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Full Length Research Paper

Possible involvement of endocyst in tolerance of the resting cyst of *Colpoda cucullus* against HCl

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Tolerance of the resting cysts of protozoa against gastric acid and proteases is a strategy for surviving in the digestive tract of animals. The present study aimed to examine the protection mechanism against HCl in resting cysts of *Colpoda cucullus*, which were surrounded by a cyst wall, which is composed of an outermost layer (ectocyst) and several inner layers (endocyst). In addition to water, paraformaldehyde, hydrogen peroxide (H_2O_2) and ethanol may diffuse across the cyst wall, because the cysts hardly showed tolerance against these molecules. However, the cysts showed tolerance against HCl (0.01 to 1 M) and pepsin. The tolerance against HCl disappeared soon after the onset of excystment induction. Electron micrographs of the excysting cells showed that digestion of the endocyst began within 30 min after the onset of excystment induction. The encysting cells surrounded by the ectocyst (3 h after encystment induction) did not show tolerance against HCl, but acquired it just after a first layer of the endocyst was formed beneath the ectocyst (6 h after encystment induction). These results suggest that the tolerance of the resting cyst of *C. cucullus* against HCl may be acquired by preventing its diffusion across the endocyst, although the possibility that cytoplasmic molecules and the plasma membrane may acquire tolerance has not been ruled out.

Key words: *Colpoda cucullus*, resting cysts, tolerance, HCl, excystment.

INTRODUCTION

Generally, terrestrial protozoa including *Colpoda* encyst promptly before the puddles in which vegetative cells of *Colpoda* grow on dry out. The encystment of *C. cucullus* is induced by an increase in the external Ca^{2+} concentration (Yamaoka et al., 2004; Matsuoka et al., 2009; Asami et al., 2010) or overpopulation of vegetative cells (Maeda et al., 2005). On the other hand, excystment is induced by the addition of cereal (wheat leaves) infusion or an artificial porphyrin analogue such as chlorophyllin-Cu (Tsutsumi et al., 2004). When the vegetative cells of *C. cucullus* are transferred into an encystment-inducing medium, the cell shape is rounded, and then surrounded by an outermost layer (a single layer termed ectocyst) within 2 to 3 h. The ectocyst formation is followed by a successive formation of several layers of endocysts (1 to 2 layers synthesized per day) until the cysts are matured for 1 to 2 weeks (Kida and Matsuoka , 2006). The cyst wall formation was accompanied by digestion of chromatin granules extruded from the macronucleus, disintegration of cell structures such as cilia, and arrest of mitochondrial activity (Kida and Matsuoka, 2006; Funatani et al., 2010).

The dried resting cysts of *C. cucullus* acquire resistance against high temperature and freezing (Maeda et al., 2005). In general, the tolerance of protozoan cysts against drying, high temperature and freezing helps survival in unstable environmental conditions in the field (Taylor and Strickland, 1936; Corliss and Esser, 1974; Maeda et al., 2005; Müller et al., 2008, 2010).

The protection of the cysts from acid (Kliescikova et al., 2011) and proteases may be a strategy for survival in the digestive tract of animals, permitting an establishment of orally transmitted infection in case of parasitic protozoa and/or a widespread distribution of protozoa including free-living protozoa. We found that resting cysts of the free-living terrestrial ciliate *C. cucullus*, which is not a pathogenic protozoa, showed resistance against HCl and pepsin. It has not been demonstrated whether the

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tolerance of the protozoan cells against hazardous environmental conditions is acquired by being surrounded by the cyst wall or by stress-resistant intracellular molecules reconstructed in the encystment process. The purpose of the present study was to examine the role of the cyst wall in protecting *Colpoda* cells against HCl.

