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Ultrastructure of the development of a species of *Encephalitozoon* cultured from the eye of an AIDS patient*

Sherwin S. Desser[1], Henry Hong[1], and Y. James Yang[2]

[1] Department of Zoology, University of Toronto, Toronto, Ontario, Canada M5S 1A1
[2] Tropical Disease Unit, Toronto General Hospital, 101 College Street, Toronto, Ontario, Canada M5G 1L7

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Abstract. Human fibroblast cell cultures inoculated with microsporidia-infected corneal scrapings from an AIDS patient were fixed in situ and examined by scanning and transmission electron microscopy. The parasite grew prolifically and all developmental stages were observed. Meronts underwent binary fission and the daughter cells transformed into elongate, chain-like sporonts that eventually separated into sporoblasts. The formation of components of the mature spores is described. The parasite, a species of *Encephalitozoon*, underwent development both in the cytoplasm and within a parasitophorous vacuole, distinguishing it from the morphologically similar species *E. cuniculi* and *E. hellem*, both of which have been described from lesions in the human eye and have been reported to develop exclusively within a parasitophorous vacuole.

Species of *Encephalitozoon*, *Enterocytozoon*, *Nosema*, and *Pleistophora* have been described from AIDS patients with enteritis, hepatitis, and peritonitis (Shadduck 1989). Recently, several cases of corneal microsporidiosis (Cali et al. 1991 a, b) and keratoconjunctivitis associated with microsporidian infections have been reported from HIV-seropositive individuals [reviewed by Cali (1991) and Orenstein (1991)]. On the basis of ultrastructural observations of organisms found in conjunctival scrapings, Friedberg et al. (1990) attributed the lesions to infection with the microsporidian *E. cuniculi*. P.J. Didier et al. (1991) cultured conjunctival and corneal scrapings from AIDS patients with keratoconjunctivitis in Madin-Darby canine kidney cells and found organisms similar to *E. cuniculi*. Marked differences observed between the parasites on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting led these authors to describe a new species, *E. hellem*, although the isolates were considered to be morphologically indistinguishable (E.S. Didier et al. 1991).

In the present report, we describe the isolation, cultivation, and ultrastructural characterization of a species of *Encephalitozoon* from lesions in the eye of an AIDS patient.

Materials and methods

Microsporidian spores isolated from corneal scrapings from a 30-year-old man with AIDS who had experienced recurrent episodes of redness and crusting of both eyes were inoculated into MRC-5 (human lung fibroblast) cells grown on plastic coverslips in plastic Leighton tubes (3393, Costar) in minimum essential medium supplemented with 10% fetal bovine serum. Monolayers were processed intact to preserve the integrity of infected cells. Specimens on coverslips were transferred to sterile centrifuge tubes (25311, Corning), fixed with 2.0% (v/v) glutaraldehyde in 0.1 M phosphate buffer (pH 7.0), postfixed in 1.0% (v/v) osmium tetroxide in 0.15 M phosphate buffer (pH 7.0), and dehydrated through a graded ethanol series.

For transmission electron microscopy (TEM), specimens on coverslips were infiltrated with Spurr's epoxy resin, fractured into small pieces with a razorblade and hammer, embedded in flat molds with the tissue culture placed face down, and cured at 65° C overnight. Ultrathin sections were stained with uranyl acetate and Reynold's lead citrate and observed with a Hitachi H-7000 TEM operating at 75 kV. For scanning electron microscopy (SEM), pieces of coverslips with specimens were infiltrated with Peldri II (Pelco International), which was then allowed to sublimate. Dried specimens were mounted on high-resolution stubs, sputter-coated with gold-palladium, and examined with a Hitachi S-2500 operating at 20 kV with a working distance of −4 mm. Measurements were taken from electron micrographs of 30 mature spores and 10 ejected polar tubules and are expressed as mean values followed by the standard deviation.

