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Dynamics of parasitophorous vacuoles formed by the microsporidian pathogen *Encephalitozoon cuniculi*

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Abstract

It has been a long-standing debate if sexual development occurs in the microsporidian lineages. Previous studies, including morphological observations, ploidy analysis, and the presence of a *sex*-related locus, provided evidence of possible extant of sexual development. This study presents another line of evidence by monitoring the parasitophorous vacuoles (PVs) formed by *Encephalitozoon cuniculi*. Time lapse observations of infection cycles of *E. cuniculi* revealed that multiple PVs can be formed in a single host cell and the PVs in the single cell can merge (fusion) or split (fission). The dynamics of PVs may provide a route for interactions between genetically distinct microsporidian isolates during host infections.

Keywords

Parasitophorous vesicles fusion and fission; sex in microsporidia; *E. cuniculi*

Introduction

Microsporidia are obligate intracellular eukaryotes that infect organisms from protists to nematodes to humans (Keeling and Fast, 2002). Infection of host cells by microsporidia involves an unusual mechanism in which eversion of the polar tube produces a syringe-like structure that pierces the target cell. Infectious contents of the spore, the sporoplasm, are rapidly injected through the polar tube into the host cell (Xu and Weiss, 2005). It has been suggested that the invagination of the host cell membrane surrounding the polar tubes and then the sporoplasm, occurs during early infection by microsporidia (Bohne et al., 2011). Intracellular development of microsporidia includes proliferation and sporogonic phases. In the proliferative phase, mono- or di-karyotic sporoplasms undergo division to increase the number of cells and/or the number of nuclei, forming sporonts. During the sporogony phase, sporonts undergo sporoblastic development to form progeny spores (Cali and Takvorian,

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2014). Due to their obligate intracellular life cycle, these developmental processes only occur in enveloped, membranous vesicles, either host- (parasitophorous vacuole) or pathogen-derived (sporophorous vesicle) (Canning and Lom 1986). Therefore, the vesicles provide biological niches for microsporidial development.

In this study, we examined the dynamics of the parasitophorous vacuoles (PVs) formed by *Encephalotoxoon cuniculi*. Our microscopic observations revealed that multiple PVs are formed in a single host cell, and the PVs undergo merging or splitting during development inside the host. These observations may imply that genetically distinct isolates can infect the same host cell, interact with each other, and possibly undergo sexual development (genetic exchange). We developed genetic markers by using tandem repeats that differ between isolates, which enabled us to examine genetic recombination of progeny. However, we did not observe any genetic exchanges between two genetically distinct isolates in the progeny set examined. Nevertheless, our study provides cellular evidence of interactions between microsporidian isolates during infections.

Results and Discussion

Fusion and fission of parasitophorous vesicles

Microsporidia are obligate eukaryotic pathogens and, therefore, the microsporidian vesicles inside host cells play a pivotal role during their life cycle. We chose the model microsporidian *E. cuniculi* to study dynamics of vesicles inside hosts. *E. cuniculi* forms host-derived parasitophorous vacuoles (PVs) and propagates to produce progeny within the PVs inside host cells.

The *E. cuniculi* PVs were monitored by time lapse microscopy to examine the dynamics inside rabbit kidney (RK13) cells. We cultured *E. cuniculi* isolate EC3 in RK13 cells. When ~20% of the cells were infected and exhibited visible PVs by microscopy, the cultures were sub-cultured into a glass bottom 6-well plate, which allowed us to perform time lapse microscopy. Suspended RK13 cells (5×10^5 cells/ml) were transferred to a well and incubated for 24 hours on the glass bottom of the 6-well plates to allow settling to occur prior to microscopic observations.

We found that *E. cuniculi* can form multiple vesicles in a single host cell in the two cell lines that were tested, including the RK13 and HeLa cell lines (Fig 1A). Furthermore, reconstructed time lapse movies verified that the vesicles in a single host cell underwent both fusion and fission (Fig 1B and C, and movie 1A and B). Six fusion and two fission events were observed in the 209 RK13 cells harboring PVs during a 320-minute observation (data not shown). A total of 17 RK13 cells were found to harbor more than one vacuole (6 fusion events in 17 RK13 cells). Interestingly, we observed a host cell containing two vesicles and undergoing cell divisions, in which each of the daughter cells inherited one vesicle (Figure 1D and movie 1C). In addition, a vesicle underwent a fission in one daughter cell. These observations may indicate that different microsporidian isolates can infect the same host cell and could interact after merging of the vesicles formed by each individual isolate. Furthermore, the segregation of vesicles during host cell division indicates that microsporidia can vertically spread during host cell divisions.