MATERIALS AND METHODS

Cell culture, induction of en- or excystment, and chemical treatment

C. cucullus was cultured in a 0.1% (w/v) infusion of dried cereal (wheat) leaves inoculated with bacteria (*Klebsiella pneumoniae*) as food. For encystment induction, vegetative cells cultured 1-2 days were collected by centrifugation (1,000 to 1,500 g, 2 min) and subsequently suspended in a solution containing 0.1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2) except in Figs. 1c and 4, where the cells were suspended in a solution containing 1 mM $CaCl_2$ and 5 mM Tris-HCl (pH 7.2). Excystment was induced by replacing the surrounding medium by 0.1% (Figure 4) or 0.2% (Figures 2, 3 and 5) cereal infusion. In Figure 2, the chemicals were dissolved in pure water, and the pH of the pepsin solution was decreased by the addition of 0.01 M HCl (final concentration). In Figure 2, 1-week-old cysts were treated with various test solutions at room temperature, rinsed in pure water several times, and immersed overnight in excystment-inducing medium (0.2% cereal infusion). The rate of excystment (viability) is expressed as a percentage of the total number of tested cells (100-300 cells). Points (columns) and attached bars correspond to the means of six identical measurements (100-300 cells per measurement) and standard errors.

Electron microscopy

The resting cysts and excysting cells were prefixed with GA fixative without OsO_4 for 6 h, rinsed in 100 mM cacodylate buffer (pH 7.2), and then postfixed in a postfixative for a week. The postfixed cells were washed several times in pure water, dehydrated through a graded ethanol series (30, 40, 50, 60, 70, 80, 90 and 100% ethanol) for 15 min each, finally suspended in acetone, and then embedded in Spurr's resin. Sections were stained with 3% uranyl acetate and then with lead citrate (10 min each). The sections were observed under a transmission electron microscope (JEOL, 1010T).

Staining for photomicroscopy

For vital staining of the cells with toluidine blue (TB), the cells were suspended in a solution containing 0.05 % toluidine blue (TB), 0.1 mM $CaCl_2$ and 1 mM Tris-HCl (pH 7.2), and kept for 10 min.

RESULTS AND DISCUSSION

When a dried mature cyst {Figure 1(a-1)} was immersed in water, it soon swelled {Figure (1a-2)}. When the mature wet cyst {Figure 1(b-1)} was transferred into hypertonic solution containing 0.25 M sucrose, it shrank {Figure 1(b-2)}. These results indicate that water can diffuse across the cyst wall. As shown in Figure 1c, the cyst wall is composed of an outermost layer (ectocyst, 'ec') and several inner layers (endocyst, 'en').

Figure 2 shows the viability (excystment rate) of the 1-week-old wet resting cysts which were treated with 2% paraformaldehyde (PA), 3% hydrogen peroxide (H_2O_2), 50% ethanol (EtOH), 0.01~1 M HCl (pH 1~2), and 0.2% pepsin (containing 0.01 M HCl). Vegetative cells were quickly killed by the addition of these chemicals (data not shown). These results indicate that paraformaldehyde, hydrogen peroxide and ethanol can diffuse across the ectocyst and endocyst, while a diffusion of HCl may be prevented by the cyst wall, or intracellular molecules and the plasma membrane of the resting cyst may be resistant against HCl, if HCl diffuses across the cyst wall. In this case, the ectocyst may be resistant against pepsin, because it was not digested despite the fact that the ectocyst was directly affected by pepsin.

The tolerance against HCl was lost within 10 min after excystment was induced (Figure. 3). This means that changes in the molecular or structural level responsible for tolerance against HCl occur soon after excystment is induced. Electron microscopy of excysting cells showed that structural changes occurred within 30 min after onset of excystment induction (Figure 4). Electron-lucent ellipsoidal bodies (Figure 4a, 'elb'), which are believed to be reserve grains (Kida and Matsuoka, 2006), accumulate in the central region in mature resting cysts. At 30 and 60 min after the onset of excystment induction, the electron-lucent ellipsoidal bodies were observed to disperse and fuse with the plasma membrane (Figures 4b, d, e, arrowheads) to excrete their contents. The endocyst (Figure 4a, en) seems to be digested by excretion of the electron-lucent material (Figure 4e). In the fastest case, cellular morphogenesis such as reconstruction of the cilia (Figures 4b, c, 'Ci') was completed within 1.5 h after the excystment was induced, followed by emergence of vegetative cell by rupture of the ectocyst (Figure 4f). Judging from electron microscopical observation, partial digestion of the endocyst induced by excretion of electron-lucent material is expected to begin within 30 min after the onset of excystment induction. Therefore, the digestion of the endocyst may be responsible for the disappearance of tolerance against HCl in excysting cells.

