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Compounds of the upper gastrointestinal tract induce rapid and efficient excystation of *Entamoeba invadens*

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Abstract

The infective stage of *Entamoeba* parasites is an encysted form. This stage can be readily generated in vitro, which has allowed identification of stimuli that trigger the differentiation of the parasite trophozoite stage into the cyst stage. Studies of the second differentiation event, emergence of the parasite from the cyst upon infection of a host, have been hampered by the lack of an efficient means to excyst the parasite and complete the life cycle in vitro. We have determined that a combination of exposures to water, bicarbonate and bile induces rapid excystment of *Entamoeba invadens* cysts. The high efficiency of this method has allowed the visualization of the dynamics of the process by electron and confocal microscopy, and should permit the analysis of stage-specific gene expression and high through-put screening of inhibitory compounds.

Keywords

Entamoeba histolytica; *Entamoeba invadens*; Amoebiasis; Cyst; Excystation; Encystation

1. Introduction

Entamoeba histolytica is a potentially invasive intestinal protozoan parasite of humans that causes an estimated 50 million cases of amoebiasis, manifesting as amebic colitis, dysentery and extraintestinal abscesses and 40,000–100,000 deaths annually (WHO/Pan American Health, 1997). Most transmission of *Entamoeba* parasites from an infected host to other potential hosts occurs via the encysted form of the parasite when it contaminates water or food. Following ingestion by a new host, the cyst is exposed to the various biochemicals found in the upper gastrointestinal (GI) tract, which results in the eventual emergence (excystment) of the ameboid trophozoite form via a process that is assumed to occur in the small intestine. The cyst form of the *Entamoeba* parasite, then, must be sufficiently resistant to the hypo-osmotic and polythermic environment outside the intestine, and to the acidic conditions of the stomach and the digestion-aiding compounds of the duodenum. At the same time, however, the encysted parasite needs to be able to sense and respond to certain aspects of these conditions so that the

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excystment process is started only upon re-ingestion and emergence from the cyst occurs in a region of the GI tract that is not toxic to the metacystic trophozoite.

Other protozoan parasites that infect the vertebrate intestine, such as *Giardia* spp. and *Cryptosporidium* spp., also travel between hosts as encysted forms, and the natural conditions that trigger their excystment have been described (Boucher and Gillin, 1990; Robertson et al., 1993; Kato et al., 2001). *Giardia* excystment is particularly responsive to host-supplied low pH conditions and proteases, whereas *Cryptosporidium* responds primarily to low pH and elevated temperature (37°C) (Fayer et al., 1998). For each of these parasites, in vitro conditions have been described that support the efficient excystation of culture- and in vivo-derived cysts or oocysts (Bingham and Meyer, 1979; Robertson et al., 1993), and in the case of *Giardia* the inclusion of other components found in the upper GI tract enhances the rates of in vitro excystation (Rice and Schaeffer III, 1981; Boucher and Gillin, 1990; Feely et al., 1991).

Trophozoites of certain strains of *Entamoeba invadens*, whose natural hosts are reptiles, will efficiently (>95%) form cysts (encyst) in vitro (McConnachie, 1955; Rengpien and Bailey, 1975; Sanchez et al., 1994). However, upon placement in axenic growth medium these culture-generated *E. invadens* cysts typically excyst with very low efficiency (1–10%) and over an extended period of time (24–72 h) (D. Eichinger, unpublished data). This has raised questions about the state of maturation of the cysts made in vitro and the relevance of the stimuli used to induce their excystment. Nonetheless, these in vitro conditions have supported studies that demonstrated or implied the involvement of various parasite-derived enzymes and biochemical pathways during excystment or the establishment of the metacystic trophozoite forms, and that documented ultrastructural changes that occurred during the relatively asynchronous process. Structural components that function during excystation and development of metacystic *E. invadens* trophozoites include those composed of actin and tubulin (Makioka et al., 2001, 2002a, 2004). Roles for calcium fluxes and calmodulin (Makioka et al., 2002b), and for signaling pathways that include protein kinase C (PKC) and phosphatidylinositol 3-kinase (PI3K) (Makioka et al., 2003b), have also been proposed. Both excystation and metacystic development are inhibited if either DNA replication (Makioka et al., 2003a) or cysteine proteinase activity (Makioka et al., 2005) is blocked. Electron microscopy (EM) studies of ultrastructural changes indicated the presence of free trophozoites 24 h after induction of excystation (Chavez-Munguia et al., 2003, 2007). Under these conditions, the final stages of excystation were most evident starting at 24 h, but it was not clear how many of the cells were excysting or how synchronous the process was.

