of Buchele et al. (1987), Schupp et al. (1988), and Boucher and Gillin (1990). For each excystation, 4–10 sterile culture tubes containing 6 ml of excystation medium (12 mM cysteine HCl/ascorbic acid in Hanks' salt solution containing 1,500 U pepsin/ml) were inoculated with 4 × 10⁵–1 × 10⁶ cysts produced in vitro. The tubes were incubated at 37 °C for 30 min, neutralized by the addition of 1.0 ml 1 N NaHCO₃, and centrifuged at 1,000 g for 5 min at room temperature. The supernatant portion was aspirated and tubes were refilled with fresh growth medium and incubated at 37 °C. In a variation of this method, cysts that had been exposed to the cysteine/ascorbic acid solution (with or without pepsin) subsequently were incubated for 30 min at 37 °C with 1 mg/ml α-chymotrypsin I-S in Tyrode's solution pH 8.0. The encystation medium then was replaced with growth medium and tubes were incubated at 37 °C and examined periodically for the presence of trophozoites.

**Production of cysts in roller bottles**

Trophozoites (strain LT) were grown to confluence in growth medium in 550-ml capacity “outside-in” roller bottles (Bellco) as described by Farthing et al. (1982). Confluent cultures were induced to encyst by pouring off the growth medium together with unattached trophozoites and replacing it with encystation medium, at pH 7.8, containing 10 mg/ml of bacteriological grade bovine bile. Roller bottles were incubated at 37 °C at 1 revolution/hr for 24 hr. Parasites were harvested by immersing the bottles in ice for 30 min, pouring the contents into sterile centrifuge bottles, and centrifuging at 150 g for 30 min. The encysting trophozoites then were resuspended in growth medium and returned to roller bottles for an additional 24 hr at 37 °C. Parasites then were harvested as above, washed, incubated in distilled water, and the number of cysts enumerated. Excystation efficiency (cyst yield/ml of encystation medium) of parasites grown in roller bottles was compared to that of parasites grown concurrently in 14-ml glass tubes.
Statistical analysis

All experiments were performed in triplicate in 15 × 125-mm borosilicate glass tubes unless otherwise indicated. All experiments were performed at least twice. Data shown are from a representative experiment, unless otherwise noted. The cyst count is the mean ± SE of the number of cysts obtained per milliliter of encystation medium. Standard errors are shown as error bars in the figures. Statistical significance was assessed by the 1-tailed, paired Student's t-test.

RESULTS

In vitro encystation

Initial attempts to induce axenically cultivated trophozoites of *G. lamblia* strain WB to encyst as described earlier (Gillin et al., 1987, 1988) yielded minimal numbers of cysts (<10⁵/ml). Likewise, in our hands, the yield obtained by the method of Schupp et al. (1988) was low, 1.75 (±0.93) × 10⁴/ml medium. Modification of these procedures, combining the factors of increased pH with increased bovine bile concentration, resulted in the production of larger numbers of cysts (1.2 ± 0.1 × 10⁵/ml). Cyst formation was observed within 24 hr of exposure to encystation medium with a progressive increase in number of cysts until a maximum was reached within 96 hr (Fig. 1). The cyst yield rose steadily with increasing pH of the encystation medium, to a peak at 7.8 followed by a rapid decline at higher pH values (Fig. 2). Increase in bile concentration from 2.5 to 5.0 mg/ml resulted in an abrupt increase in the number of cysts, with a small but not statistically significant increase at higher concentrations (Fig. 3). As 2 commercial preparations

![Figure 1](image1.png)  
**Figure 1.** Time course of encystation. Confluent cultures of *Giardia lamblia* trophozoites strain WB were incubated in encystation medium containing 10 mg/ml of bacteriological grade bovine bile, adjusted to pH 7.8, for various times, then harvested as described in Materials and Methods. *P* values are for cyst counts compared to those obtained at 96 hr. *P* < 0.05.