If the endocyst prevents diffusion of HCl, then the timing of endocyst formation during encystment and the acquiring of tolerance is expected to coincide. It has been reported that ectocyst formation was completed within 2 to 3 h, and the first layer of the endocyst was formed within 4~5 h after encystment was induced (Kida and Matsuoka, 2006; Funatani et al., 2010). As shown in Figures 5a and b, the timing of endocyst formation was photomicroscopically visualized by toluidine blue (TB)-staining (Figures 5a and b). The rates (%) of cells surrounded by the endocyst (TB-positive cells) are indicated in the photomicrographs (Figs. 5a, b). When 3-h-old immature cysts (most of the cells were surrounded by only the ectocyst) were treated with 0.01 M HCl for 10 min, no viable excysted cell was observed (Figure 5c, '3h, Cells with Ec'), although this sample contained a small number of TB-stained cells (8%). Presumably, the

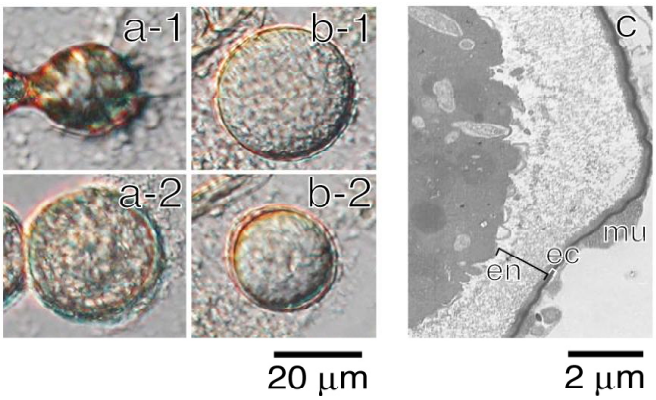

Figure 1. Swelling of a dried mature cyst induced by being immersed in water and shrinking of a wet cyst induced by being immersed in hypertonic solution (0.25 M sucrose). **(a-1).** 2-week-old dried cyst. 1-week-old wet cyst was desiccated for 1 week; (**a-2).** The same cyst in 2 min after it was immersed in water. **(b-1).** 1-week-old wet cyst; **(b-2).** The same cyst in 10 min after the surrounding medium was replaced by 0.25 M sucrose solution. **(1c).** Electron micrograph of 2-day-old resting wet cyst, showing cyst wall composed of an outermost single layer of ectocyst (ec) and several layers of endocyst (en). The cyst was enveloped by sticky mucous ('mu').

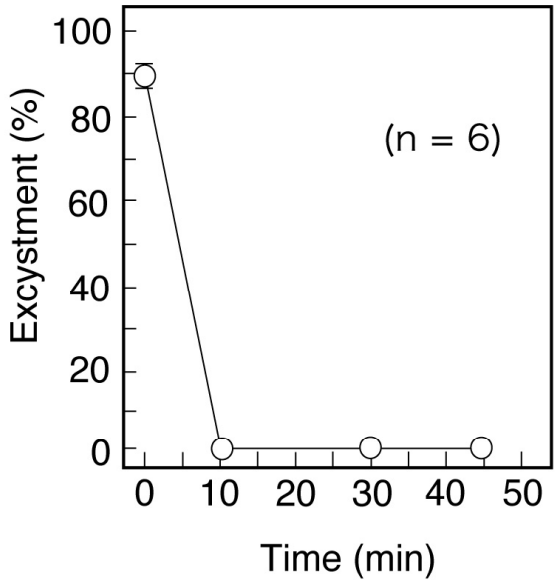

Figure 3. Tolerance of excysting cysts against 0.1 M HCl. The 1-week-old wet cysts were induced to excyst, and the excysting cells were treated with 0.1 % HCl for 10 min at 10, 30 and 45 min after the onset of excystment induction.