The replicative stages of *Giardia* and *Cryptosporidium* establish residency in the small intestine, and the cysts and oocysts of these parasites must therefore be able to rapidly excyst upon passage through the stomach. In fact, both parasites can be induced to excyst within time-frames of minutes to less than 2 h (Feely et al., 1991). In contrast, the multiplicative phase of *Entamoeba*'s life cycle occurs in the host's large intestine. This more downstream colonic site of infection by *Entamoeba* may seem to offer greater flexibility in the timing of its excystment, but colonic filling in humans can begin as early as 4 h following intake of solid or liquid foodstuffs (Camilleri et al., 1989). To identify the likely excystment stimuli, to examine the flexibility of the timing of the *E. invadens* excystment process, and to establish a more rapid and complete process useful for analysis of ultrastructural and gene expression changes, we tested the excystation-enhancing properties of molecules that the cyst encounters upon ingestion by a host. We report here conditions that produced metacystic amoebae of *E. invadens* in as little as 2 h, with 50% excystation by 6 h and up to 90% excystation by 24 h. Changes in ultrastructure consistent with previous reports occurred at an accelerated rate, and the process was sufficiently synchronous to arrange the ultrastructural changes in a defined sequence and to directly visualize multiple occurrences of the excystment process.

2. Materials and methods

2.1. Cells and reagents

Entamoeba invadens (strain IP-1) was obtained from the American Type Culture Collection (Rockville, USA) and maintained at 25°C in T25 culture flasks containing 50 ml of low glucose (LG) medium (Sanchez et al., 1994) supplemented with 10% adult bovine serum (ABS). Sodium acetate, sodium propionate, sodium n-butyrate, α -amylase, sodium bicarbonate, bile (bovine and ovine), sodium taurodeoxycholic acid, trypsin and α -chymotrypsin were purchased from Sigma-Aldrich (Saint Louis, MO, USA). Sodium taurocholate hydrate was purchased from Alfa Aesar (Ward Hill, MA, USA). Propidium iodide, Hoechst 33342 and SYTO 11 were purchased from Invitrogen (Cleveland, OH, USA).

2.2. In vitro encystment

Late log-phase *E. invadens* trophozoites were chilled and harvested by centrifugation at 160 g, washed once with ice-cold LG medium, suspended at a density of 2×10^5 cells/ml in 47% LG supplemented with 5% serum or other pharmacological agents and incubated at 25°C. After 3 days, culture flasks were chilled on ice, cells were harvested by centrifugation and treated with 0.05% Sarkosyl for 10 min in PBS (12 mM phosphate buffer, 2.7 mM KCl, 136.8 mM NaCl, pH 7.4). The cells were centrifuged (500 g for 3 min), washed three times with distilled water and cyst counts were determined using a haemocytometer.

2.3. Effects on excystation of medium pH, vortexing with glass beads or pre-treatment with acid or sodium hypochlorite

TYI-S-33-based media (TYI) (Diamond et al., 1978), with one pH-unit increments ranging from 4 to 10, were prepared by adjusting the pH with 1 M HCl or 10 N NaOH. Approximately 8×10^5 cysts were suspended in 6.5 ml medium in 13×100 mm² screw-capped borosilicate glass tubes and incubated for 24 h at 25°C to allow excystment to occur. To determine the effect of physical agitation on excystment efficiency, 1.5 g glass beads (425–600 μ m; Sigma-Aldrich) were placed in 15-ml polypropylene conical tubes with 8×10^5 cysts in PBS and vortexed for 30 to 60 s at room temperature (RT). The supernatant was removed, the glass beads and pelleted cells washed twice with PBS, and the pelleted cells suspended in TYI to allow excystment to occur. For acid pre-treatment, $\sim 8 \times 10^5$ cysts were pelleted in a 1.5-ml microfuge tube, the supernatant was removed and the cysts suspended in 1 ml of 0.01–0.02 N HCl or H₃PO₄ (pH 2.0–3.0) for 30 min in a 37°C heat block. Cysts were centrifuged at 2,600 g for 1 min in a 1.5-ml microfuge tube, suspended in TYI and incubated as mentioned above. To determine the effect of sodium hypochlorite on excystation, 8×10^5 cysts were suspended in 1% sodium hypochlorite for 30 s to 10 min, washed with TYI medium and suspended in the same medium for excystment.

