NOTE

The Parasitophorous Vacuole Membrane of *Encephalitozoon cuniculi* Lacks Host Cell Membrane Proteins Immediately after Invasion

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Microsporidia of the genus *Encephalitozoon* develop inside a parasitophorous vacuole (PV) of unknown origin. Using colocalization studies, the PV was found to be absent from the endocytic pathway markers early endosomal autoantigen 1, transferrin receptor, and lysosome-associated membrane protein 1 and for the endosomal reticulum marker calnexin. The nonfusiogenic characteristic of the PV appears to be acquired as early as 1 min postinfection and is not reversed by drug treatment with albendazole or fumagillin.

Microsporidia are obligate intracellular protists that have been recognized as emerging opportunistic pathogens causing diarrhea and disseminated infections in immunocompromised patients (12, 14, 15). The mature spore contains a coiled hollow tube, the polar filament, which is explosively extruded during invasion and can penetrate the cytoplasmic membrane of host cells (7, 9, 16). The sporoplasm is then directly injected into the host cell while the empty spore remains outside (1, 18). *Encephalitozoon* species spend their entire intracellular life cycle inside a nonacidified (8, 17) parasitophorous vacuole (PV), whose precise genesis is unclear so far. The aim of this study was to characterize the fate and fusion capabilities of the PV.

A detailed analysis of the molecular characteristics of the PV, particularly at early time points of the infection, is complicated by the fact that beside active invasion, spores can also be internalized by phagocytosis (4, 6). A recent study revealed that even nonprofessional phagocytes internalize substantial amounts of spores by a zipper-type phagocytosis, which then move into a late endosomal-lysosomal compartment (4).

First, we investigated whether phagocytic uptake of spores also occurs in fibroblasts. Human foreskin fibroblasts (HFF) and murine L929 cells were incubated with *Encephalitozoon cuniculi* spores and fixed with 4% paraformaldehyde 2 h postinfection (p.i.). Samples were doubly immunostained with MAb 6G2 and an antibody, which was detected with Cy3-conjugated anti-mouse IgG. SWP1-positive spores were located inside LAMP1-positive vacuoles in 35% of the host cells, confirming that phagocytic uptake of *E. cuniculi* spores also occurs in fibroblasts (Fig. 1a to d).

In order to distinguish between active invasion and phagocytosis, we used an indirect immunofluorescence assay (IFA). In a screen for stage-specific monoclonal antibodies, MAb 6G2 was found to recognize a cytoplasmic antigen in *E. cuniculi* meronts but did not label spores and sporonts. Since organisms at the periphery of the *E. cuniculi* PV are maintained in the merontic stage while sporont and spore differentiation takes place in the center of the vacuole, a characteristic ring-like staining pattern is obtained when MAb 6G2 is used in IFA on more-mature PVs (Fig. 1e and f). In tissue cultures, which are fixed immediately after infection with *E. cuniculi* spores (~1 min), MAb 6G2 reacts with the extruded sporoplasm, indicating that the recognized antigen is part of the injected sporoplasm (Fig. 1g). Inside spores, the sporoplasm appears to be protected from recognition by MAb 6G2 due to the impermeability of the spore wall to antibodies. MAb 6G2 is thus a highly specific tool for the detection of sporoplasm and meronts at early time points of the infection, while phagocytosed spores remain unlabeled. The combination of MAb 6G2 and anti-SWP1 antiserum in double-immunofluorescence analysis allowed a clear discrimination between the PVs that emerged from active invasion and spore-containing vacuoles derived from phagocytosis (Fig. 1h).

Antibodies against marker proteins of the endocytic pathway and the endosomal reticulum (ER) were used for colocalization studies with the *E. cuniculi* PV. The markers employed include the following: (i) transferrin receptor (TIR), which is located on the cell surface and on early endosomes; (ii) early endosomal autoantigen 1 (EEA1), a marker for early endosomes; (iii) LAMP1, which is associated with late endosomes and lysosomes; and (iv) the ER marker protein calnexin.

L929, HFF, and murine RAW monocytes/macrophages were infected with *E. cuniculi* spores and analyzed at 1 min, 5 min, 20 min, 1 h, 2 h, and 24 h p.i. by IFA and confocal laser-scanning microscopy for colocalization of the PV with the marker proteins employed. The PV was clearly detected at early infectious stages by using MAb 6G2 in a double-immunofluorescence analysis. Vacuoles containing microsporidia were classified as positive for TIR, EEA1, LAMP1, or calnexin.
if they displayed a rim of fluorescence staining around the vacuole containing microsporidia. Samples from at least three different experiments were scored independently by two investigators.

At all time points investigated, the great majority of MAb 6G2-positive PVs (>95%) did not colocalize with TIR, EEA1, LAMP1, or calnexin (Fig. 2), regardless of the host cell type. This indicates that at no time is the PV part of the endosomal
In addition, colocalization studies of *E. cuniculi* PVs with phagocytosed Texas Red- and zymosan particles were performed in macrophages derived from bone marrow and in HFF. Host cells were incubated with Texas Red- and zymosan (0.02 mg/ml) 30 min prior to infection with *E. cuniculi* and immunostained with MAb 6G2 24 h.p.i. Colocalization of MAb 6G2-positive PVs with Texas Red-zymosan particles was not observed, confirming that the PV does not fuse with endocytic vesicles (data not shown). Therefore, in its fusiogenic behavior, *E. cuniculi* PV is most similar to the *Toxoplasma gondii* PV, which also lacks host cell transmembrane proteins and is fusion incompetent (11).

To investigate whether regular development and replication of *E. cuniculi* are necessary to maintain the inability to fuse, we inhibited microsporidial growth by the addition of albendazole (100 ng/ml) or fumagillin (10 ng/ml). Samples were analyzed 24, 48, and 72 h.p.i. by immunostaining with both MAb 6G2 and anti-LAMP1 antibody. The size of the PVs and the number of meronts inside the vacuoles were much lower in drug-treated samples than in untreated controls. As in untreated

![Diagram](image_url)
samples, colocalization of the microsporidian PV with the lysosomal marker LAMP1 was not observed in drug-treated samples (data not shown), suggesting that normal growth and development are not necessary to maintain the inability of the PV to fuse.

An important aspect for understanding the fusogenic capability of the *E. cuniculi* PV is the origin of the PV membrane. It is generally believed that the *Encephalitozoon* PV membrane is derived from the host cell (1, 3, 13); however, there is no direct evidence for this assumption. On the basis of electron micrographs, an alternative model of host cell invasion for *Encephalitozoon intestinalis* was recently proposed (1, 5, 10). According to this model, the sporoplasm is not directly injected into the cytoplasm but internalized from germinated spores by a phagocytic process. The origin of the PV membrane would thus be the invaginated host cell cytoplasm membrane. If this uptake mechanism is verified for *E. cuniculi*, the host cell transmembrane proteins and the transferrin receptor as well as fusion-mediating proteins must be removed very rapidly (<1 min) from the emerging PV membrane in order to explain the absence of these markers. Further investigations of the origin and genesis of the *Encephalitozoon* PV are necessary in order to understand the properties of this important host cell-pathogen interface.

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