EUKARYOTIC CELL, Jan. 2005, p. 221–224 Vol. 4, No. 1
1535-9778/05/$08.00+0 doi:10.1128/EC.4.1.221–224.2005


# NOTE

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

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Received 2 September 2004/Accepted 5 November 2004

**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 endoplasmic 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 rabbit antiserum against the spore wall protein SWP1 (2), which was detected with a Cy2-conjugated anti-rabbit immunoglobulin G (IgG) and a mouse anti-lysosome-associated membrane protein 1 (LAMP1) (RDI Research Diagnostics) 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).

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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 endoplasmic reticulum (ER) were used for colocalization studies with the *E. cuniculi* PV. The markers employed include the following: (i) transferrin receptor (TfR), 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 TfR, EEA1, LAMP1, or calnexin

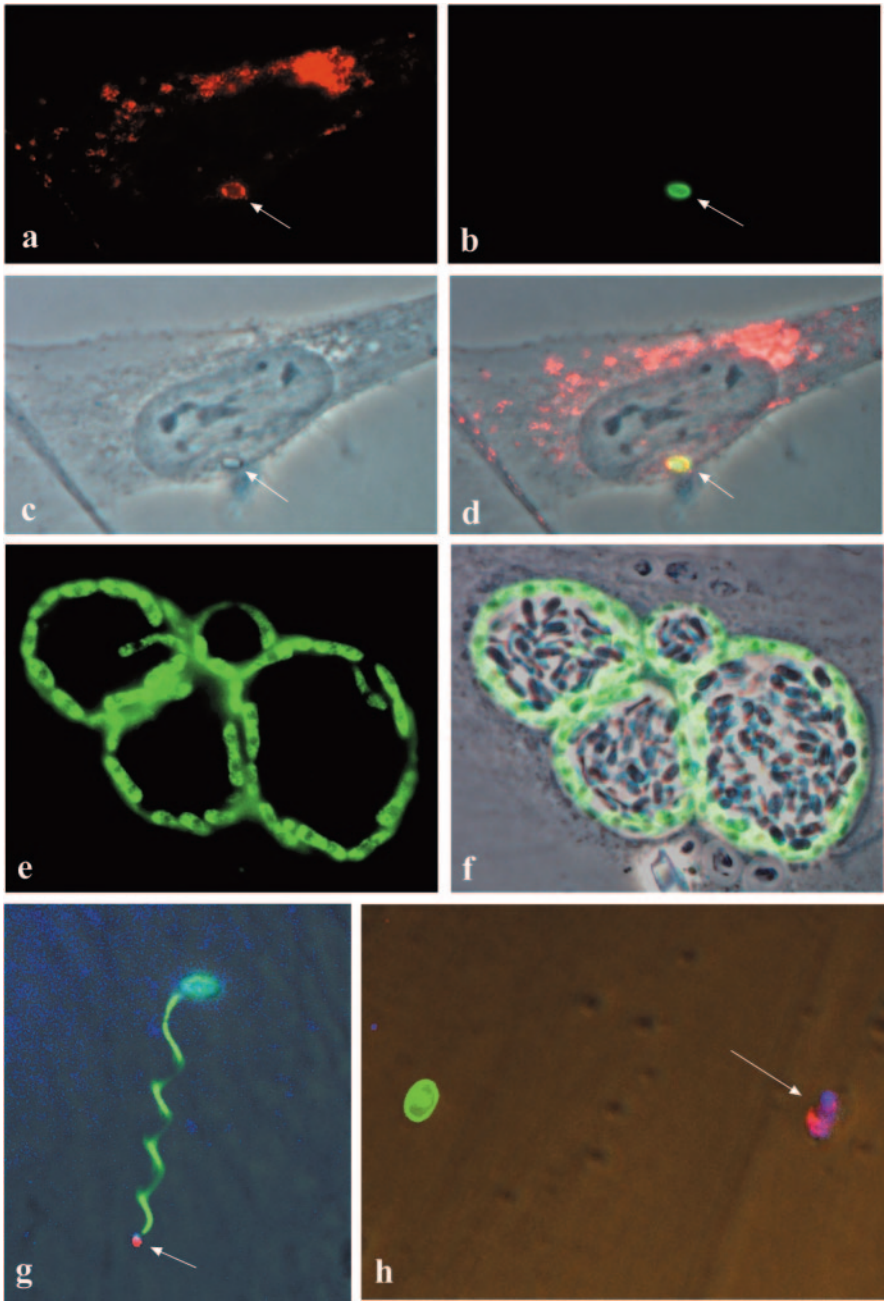


FIG. 1. Distinction between phagocytosis and active invasion. Panels a to d show the same view, but panels a and b show LAMP1 (a) or spore wall protein SWP1 (b), while panel c was visualized by phase-contrast microscopy, and panel d is a merged picture of panels a to c. The association