2.4. Effects of trypsin, chymotrypsin and short-chain fatty acids (SCFAs) on excystment

To examine the effect of trypsin, chymotrypsin or SCFAs on excystation, approximately 8×10^5 cysts were incubated in TYI either with 0.25–2.0 mg/ml trypsin, mg/ml 0.1–1.0 mg/ml α -chymotrypsin, 7.0–280.0 mM sodium acetate, 2.0–80.0 mM sodium propionate or 1.0–40.0 mM sodium n-butyrate and incubated at 25°C.

2.5. Effect of water pre-treatment and different concentrations of sodium bicarbonate, taurocholate, taurodeoxycholate or bile on excystation

To study the effect of water pre-treatment, detergent-resistant cysts were incubated in distilled water at 25°C for 0, 2, 4, 6, 8, 24 and 32 h, pelleted and suspended in TYI for excystation. To determine the optimal concentrations of sodium bicarbonate, taurocholate, taurodeoxycholate or bile for excystation, 8×10^5 cysts were suspended in TYI medium with varying

concentrations of sodium bicarbonate (5.0–160.0 mM), taurocholate (2.5–40.0 mM), taurodeoxycholate (1.25–40.00 mM) or bile (0.25–16.00 mg/ml) for 24–48 h and excysted cells were counted.

2.6. Induction of excystation using water pre-treatment together with a combined mixture of sodium bicarbonate and bile or bile salts

Based on results from individual treatments, we developed a two-step method that reproducibly yielded the highest excystation efficiency. First, sarkosyl-resistant cysts from 72 h encystation cultures were suspended in distilled water and incubated at 25°C for 6 h. Second, an excystation induction medium was prepared by supplementing LG medium with 10% serum, 2.8% vitamin mix, 1% streptomycin-penicillin, 1% glucose, 1 mg/ml bile or 1.25–2.50 mM bile salts and 40 mM sodium bicarbonate. For each excystment condition, duplicate aliquots of 0.8×10^6 water-treated cysts were suspended in excystment medium in glass tubes and incubated in a slanting position at 25°C for 6 to 48 h. At defined time points the culture tubes were chilled on ice, centrifuged for 2 min at 500 g and all but 1 ml of the medium was discarded. Pelleted cells were suspended by gentle vortexing, and 10 μ l of the sample were examined in a haemocytometer for the percentages of excysted trophozoites and intact cysts.

2.7. Electron microscopy

For the examination of cellular morphology during excystation, water-treated cysts were induced to excyst with the combination of sodium bicarbonate and bile for 0, 2, 4, 6 and 8 h, and fixed in 1% glutaraldehyde/4% paraformaldehyde in PBS for 24 h at 4°C. Specimens were post fixed in 1% osmium tetroxide and 1.5% $K_3Fe(CN)_6$ in PBS for 2 h at RT, followed by incubation in 0.5% uranyl acetate for 1 h. The specimens were dehydrated with increasing concentrations of ethanol and then incubated for 1 h in propylene oxide, followed by incubation for 1 h in a 1:1 mixture of propylene oxide and Epon (Electron Microscopy Sciences, Hatfield, PA, USA). Specimens were subsequently embedded in Epon at 60°C for 2 days. Semi- and ultra-thin sections were obtained using an ultramicrotome RMC MT-7000. Semi-thin sections were dried on slides at 60°C, stained with a mixture of 0.5% methylene blue and 0.5% AzurII (Sigma-Aldrich, Taufkirchen, Germany) for 1 min, and examined using a Zeiss Axiophot microscope with 1,000-fold magnification in combination with an Axiocam (Zeiss, Oberkochen, Germany). Ultra-thin sections were post-stained with 1% uranyl acetate for 30 min. Photographs were taken with a Zeiss EM10 transmission electron microscope and scanned images were processed using Adobe Photoshop 6.0 software.