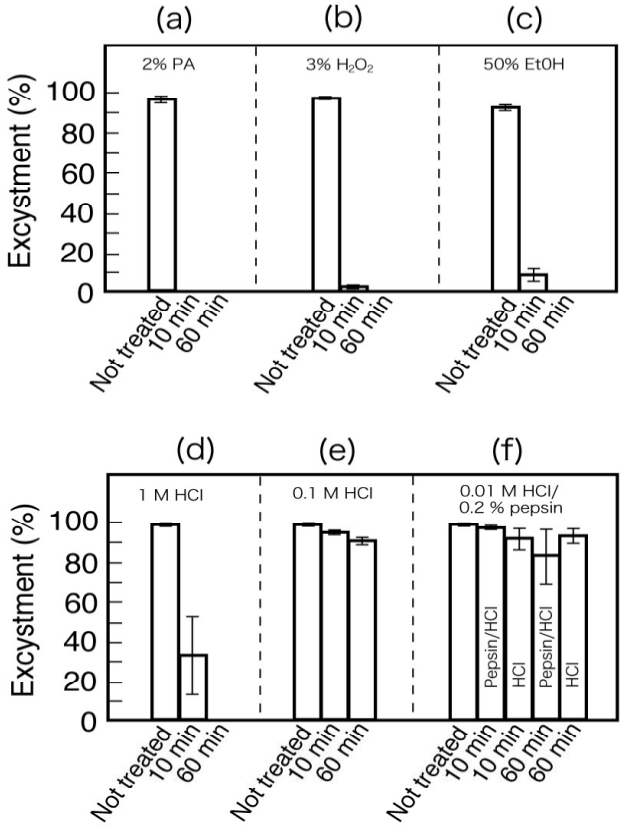

Figure 2. Tolerance of wet resting cyst (1-week-old) against 2% paraformaldehyde (PA), 3% hydrogen peroxide (H_2O_2), 50% ethanol (EtOH), 0.01 to 1 M HCl and 0.2 % pepsin (containing 0.01 M HCl). The cyst was incubated in these test solutions for 10 or 60 min.

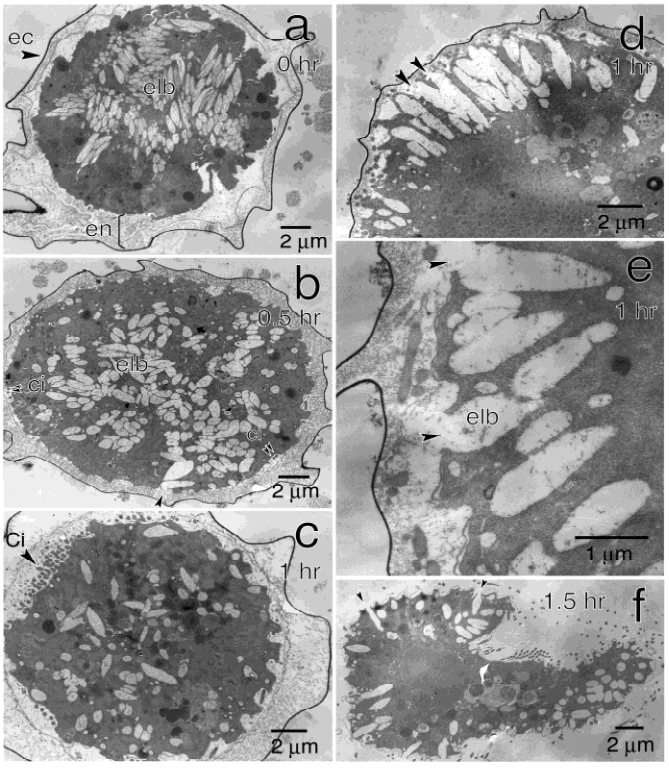

Figure 4. Transmission electron micrographs of excysting cells after onset of excystment induction. (**4a)** A wet resting cyst aged more than 2 weeks; (**4b-e)** Excysting cells at 30 and 60 min after onset of excystment induction, showing dispersal and excretion (indicated by arrowheads) of electron-lucent bodies ('elb') and reconstruction of cilia ('Ci'). (**4f)** An emerged vegetative cell.

