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Electron microscopic observation of the early stages of *Cryptosporidium parvum* asexual multiplication and development in *in vitro* axenic culture☆

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Abstract

The stages of *Cryptosporidium parvum* asexual exogenous development were investigated at high ultra-structural resolution in cell-free culture using transmission electron microscopy (TEM). Early *C. parvum* trophozoites were ovoid in shape, $1.07 \times 1.47\ \mu m^2$ in size, and contained a large nucleus and adjacent Golgi complex. Dividing and mature meronts containing four to eight developing merozoites, $2.34 \times 2.7\ \mu m^2$ in size, were observed within the first 24 h of cultivation. An obvious peculiarity was found within the merozoite pellicle, as it was composed of the outer plasma membrane with underlying middle and inner membrane complexes. Further novel findings were vacuolization of the meront's residuum and extension of its outer pellicle, as parasitophorous vacuole-like membranes were also evident. The asexual reproduction of *C. parvum* was consistent with the developmental pattern of both eimerian coccidia and Arthrogregarinida (formerly Neogregarinida). The unique cell-free development of *C. parvum* described here, along with the establishment of meronts and merozoite formation, is the first such evidence obtained from *in vitro* cell-free culture at the ultrastructural level.

Keywords: *Cryptosporidium parvum*; TEM; Host cell-free culture; Extracellular development; Asexual life cycle

Introduction

Cryptosporidium is recognized as an important ubiquitous pathogen for a wide range of vertebrate hosts. In humans, the parasite is responsible for acute self-limiting diarrheal disease, which can be life threatening, especially in immunocompromised individuals. Infection occurs with the ingestion of oocysts, followed by specific developmental phases: excystation (the release of infective sporozoites), merogony (asexual multiplication), gamogony (sexual reproduction), fertilization and sporogony (Fayer, 2008). Despite intensive efforts in *Cryptosporidium* research during the last three decades, there is no effective treatment or vaccine to date against this protozoon, which may contribute to the lack of successful cultivation of the parasit (Karanis and Aldeyarbi, 2011). Recent advances in *in vitro* cultivation

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of *C. parvum* and *C. hominis* have revealed the possibility of parasite development in media devoid of host cells (Hijjawi et al., 2004, 2010). Previously, the visualization and detection of development *in vitro* and in cell-free systems have been accomplished using the *Cryptosporidium*-specific polyclonal antibody Sporo-Glo™, immunofluorescence and confocal microscopy (Boxell et al., 2008; Hijjawi et al., 2010; Koh et al., 2013, 2014). However, in general, these methods have failed to distinguish the invasive smaller stages, with a lack of fine structural information. Therefore, a TEM-based approach of monitoring *Cryptosporidium* in cell-free culture was used to image and confidently identify a variety *C. parvum* developmental stages at high ultrastructural resolution. This study aimed to provide additional insight into the development of *C. parvum* during its extracellular existence to improve the understanding of *Cryptosporidium* biology.

Material and methods

Cryptosporidium oocysts and genotyping

The purified *Cryptosporidium parvum* isolate used in the present study was originally obtained from the Leipzig Parasitology Institute, Germany, and genotyped by SSU rRNA sequence analysis, as previously described (Xiao et al., 1999). According to the provider, the oocysts were purified using ether extraction and a saturated NaCl flotation method. The parasite suspension was stored in 50 ml phosphate-buffered saline (PBS) with amphotericin B (0.0025 mg/ml) and penicillin/streptomycin (100 U/ml; 0.1 mg/ml) at 4 °C.

Decontamination (bleaching) of oocysts

Purified *C. parvum* oocysts were bleached before use by placing them in 10% sodium hypochlorite solution for 30 min on ice at 4 °C. Subsequently, the suspension was washed and centrifuged at 5000 × *g* for 3 min at room temperature. As a control, oocysts were fixed directly in 2.5% glutaraldehyde after decontamination and then processed for embedding and sectioning for TEM, as described below.

Axenic *in vitro* cultivation

Each well in a 12-well culture plate (Greiner Bio-One, CELLSTAR), contained 1.5 ml of a sterile, pre-warmed, cell-free maintenance medium, Express Five® Serum Free Medium (SFM) (×1). The wells were inoculated with a suspension of unexcysted *C. parvum* oocysts (~1×10^6). The cultures were incubated at 37 °C and 5% CO_2 for 2, 4, 6, 8, 12 and 24 h up to 7 days. The spent medium was exchanged with fresh 100% Express Five SFM every 2–3 days. The wells were inspected daily for contamination.

Embedding and sectioning for TEM

Aliquots of *C. parvum* culture were pelleted and fixed in 2.5% glutaraldehyde for at least 24 h. After centrifugation, this suspension was then mixed with 2% low-melting-temperature agarose. After cooling, the agarose block was cut and re-suspended in 2.5% glutaraldehyde fixative overnight at 4 °C. Post-fixation was carried out with agarose pieces in 1% osmium tetroxide for 3 h, followed by dehydration in a graded series of ethanol, embedding in pure Epon epoxy resin by conventional procedures and polymerization at 36.9 °C for 96 h. Ultra-thin sections (75 nm) were stained with uranyl acetate and lead citrate and examined using a Zeiss EM-902 TEM operated at 80 kV.

Results

The findings of the current study are representative of a careful serial ultra-sectional analysis of micrographs of all *C. parvum* developmental stages in axenic culture. The oocysts contained four sporozoites measuring 3.2 (2.4–4.8) × 3.7 (2.8–5.7) μm (SD = ± 0.39 × 0.46) (n = 121, in the sectional plane) (Fig. 1a, b). After incubation, most of the oocysts were void leaving empty or partially empty oocysts with a residuum, and variation in the rate of excystation was observed (data not shown).

The sporozoites displayed a conical-shaped apical complex, a single rhoptry, electron-dense granules, micronemes and a large crystalloid body posterior to the nucleus (mean = 1.33 × 2.16, SD = ± 0.1 × 0.25, range = 1.23–1.43 × 1.91–2.40 μm, n = 3, in the sectional plane). The crystalloid body was closely packed with electro-dense 'vesicular spheres' enveloped by limiting membranes approximately 29–36 nm in diameter (Fig. 1c). The trophozoites were ovoid or round, 1.07 (0.8–1.42) × 1.47 (1.02–2) μm in size (SD = ± 0.18 × 0.3) (n = 12, in sectional plane), and observed from the first 24 h post-cultivation. At different stages of maturation, several modifications were detected. First, the inner two pairs of membrane complexes of the pellicle disappeared, leaving the outer plasma membrane covering the parasite. Second, dedifferentiation of the apical organelles and anterior vacuolation of the cytoplasm had occurred. Simultaneously, the nucleus and nucleolus enlarged, filling up almost 1/2 of the parasite. Early trophozoites contained a distinct large nucleus (~1.07 × 1.47 μm^2), with a prominent nucleolus and Golgi complexes located near the nucleus and endoplasmic reticulum (Fig. 2). Fig. 2b shows the two double electro-dense thick membranes separated by a translucent space, emanating for new merozoite development. Notably, trophozoites were observed inside of unexcysted oocysts at 72 h after the separation of their apical ends and ER (Fig. 2c, d).

The mature rounded trophozoite underwent marked cytoplasmic proliferation and two to four nuclear divisions, becoming an early meront (Fig. 3). At the beginning,