2.8. Effect of bile and sodium bicarbonate on trophozoite growth

Trophozoites (1.5×10^4 /ml) of *E. invadens* were incubated at 25°C for 72 h in 13×100 mm² screw-capped borosilicate glass tubes in LG medium containing 1 mg/ml bile, 40 mM of sodium bicarbonate or a mixture of 1 mg/ml bile and 40 mM of sodium bicarbonate. Every 24 h aliquots of cells were harvested by chilling and the cell density was determined using a haemocytometer. At each time point the cell density was determined using triplicate cultures.

2.9. Nuclear staining of cysts and metacystic trophozoites

To visualize nuclei, chromatin staining was performed with Hoechst 33342 dye. Cysts or trophozoites (1×10^6) were washed with PBS and centrifuged. The cells were suspended in 1 ml of PBS with 1 μ g/ml of Hoechst dye, mixed gently, wrapped with aluminum foil and incubated for 30 min at 25°C. The cells were washed with PBS, suspended in 30 μ l of PBS and observed using a Nikon Eclipse E600 fluorescence microscope equipped with a 40 $\times$ /0.75 Plan Fluor objective lens and an excitation wave length range of 330–380 nm. The average number of nuclei per cell was determined from 500 randomly chosen cells.

2.10. Cyst viability

Entamoeba invadens cyst viability was determined based on visualized morphology using phase-contrast microscopy and by propidium iodide (PI) staining (Schupp and Erlandsen, 1987). A 200 µl aliquot of cyst suspension was incubated with 10 µl of PI (0.06 mg/ml) in PBS for 10 min at 25°C, washed, suspended in PBS and examined with a fluorescence microscope using an excitation wave length range of 450–490 nm.

2.11. Confocal microscopy

Cultures encysting for 72 h in 47% LG were collected by centrifugation, suspended in 0.05% sarkosyl for 20 min and washed with water. Cysts were treated with water for 6 h and then transferred to LG medium containing the optimal amounts of sodium bicarbonate and bile. Cyst nuclei and walls were labeled by adding 100 µM SYTO 11 (Invitrogen) and 0.2% calcofluor fluorescent dye (Sigma), respectively, to the excystation medium. Labeled cysts were transferred to glass-bottomed dishes (50 mm Ø; Willco Wells, Amsterdam, The Netherlands). After sealing with high vacuum grease, the dishes were examined with a Leica TCS SP2 AOBS confocal microscope. A Ludin chamber was used to adjust the temperature to 25°C. Time series were recorded at 1.629 ms intervals using 488 nm and 405 nm laser lines for the membrane-permeable nuclear dye SYTO 11 and calcofluor-labeled cyst walls, respectively. The laser power was reduced to a minimum to avoid phototoxicity and bleaching (Frevert et al., 2009). Leica Confocal Software (version 2.61 Build 1537), Image-Pro Plus and AutoDeBlur software (both from Media Cybernetics, Silver Spring, MD, USA) were used for image processing. Figure panels were assembled with Adobe Photoshop.

3. Results

3.1. Preparation of cysts and TYI

All cysts of *E. invadens* IP-1 used in the following experiments were harvested from cultures that were induced to encyst for 72 h in 47% LG (Sanchez, 1994) followed by treatment with 0.05% sarkosyl to eliminate trophozoites. Cysts were then treated in various ways as described below and transferred into TYI medium to allow excystation to occur. To first determine whether the pH of the medium into which the excysting cells were placed had an effect on the ability to recover metacystic trophozoites, TYI media with pH values of 4, 5, 6, 7, 8, 9 or 10 were tested. The optimal pH for recovery of excysting trophozoites was between 6 and 7 (data not shown), which encompasses the normal pH (6.4) of unadjusted TYI growth medium, and this was used in all subsequent excystment experiments.

3.2. Exposure to water, acids, proteases and SCFAs

As water is the first extraintestinal condition that cysts released from a host are likely to be exposed to, we tested the effect of exposure to water for different lengths of time on excystation efficiency. Compared to control conditions in which cysts were transferred directly to TYI, pre-incubation in water at 25°C before transfer to TYI increased the recovery of trophozoites, with maximal excystation (of approximately 40%) occurring after treatment with water for a period of 6–8 h (Fig. 1A). Exposure for as little as 2 h substantially increased the excystment efficiency above control levels, and the level remained elevated after exposure for as long as 32 h, but was trending downwards at this time. Treatment with acids, which the ingested cyst would encounter in the stomach, had only a minor enhancing effect on excystation. Both hydrochloric and phosphoric acids stimulated excystation approximately 10–15% above background, whereas acetic and oxalic acids were toxic to the cysts (data not shown). There was no enhancement of excystation following treatment with trypsin, chymotrypsin or the three main SCFAs found in the distal intestine (data not shown).