![Figure 2](image2.png)  
**Figure 2.** Effect of pH of encystation medium on cyst yield. Trophozoites (*Giardia lamblia* strain WB) were incubated for 96 hr in medium containing 5 mg/ml of bacteriological grade bovine bile, adjusted to various pHs as described in Materials and Methods. *P* values are for counts compared to those obtained at pH 7.8. *P* < 0.05.

![Figure 3](image3.png)  
**Figure 3.** Effect of bile concentration of encystation medium on cyst yield. Trophozoites (*Giardia lamblia* strain WB) were incubated for 96 hr in medium containing various amounts of bile as described in Materials and Methods. Each point shows the mean of 3 experiments each done in triplicate. *P* values are for counts compared to those at bile concentration 10 mg/ml. *P* < 0.1, **P* < 0.05, ***P* < 0.001.
of bovine bile are available from Sigma (crude [catalogue no. B3883] and bacteriological grade [B8381]), we compared their ability to promote encystation. The bacteriological grade, which we use routinely in the growth medium, was 5 times as effective as the crude extract and therefore was used for all subsequent experiments.

Cysts obtained by this method were resistant to hypotonic lysis and reacted with a cyst-specific monoclonal antibody using an immunofluorescence assay, but they were not viable as determined by fluorogenic dye staining. We therefore determined the length of exposure to encystation medium required to initiate viable cyst production. Brief exposure to encystation medium (1, 2, or 4 hr) failed to yield cysts when cultures were harvested 48 hr after initial exposure. However, replacement of the encystation medium with growth medium after 8 or 24 hr yielded increasing numbers of cysts, with a maximum yield after 24 hr of exposure of 2.1 ± (±0.2) × 10^5/ml and a viability of 21% ± (±1%) as assessed by fluorogenic dye staining.

Because earlier experiments (Fig. 1) had indicated that cyst numbers increase with time, we examined the effect of longer incubation on cyst yield and viability. Incubation of encysting parasites longer than 48 hr increased yield but also decreased viability, which rapidly declined with continued incubation at 37 C, from 42% (±1%) after 48 hr to 18% (±3%) after 96 hr.

Cysts obtained by this modification (Fig. 4a) were resistant to hypotonic lysis by water and stained with the cyst-specific monoclonal antibody as shown in Figure 4b. In addition, up to 50% of these cysts stained positive by FDA uptake (Fig. 4c, d). We were unable to excyst these parasites by exposing them to medium containing cysteine and ascorbic acid as described by Schupp et al. (1988). However, the addition of either pepsin or chymotrypsin resulted in the appearance of excysted trophozoites within 1 hr after cysts were returned to growth medium. Trophozoites counts 5 days after encystation were highest in tubes excysted with both enzymes (3.3 × 10^5 ± 0.9 vs. 1.4 × 10^5 ± 0.8 for tubes with chymotrypsin alone vs. 0.8 × 10^5 ± 0.8 for pepsin alone). All 5 tubes containing cysts excysted with chymotrypsin achieved confluency within 8 days, whereas some of those excysted with pepsin alone required 10 days.

Our current protocol for obtaining viable cysts is shown in schematic form in Figure 5. Using this method we have obtained cysts whose viability by FDA uptake was up to 51.3%, with a mean of 36.2 ± 0.9 (n = 35).

Large-scale production of in vitro cysts

We then scaled up the procedure for obtaining viable cysts using "outside-in" 550-ml capacity roller bottles. There was no significant difference in the number of cysts obtained per milliliter of medium compared to the yield from tubes. Cyst yield per roller bottle of G. lamblia (strain LT) was up to 1.6 × 10^8.

Strain and clone differences in relative encystation efficiency

Various strains of G. lamblia as well as several clones of G. lamblia strain WB were compared for their relative cyst production. As cyst yields showed some batch-to-batch variation, cultures to be compared were grown concurrently and encysted from the same batch of encystation medium. As seen in Figure 6, of the several strains examined, LT resulted in the highest numbers per tube (P = 0.0001). Of the 4 clones of WB tested, clone A consistently produced higher numbers of cysts (P = 0.001). Viability of cysts derived from various strains and clones did not differ significantly within an experiment. To confirm that differences in encystation efficiency were not due to differences in the number of attached trophozoites at the start of encystation, the encystation rates of strain WB clone E, strain WB clone A, and strain LT were compared by encysting equal numbers of log phase trophozoites. The encystation rates (number of resulting cysts ± number of initial trophozoites) were 2.2%, 24.4%, and 56.1%, respectively. Generation times (hr/doubling) for these cultures determined immediately prior to encystation were 7.8, 9.9, and 12.0 hr, respectively.