of SWP1-positive vacuoles (arrowheads) with the lysosomal marker LAMP1 is indicative of phagocytic uptake of spores in HFF. (e) MAb 6G2 detected with Cy2-conjugated donkey anti-mouse IgG. (f) Merged picture of panel e and phase contrast. MAb 6G2 specifically detects meronts. (g) Double-immunofluorescence staining of a sample fixed immediately after infection (~1 min). MAb 6G2 was detected with a Cy3-conjugated anti-mouse IgG. The spore wall and polar filament were stained with a polyclonal rabbit anti-*E. cuniculi* antibody and detected with a Cy2-conjugated donkey anti-rabbit IgG. 4′,6′-Diamidino-2-phenylindole (DAPI) staining was used to detect nuclei. The arrowhead shows the extruded sporoplasm (red) with the nucleus (blue) at the tip of the polar filament. (h) Double-immunofluorescence staining of a sample fixed 2 h p.i. MAb 6G2 was detected with a Cy3-conjugated donkey anti-mouse IgG. A polyclonal rabbit antibody against the spore wall protein SWP1 was detected with a Cy2-conjugated donkey anti-rabbit IgG, and DAPI staining was used to detect nuclei. Extra- or intracellularly located spores (green) can be clearly distinguished from MAb 6G2-positive meronts (red), which entered host cells by active invasion (arrowhead).

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 TfR, 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