3.3. Role of sodium bicarbonate, bile or bile salts in excystation

A variety of compounds made by the host that have potential metabolism-altering effects are added to the acidified ingested material as it exits the stomach. Whether the cysts were responsive to bicarbonate was examined by exposing them to a range of concentrations of sodium bicarbonate typical of the upper GI tract. In contrast to acids, bicarbonate had a strong excystation-stimulating effect, with 40–80 mM amounts yielding up to 48–60% excystation in 24 h (Fig. 1B). As little as 5 mM bicarbonate induced 45% excystation by 48 h, versus 8% in the untreated controls at the same time point.

Ingested materials passing out of the stomach also receive a mixture of various bile salts. The taurine-conjugated forms of cholate and deoxycholate, in concentrations ranging from 1.25 to 40 mM, were added to cysts and the levels of excystation determined after 24 and 48 h. Both of these bile salts had strong stimulating effects on excystation (Fig. 1C, D), with the lowest amounts tested in each case yielding the greatest numbers of excysted trophozoites.

Taurocholate (2.5 mM) and taurodeoxycholate (1.25 mM) induced 53 and 70% excystation, respectively, by 24 h and this yield increased only slightly at 48 h. Whole commercial bile, which is a mixture of bile salts and ions, was also tested and TYI medium containing 1 mg/ml bile supported excystation of up to 62% at 24 h and greater than 80% at 48 h (Fig. 1E).

3.4. Combined effects of water pre-treatment, sodium bicarbonate and bile or bile salts on excystation

Various combinations of the different individual stimuli, including a combination of water pre-treatment and sodium bicarbonate, sodium bicarbonate and bile, sodium bicarbonate and taurocholate or sodium bicarbonate and taurodeoxycholate, enhanced excystation after 24 h up to 53–67%, similarly to the individual treatments. (Fig. 2A). However, when cysts were pre-treated with water for 6 h and then cultured in the presence of sodium bicarbonate plus bile or bile salts, excystation was increased dramatically to 88–90% in 24 h, compared with only 8% of the untreated controls excysted (Fig. 2A). To explore the kinetics of excystment during the initial 24 h in response to individual stimuli or to the combinations of stimuli, samples of cells induced to excyst were collected at 6 h intervals and the numbers of excysted cells counted. In cultures exposed to the water/bicarbonate/bile combination treatment, at 6, 12 and 24 h time points the excystation yields were 52, 74 and 92 %, respectively, whereas in untreated controls they were 0, 3 and 7% (Fig. 2B). Exposure for as short a time as 15 min to the combination of bicarbonate and bile, which was then removed, was sufficient to increase excystation above that resulting from water treatment alone, and a 60 min exposure, followed by removal of the compounds, yielded maximal excystment (data not shown).

3.5. Number of nuclei in cysts and excysted trophozoites

To follow the conversion of cysts to metacystic trophozoites and the extent of cell division of trophozoites, the numbers of nuclei were counted in detergent-resistant cysts before excystment and in excysted trophozoites at progressive time points. As shown in Fig. 3A and B, approximately 61% of the starting cysts had four nuclei, and the remaining 39% were evenly distributed amongst those with one to three nuclei per cyst. (There were no free trophozoites at this point). After 6 h in excystment medium, approximately equal percentages (30%) of the excysted trophozoites had either four, three or two nuclei (Fig. 3C). The number of binucleate cells continued to increase at 12 h, primarily at the expense of the tetranucleate cells (Fig. 3D), and by 24 h very few tetranucleate cells remained (Fig. 3E). At 48 h, 60% of the trophozoites had a single nucleus (Fig. 3F).