DISCUSSION

The ability to reproduce the entire life cycle of G. lamblia in vitro has provided an effective means of studying the biochemical and molecular events involved in differentiation. Recent studies have shown that encystation can be triggered in vitro by providing factors characteristic of the upper small intestine, specifically a high pH and increased bile concentration (Gillin et al., 1987, 1988, 1989; Schupp et al., 1988). The present study confirms these findings, outlines a simple method for the production of large numbers of in vitro cysts, and demonstrates differences in encystation efficiency among various
strains as well as different clones of the same strain.

Our initial experiments confirmed the findings of Gillin et al. (1989) that a pH of 7.8 (Fig. 2) was optimal for cyst production. The optimal type and concentration of bile however varied among studies. We found that bacteriological grade bovine bile resulted in the highest number of cysts, with 10 mg/ml being the optimal concentration (Fig. 3). The finding that different commercial preparations of bovine bile differ markedly in their efficacy of cyst production may explain the various results with this source of bile reported in other studies. A comparison of the bile salt profile of these 2 preparations, similar to that done by Gillin et al. (1989) that compared human, porcine, and bovine bile, might suggest which components are critical in triggering the encystation process.

Our initial time course experiments also confirmed the earlier findings of Gillin et al. (1987) that maximal cyst production was achieved after 4 days of incubation in encystation medium. However, the majority of these cysts were of the type II variety (Gillin et al., 1989) with a “cytoplasm which is distorted or shrunken away from the cyst wall” and were nonviable as determined by fluorogenic dye staining. As it is known that high concentrations of bile (Farthing et al., 1983) and high pH (Gillin and Reiner, 1982) inhibit the growth of trophozoites, we postulated that although these conditions were necessary for induction of encystation, they may, however, also decrease the viability of the newly formed cysts.
Earlier studies (Reiner et al., 1989; Ward et al., 1990) have shown that cyst-specific antigens are expressed as early as 8 hr after exposure to encystation medium, well before the appearance of mature cysts, suggesting that commitment to encystation is an early event. We therefore reasoned that a limited exposure to encystation medium followed by return to the nontoxic growth medium may be sufficient to induce differentiation without affecting viability of the encysting parasite. The results of this study suggest that this is the case. In addition, we have shown that although cyst numbers increase with increase in incubation time, viability decreases. This is not unexpected in view of the findings of Bingham et al. (1979) who showed that viability of in vivo cysts held at 37°C declined rapidly. Therefore we harvest cysts 48 hr after initiation of encystation.

Up to 50% of cysts produced by this modified method resemble type I cysts as described by Gillin et al. (1989), “with a smooth oval shape” and “phase bright.”

A major interest of this study was to obtain large numbers of cysts for biochemical and molecular investigations. In the present study we have shown that the roller bottle culture method can be used to produce large numbers (up to 1.6 × 10^8 per roller bottle) of cysts in vitro.

An earlier study by Schupp et al. (1988) alluded to differences in encystation efficiency among various strains of *Giardia*, but details were not provided. Their maximal reported cyst production was by the muskrat strain MR4—1.5 × 10^5 cysts/ml medium. In this study we have shown consistent differences among various strains, with the LT strain being the most efficient of 4 strains tested. In addition we have found clone-to-clone variation. Thus of several clones of the WB strain tested, clone A was the most efficient. These differences are consistent with reported strain and clone differences in a number of other parameters such as infectivity and antigenic composition (Nash, 1989). The reason as well as the biological relevance for these differences remain to be determined. We noted that cultures with longer generation times exhibited
higher encystation rates, which may explain the difference in encystation efficiency among strains and clones.

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