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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-throughput screening of inhibitory compounds.

1. Introduction

Entamoeba histolytica is a potentially invasive intestinal protozoan parasite of humans that causes an estimated 50 million cases of amoebiasis, manifesting as amoebic colitis, dysentery and extra-intestinal 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 amoeboid 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 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, 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

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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, Hoechest 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 mm^2 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 μm; Sigma–Aldrich) were placed in 15-ml polypropylene conical tubes with 8×10^5 cysts in PBS and vortexed for 30–60 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 H_3PO_4 (pH 2.0–3.0) for 30 min in a 37 °C heat block. Cysts were centrifuged at 2600*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, 0.1–1.0 mg/ml α-chymotrypsin, 7.0–280.0 mM sodium acetate, 2.0–80.0 mM sodium propionate or with 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 6–48 h. At defined time points the culture tubes were chilled on ice, centrifuged for