3.6. Effect of excystment-enhancing compounds on growth of *E. invadens* trophozoites

To determine whether excystment efficiency was being underestimated due to possible toxic effects of sodium bicarbonate, bile or both on metacystic trophozoites, *E. invadens* trophozoites were continuously cultured in TYI containing the various compounds for 72 h. At the 24 and 48 h time points trophozoite growth in the treated cultures was not significantly different from the untreated control (Fig. 4), but after 72 h cell growth had noticeably decreased. This result suggests that up until 48 h the effects of these compounds on excystment are likely accurate, but not beyond that time.

3.7. Effect of storage conditions on cyst viability and percentage of excystation

To start to determine how long a cyst may be infective, biological activity of cysts was monitored for up to 20 days by determining cyst viability and the percentage of excystation. At 4 day intervals cyst samples were harvested from five different storage conditions: 4°C in 47% LG, distilled water or PBS, 25°C in 47% LG, or in liquid N₂. In distilled water or liquid N₂, cyst viability was reduced 95% by 8 days. In PBS, 47% and 28% of the cysts lost their viability in 4 and 20 days, respectively. In 47% LG medium cysts maintained at 4°C and 25°C slowly lost their viability and by 20 days only 50–53% of the cysts remained viable. However, although apparently viable, only 8.4% of the cysts stored at 4°C were able to excyst.

3.8. Proposed sequential events of excystation

Sequential ultrastructural changes occurring during excystment were examined by EM. Cysts were induced for excystment by incubating water-pre-treated cysts in TYI medium containing bile and sodium bicarbonate, and samples were fixed every 2 h during the first 8 h and embedded. Semi-thin sections from each time point were examined to verify the relative numbers of cysts and emergent trophozoites (Fig. 5A, B). In high resolution thin sections at the zero hour time point cysts showed a consistent, poorly-vesiculated cytoplasm containing chromatoid bodies and multiple nuclei (Fig. 6, A, B), typical of normal cysts. The plasma membrane of the encysted trophozoite was in uniform contact with the overlying cyst wall. The first obvious changes at 2 h were the presence of increased numbers of cytoplasmic vesicles and the localized separation of the plasma membrane from the cyst wall (Fig. 6C). As the separation of these two surfaces progressed, the appearance of an invagination of the plasma membrane in a single area of the trophozoite was found in a large number of cysts, as previously described by Chavez-Munguia (Chavez-Munguia et al., 2003) (Fig. 6D, E). Eventually, the majority of cells showed a near complete separation of the plasma membrane and cyst wall (Fig. 6F), although a few cells maintained a single contact point, the visualization of which likely depended on the plane of section (Fig. 6G). Fig. 6H shows a trophozoite that appears to be exiting through an opening in a cyst wall. Metacystic trophozoites, some still with multiple nuclei (Fig. 6I, J), eventually developed the large number of morphologically diverse vesicles typical of vegetative trophozoites.

3.9. Visualization of the dynamics of excystment

The relatively rapid rate of excystment following transfer of cysts to bile/bicarbonate medium suggested the possibility of viewing the excystment process in real time to determine how the trophozoites emerged from the cyst capsules. Mature (72 h) cysts were treated with water/bile/bicarbonate, stained with calcofluor to label the cyst wall and with SYTO11 to label the nuclei, and viewed with a confocal microscope with time lapse recording. Multiple excystment events were visualized, a representative of which is depicted in Fig. 7A. The first apparent change in cyst structure was the loss of calcofluor staining of the cyst wall in an area adjacent to one defined region of the trophozoite surface. The trophozoite appeared to then extend a portion of its membrane beyond the original boundary of the cyst wall in this region, while the rest of the cell remained within the calcofluor-stained compartment. Eventually, empty space

developed between the opposite, encysted portion of the trophozoite and the remaining cyst wall, effectively separating the cyst wall from the trophozoite. This process continued until the trophozoite was completely excysted, at which point the parasite discarded the empty, fishbowl-shaped cyst wall casing. With time, many of these casings accumulated (Fig. 7B) as excystment of other cells took place. The entire process shown in Fig. 7A occurred in 13.5 min.