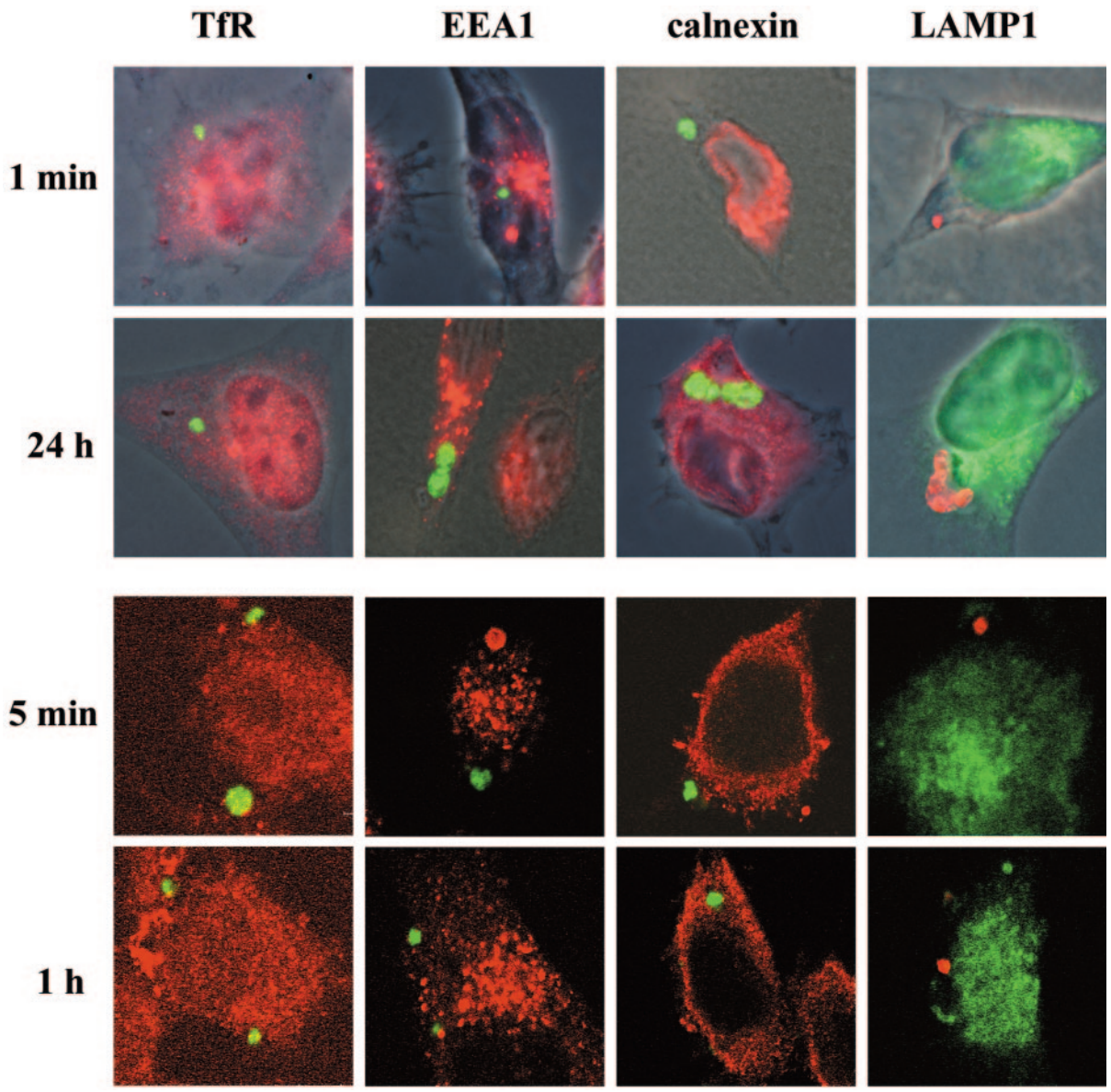


FIG. 2. The *E. cuniculi* PV membrane does not contain markers for endosomes, lysosomes, and the ER. L929 murine fibroblasts were immunostained at 1 min, 5 min, 1 h, and 24 h p.i. with the following antibodies: rabbit anti-TfR (1:100), goat anti-EEA1 (1:500), goat anti-calnexin (1:500), or goat anti-LAMP1 (1:500). Primary antibodies were obtained from Santa Cruz Biotechnology and detected with a Cy3-conjugated anti-goat or anti-rabbit IgG. The *E. cuniculi* PV was stained with MAb 6G2 and detected by the Cy2 conjugate. Note that Cy2 and Cy3 labeling is reversed for LAMP1. Samples were analyzed with a Leica confocal laser-scanning microscope (model TCS SP2) or with a conventional fluorescence microscope (model DM R; Leica) in combination with digital camera equipment (AxioCam; Zeiss). Merged pictures (pictures using Cy2- and Cy3-conjugated antibodies plus the picture from phase-contrast microscopy) from conventional microscopy are presented for the 1-min and 24-h samples. Merged pictures (pictures using Cy2- and Cy3-conjugated antibodies) from confocal microscopy are shown for the 5-min and 1-h samples.

or lysosomal vesicle network or part of the ER or Golgi vesicle network.

In addition, colocalization studies of *E. cuniculi* PVs with phagocytosed Texas Red-zymosan particles were performed in macrophages derived from bone marrow and in HFF. Host cells were incubated with Texas Red-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

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 fusiogenic 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.

This work was supported by a grant from the Deutsche Forschungsgemeinschaft (GR 906/11-1).

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