In addition to cell division and progeny formation, one possible mechanism that occurs inside parasitophorous vacuoles is generation of genetic variation, resulting from physical interactions. A study revealed that the *E. cuniculi* genome encodes four ATP transporters that were horizontally acquired from *Chlamydia* spp. (Katinka et al., 2001). Both species are obligate intracellular pathogens and, therefore, the only opportunity for them to interact would be during host infection. That we previously found that they are able to infect the same host cells (Lee et al., 2009), provides evidence that the genetic exchange might have occurred during infection of the same host by ancestors of both organisms.

The intracellular vesicles formed by microsporidia may serve as a haven for sexual development. Reports suggest some microsporidian species may have extant sexual cycles based on morphological observations (Hazard and Brookbank, 1984; Sweeney et al., 1988). For example, a survey of genomes of multiple *E. cuniculi* isolates identified loci that exhibit heterozygosity, implying this species is diploid and may have an extant sexual cycle (Katinka et al., 2001; Pelin et al., 2015; Pombert et al., 2013; Selman et al., 2013). Some microsporidia transition their karyotype from diplokaryotic to monokaryotic during development inside of hosts, also possibly consistent with sexual reproduction.

Evidence for gametogenesis and syngamy is also observed in some microsporidia (Becnel, 1992; Becnel et al., 1987; Hazard et al., 1985). For example, the mosquito microsporidian *Culicospore magna* forms enlarged multi-nucleated plasmodia after binary fission between mononucleate sporoplasms, which is similar to syngamy in sexual development (Becnel et al., 1987). Multi-nucleate plasmodia undergo development into mononucleate cells to form gametogony, which mimics gametogenesis.

In addition, microsporidian genomes encode a *sex*-related locus, which is analogous to the *sex* locus found in the Mucorales fungi (Lee et al., 2008; Lee et al., 2010a; Lee et al., 2010b). The *sex* locus of Mucorales fungi encode two idiomorphic high-mobility group (HMG) transcription factors, SexP or SexM, depending on mating types, (+) or (–), respectively. The *sex* gene of each mating type is flanked by conserved genes encoding a triose phosphate transporter (Tpt) and RNA helicase in either side. The similar microsynteny encoding a set of Tpt/HMG/RNA helicase is also found in several known microsporidian genomes, indicating the *sex*-related locus may control sexual development.

Genetic marker development and measure of genetic exchange

We therefore examined genetic recombination between isolates that might have resulted from sexual development. To do so, we developed genetic markers by using tandem repeats (TRs). A web-based software (<http://tandem.bu.edu/trf/trf.html>) (Benson, 1999) was used to identify TRs in the *E. cuniculi* genome (GB-M1 strain sequence in NCBI) as described previously (Lee et al., 2009). Two TRs were chosen per chromosome (total of 11 chromosomes) and amplified from the genome of EC1, EC2, and EC3 with primers designed based on the GB-M1 genome. There are several TRs exhibit substantial differences between the three strains, especially the size difference of TRs in EC1 and EC3 was clear in several TR loci (Fig 2).

We then examined if there is genetic recombination between EC1 and EC3 strains after 10 rounds of vegetative passages. To isolate progeny, RK13 cells infected with EC1+EC3 were sorted by a cell sorter and placed in a well of 96 well plates. Among 960 sorted RK13 cells (10 96-well plates), 9 wells were found to contain live RK13 cells infected with *E. cuniculi*. However, analysis of TRs in the isolates did not indicate that genetic recombination occurred between EC1 and EC3, where only EC3 TR patterns were observed (data not shown), indicating a fast-growing-phenotype or domination growth of the isolate.

It is possible that the genetic recombination ratio is low so examination of 9 progeny would not be sufficient to observe recombination. Alternatively, *E. cuniculi* may need a specific condition and/or host cells to undergo sexual recombination that has yet to be determined. Although we did not observe bona fide genetic recombination in *E. cuniculi*, the dynamics of PVs in host cells such as fusion and fission are intriguing because it can provide evidence that interactions can occur between isolates. The development of TRs as a genetic marker can be further applied in future studies, incorporating a larger progeny set to examine recombination, which is thus far limited by current techniques.