4. Discussion

The studies reported here used in vitro conditions to examine excystment of the reptile parasite *E. invadens*. Another report previously described the use of compounds found in the upper GI tract on *E. invadens* excystment (Makioka, 2006). In that study excystment was increased in response to exposure to combinations of compounds that are different from those described here, and the resulting rates of excystment were considerably slower than those obtained with the water/bicarbonate/bile treatment described here. Whether cysts of the human parasite, *E. histolytica*, respond in a similar manner to bile and bicarbonate or to distinct compounds of the upper GI tract is being tested. Preliminary results indicate that cysts isolated from infected patients respond to the combined treatment protocol and excyst (Julio C. Villagomez-Castro, personal Communication). Similar digestion-aiding compounds are released into the small intestine by both reptiles and humans, so both species of *Entamoeba* parasite will be exposed to bile acids and bicarbonate as cysts enter the proximal small intestine. How rapidly each species will exit the cyst in vivo is hard to predict. A majority of the *E. invadens* cyst preparations followed here were capable of excysting in less than 2 h, and in early experiments free trophozoites were typically visible within 10–15 min following exposure to bicarbonate and bile, as was later verified with confocal analysis. Although ingested liquids and solids can reach the human large intestine in 3–4 h (Camilleri et al., 1989), these materials will remain in the colon for considerably longer times, so exit from the cyst need not occur prior to entry into the colon. Some of the *E. invadens* excystment activity described here in fact did appear to continue, albeit at lower rates, beyond 12 h. Retention of the parasite within the colon, however, does presumably require the surface expression of gal/galNAc-specific lectins on free trophozoites, so excystment prior to entry into the colon is a likely scenario. Kinetics of *Entamoeba* excystment may in fact be similar to those for *Giardia* and *Cryptosporidium*, which are adapted to reside in the upper GI tract. Whether the rate of excystment of *E. invadens* seen here reflects distinct differences in progression of material through the reptilian digestive system, or is aberrant because of the in vitro origin of the cysts, awaits comparison with rates of in vivo-derived samples.

The in vitro-generated *E. invadens* cysts used here for excystment contained varying numbers of nuclei. *Entamoeba invadens* cysts, like those of *E. histolytica*, are classified as tetranucleate (Clark and Diamond, 1997), but after 72 h in encystment medium only 60% of the *E. invadens* cysts were tetranucleate. This range of nucleus number is similar to that in cysts of *E. histolytica* obtained from infected humans (Julio C. Villagomez-Castro, personal communication). We did not detect an increased rate of excystment of cysts with four nuclei relative to those with lesser numbers, suggesting that all forms are capable of (in vitro) excystment.

It is not yet clear how the three treatments (water, bicarbonate, bile) served to stimulate excystment. Each of the stimuli alone increased excystment levels above background, suggesting that the cyst population may have been variable in its sensitivity to the three stimuli. Water treatment by itself, followed by return to growth medium (which contains salts, amino acids, peptides, etc.), was sufficient to induce some level of excystment. Water exposure also made the cysts more responsive to bicarbonate or bile acids. Each of these latter two compounds was added in the form of a sodium salt, but increased Na⁺ alone was not effective, and not all

bile acids or charged detergents tested were equally effective (not shown). Bile acids are normally deconjugated from their amino acids in the ileum and colon, reabsorbed by enterocytes, and then taken up from circulation by hepatocytes (Hamilton et al., 2007). There they induce changes in gene expression after binding to nuclear receptor proteins (Chiang, 2002). Taurine-conjugated forms of the two bile salts were used here, and in that form they would be less prone to passive diffusion across the amoeba membrane. It is therefore not clear whether they would have effects on gene expression in trophozoites unless they were deconjugated by the amoebae, and this is being tested. Previous studies have reported the effects of bile on the physical properties of *Entamoeba* trophozoites (Yadava and Dutta, 1976; Smith and Meerovitch, 1980), but did not examine changes in protein or gene expression.

In these experiments the two bile acids and whole bile were added to excystation media below their micellar concentrations, so an extracellular detergent-like effect is not likely to account for their activity. However, displacement of cyst wall components, via hydrophobic interactions, could serve to increase the permeability of the wall. In the simplest scenario a physical alteration of the cyst wall and/or the underlying plasma membrane that allowed for increased permeability to, or triggered release of, ions or peptides could account for the response of the cells, and would be consistent with previous descriptions of the roles for Ca^{++} and calmodulin-like proteins during excystment of *Entamoeba* and *Giardia* (Makioka et al., 2002b; Reiner et al., 2003).