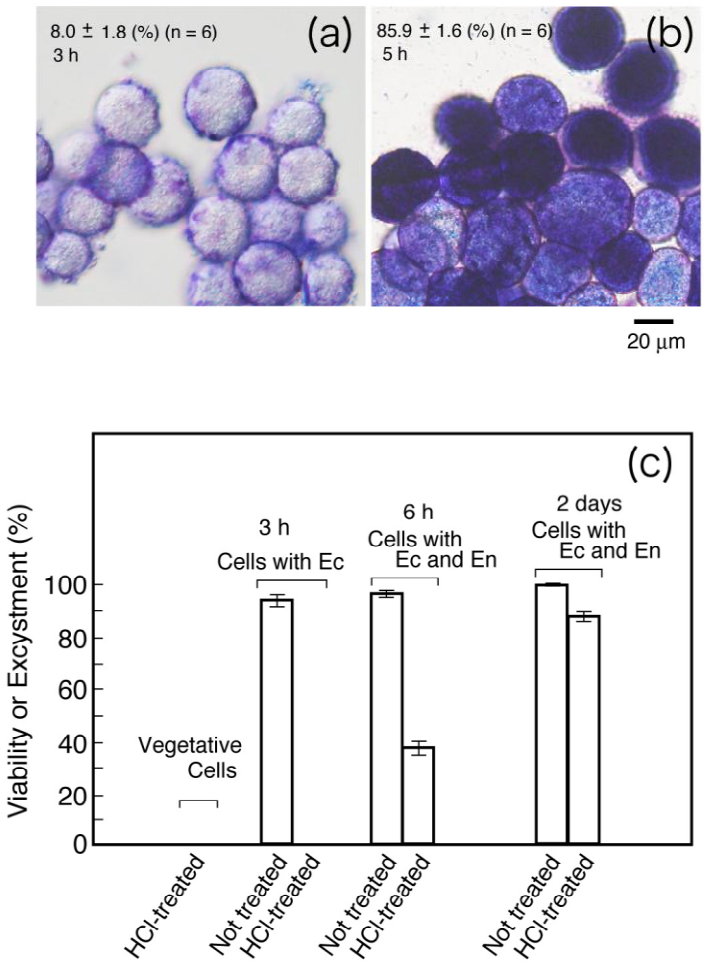

Figure 5. Tolerance of immature cysts against HCl. (**a and b).** Toluidine blue (TB)-stained cells at 3 and 5 h after onset of encystment induction. (**c).** Tolerance of vegetative cells, and 3-h-, 6-h- and 2-day-old immature cysts against 0.01 M HCl. These different-aged cysts were incubated in 0.01 M HCl for 10 min.

endocyst layer of this stage may be too incomplete to prevent diffusion of HCl. When 6-h-old immature cysts (85.9% of the cells surrounded by the ectocyst and endocyst) were treated with 0.01 M HCl for 10 min, 38% of the cysts were viable (excysted) (Fig. 5c, '6 h, Cells with Ec and En'). A two-day-old immature cyst, in which several layers of endocyst had been formed (Figure 1c), showed an extensive tolerance against 0.01 M HCl (10-min treatment) (Fig. 5c, '2 days, Cells with Ec and En'). These results suggest that the tolerance of the resting cyst of *C. cucullus* against HCl may be involved in endo-cyst formation. However, it still remains the possibility that cytoplasmic molecules and plasma membrane may acquire tolerance during encystment. The cysts of *C. cucullus* were also resistant against pepsin in the acidic condition (Figure 2f). Unfortunately, in the present study, whether or not pepsin diffuses across the cyst wall could not be elucidated, because the enzymatic activity of pepsin requires an acidic condition.

The tolerance of *C. cucullus* against 0.01~0.1 M HCl and 0.2% pepsin, which approximately correspond to concentrations of HCl and pepsin in the gastric juices of animals, may enable *Colpoda* cysts to survive in digestive tracts. The fact that the cysts of non-parasitic ciliate *C. cucullus* are tolerant against HCl and pepsin implies that the tolerance may be related to their widespread distribution through animals.

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