Materials and Methods

Stains and culture conditions

Three *Encephalitozoon cuniculi* strains, EC1, EC2, and EC3 (Pombert et al., 2013) were used in this study. The rabbit kidney cell line, RK13, was used to maintain the microsporidian strains. The cells were grown in minimum essential media (MEM) containing fetal bovine serum (10%) in a 5% CO₂ environment. The media was replaced 2 to 3 times per week. For subculture of RK13 cells or RK13 cells infected with *E. cuniculi*, the tissue culture media was aspirated and monolayers of cells were washed with sterile pre-warmed PBS. The cells were then detached with 0.05% trypsin/EDTA (Gibco) and the suspended cells were diluted 1:6 with media for subculture in tissue culture T-75 flasks. HeLa cell lines were also cultured and maintained in a similar way.

To examine genetic recombination in *E. cuniculi*, EC1 and EC3 were co-cultured in RK13 cells. In brief, when ~20% of the RK13 cells in the monolayer were infected by each strain, the cultures were trypsinized in 6 ml of 0.05% trypsin/EDTA solution (Gibco). The suspended RK13 cells infected with EC1 and EC3 then were mixed and sub-cultured at a 1:6 ratio (2 ml of cell suspension + 10 ml of tissue culture media) in T75 flasks. The cultures were sub-cultured once a week at a 1:6 ratio for up to 10 rounds of sub-culture cycles. The RK13 cells were then trypsinized and were sorted into 10 96-well plates by a BD FACSDiVA housed in the Duke Flow Cytometry Facility. After sorting, the cells were grown for 3 weeks and transferred into 6 well plates. The cells were grown for 6 weeks to collect spores that are enough to extract genomic DNA.

Microscopy

RK13 cells infected with the *E. cuniculi* EC3 strain were transferred into a glass bottom 6-well plate. After 24 hours of incubation, the plate was placed on a motorized stage of a microscope (Axio Observer Z1, Zeiss) equipped with an on-stage tissue culture chamber

housed in the Duke University Light Microscopy Core Facility. Infection cycles of *E. cuniculi* in the RK13 cells was recorded. Images were acquired every 1 min and the obtained images were process with MetaMorph.

Identification of tandem repeats

Tandem repeats were identified in the genome of the GB-M1 strain as described previously (Benson, 1999; Lee et al., 2009). Primers to amplify the TRs from each chromosome of the EC1, EC2, and EC3 strains are listed in Supplemental table 1.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- *E. cuniculi* can form multiple parasitophorous vesicles (PVs) in a single host cell that undergo fusion or fission.
- Genetically distinct *E. cuniculi* isolates could infect a same host cell and interact with each other.
- Genetic exchange (sexual development) therefore could occur during host infection in the microsporidian lineage.

Figure 1.

Dynamics of parasitophorous vesicles (PVs) in host cells formed by *E. cuniculi*. More than two PVs are formed in the RK13 cells (left) and HeLa cells (Right) (A). The vesicles formed in the RK13 cells undergo merging (fusion) (B) or splitting (fission) (C). An RK13 cell harboring two vesicles undergoing cell division, in which each vesicle segregated into a different daughter cell. A vesicle in one daughter cell subsequently went fission (D). Scale = 20 μ m.

Figure 2.

Tandem repeat (TR) markers in three *E. cuniculi* strains. Two tandem repeats (designated A or B) throughout 11 chromosomes. Designations of chromosome follow the TR designation, i.e. A1 and B1 indicate two TRs found in the chromosome 1. The primers to amplify TRs from the EC1, EC2, and EC3 strains were designed based on the genome of GB-M1 strain. In some cases, the PCR reaction did not amplify an apparently visible band and this may be due to the tandem-repeat being too long for PCR or PCR artifacts. In the case of multiple bands in B5 and A11, a segmental DNA duplication was observed in Chromosome 5 and 11 (Katinka et al., 2001) and the tandem-repeats we chose are within the duplicated area; therefore, it is possible that each segmental duplicon on Chromosomes 5 and 11 has a different number of tandem repeats. DNA ladder marker was purchased from New England Biolabs (Ipswich, MA).