The shortened time frame of *Entamoeba* excystment achieved with the described method has allowed for a more precise ordering of the ultrastructural changes that occur during this differentiation process. This protocol is also proving useful for the in vitro screening of compounds that inhibit the excystment process, and should facilitate the analysis of changes in excystation-specific gene expression in both the human and reptile-infecting species of *Entamoeba* parasites.

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Fig. 1.

Effect of various individual treatments on the excystation of in vitro generated *Entamoeba invadens* cysts. (A) 8×10^5 detergent-resistant cysts pre-treated with distilled water for the indicated times were placed in TYI-S-33-based media (TYI) and excystment was examined at 24 and 48 h. To optimize the concentration of sodium bicarbonate (B), taurocholate (C), taurodeoxycholate (D) and bile (E), detergent-resistant cysts were transferred directly to TYI containing the indicated concentrations of each substance, and excystment was examined at 24 and 48 h. Error bars represent the S.E.M. of three independent experiments. C, control.

Fig. 2.

Combined effects of water pre-treatment, sodium bicarbonate (SBC) and bile/bile salts on *Entamoeba invadens* excystation. (A) Approximately 8×10^5 cysts were incubated in TYI-S-33-based media after pre-treatment of cysts in water for 6 h, with or without subsequent addition of SBC, bile/bile salts or a combination of the two. The excystation percentage was determined after 24 h. (B) Kinetics of excystation. Cysts were incubated as in (A) and at the indicated times, cysts and trophozoites were harvested and the excystation percentage was determined.

Fig. 3.

Numbers of nuclei per cell before and during excystation of *Entamoeba invadens*. Seventy-two h detergent-resistant cysts were excysted for the indicated times (B–F), collected by centrifugation, stained with Hoechst dye, and the numbers of nuclei per cell were determined using a Nikon Eclipse E600 fluorescence microscope. A) A photograph of the stained cysts corresponding to B. Error bars represent the S.E.M.

Fig. 4. Effect of sodium bicarbonate and bile on growth of *Entamoeba invadens* trophozoites. Trophozoites (1.5×10^4 cells/ml) were placed in TYI-S-33-based media with sodium bicarbonate (40 mM), or bile (1 mg/ml) or both. At 24 h intervals cells were counted. Error bars represent the S.E.M. of two independent experiments.

Fig. 5.

Visual quantification of excystment of *Entamoeba invadens*. (A) Light micrographs of semi-thin Epon sections. After 6 h water pre-treatment, cysts were incubated in TYI-S-33-based media in the presence of sodium bicarbonate and bile acid. Samples were collected at 0, 2, 4, 6 and 8 h time points, fixed and embedded, and semi-thin sections were stained and examined by light microscopy. Scale bar = 10 μ m. (B) Quantitative analysis of excysted trophozoites and intact cysts of semi-thin sections as shown in A. Error bars represent the S.E.M.

Fig. 6.

Sequential events of *Entamoeba invadens* excystation. Transmission electron micrographs of typical ultrastructural features of cysts and excysting cells during the first 8 h following excystment induction. A and B) Cysts prior to excystment, containing chromatoid bodies (CB) and multiple nuclei (N). Note that the plasma membrane is closely apposed to the inner aspect of the cyst wall. As excystment progresses, spaces (arrows) between the parasite and the cyst wall develop (C–G). D and E) Cells with an invagination of the trophozoite located between flanking points of contact with the cyst wall (arrow heads). Depending on the plane of section, the rest of the trophozoite appears to be completely separated from the cyst wall (F) or attached in a very limited way (G). H) A trophozoite that appears to be exiting from the cyst capsule. I and J) Excysted trophozoites.

Fig. 7. Live cell imaging of excysting *Entamoeba invadens* trophozoites. Cysts were labeled with calcofluor (cyst wall, blue) and SYTO11 (nuclei, green) following exposure to the water/bile/bicarbonate excystment medium, and viewed by confocal microscopy. Differential interference contrast was used to visualize the outline of the cells. A) A typical excystment sequence, with the arrow indicating the cyst wall of the excysting cell. At 244 s, all four nuclei of the emerging trophozoite are visible in the optical plane. Numbers represent seconds elapsed after the first frame, and the scale bar represents 20 μm . B) Discarded cyst walls from trophozoites that have already excysted. Scale bar represents 15 μm .