Results

Heavily infected MRC-5 cells were hypertrophied and contained a variety of stages, including meronts, daughter cells, sporonts, and sporoblasts as well as developing and mature spores (Fig. 1). In some cells, the parasites

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Correspondence to: S.S. Desser

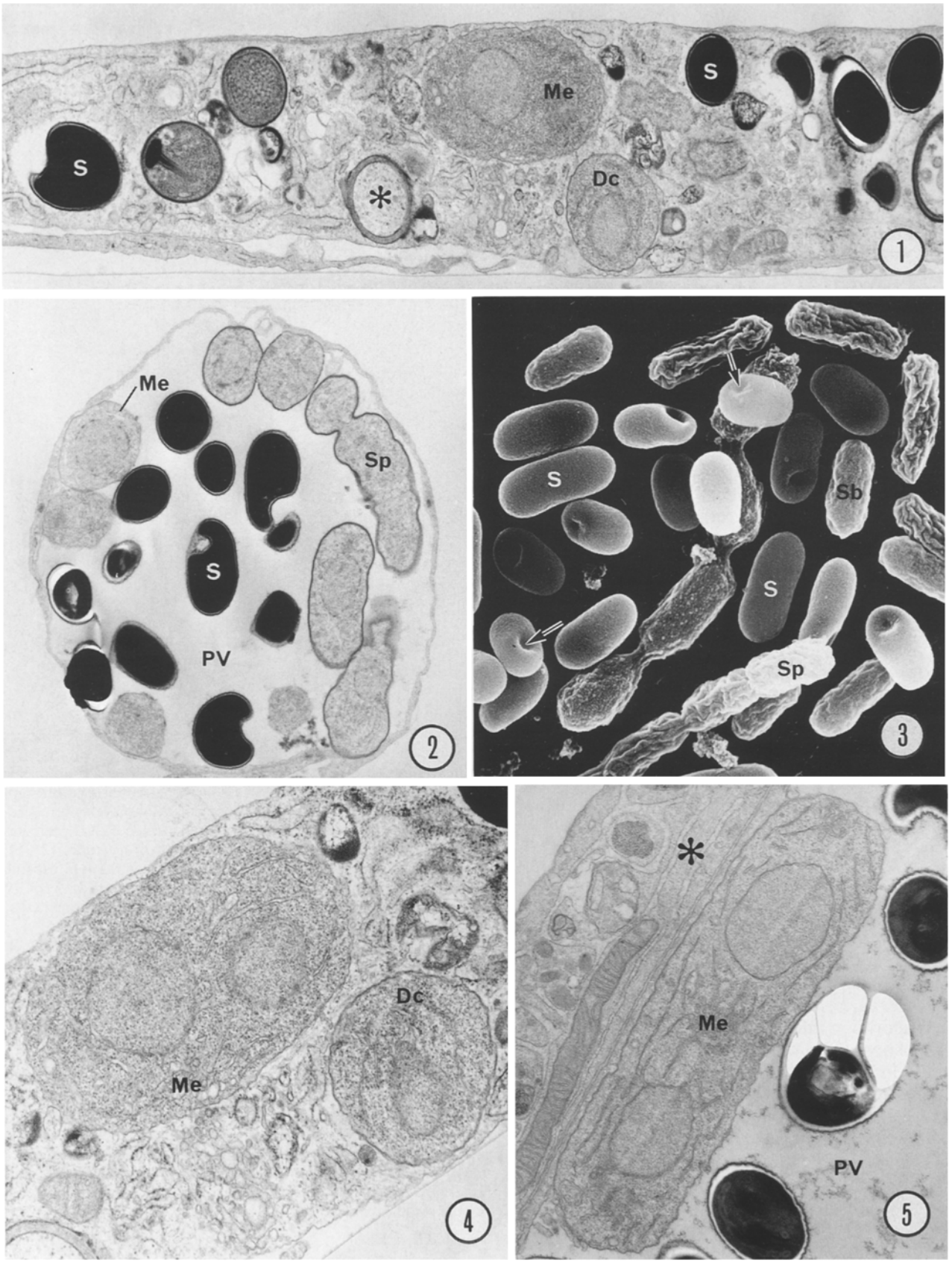


Fig. 1–18. Electron micrographs of developmental stages of *Encephalitozoon* sp. in human fibroblast cells

Fig. 1. Various stages of development, including a meront (*Me*), a daughter cell (*Dc*), dense mature spores (*S*), and a discharged spore (*asterisk*), lie in direct contact with the cytoplasm of the host cell. ×14000. **Fig. 2.** Mature spores (*S*), a meront (*Me*), and linked dense-walled sporonts (*Sp*) within a parasitophorous vacuole (*PV*). ×10500. **Fig. 3.** SEM of an infected cell containing smooth, ellipsoidal mature spores (*S*), wrinkled sporonts (*Sp*), and a smooth-walled sporoblast (*Sb*). Note the dimple (*arrow*) apparent in the subapical region of mature spores. ×7000. **Fig. 4.** A binucleate meront (*Me*) and a daughter cell (*Dc*) with a thicker wall composed of two membranes are in direct contact with the host-cell cytoplasm. ×26400. **Fig. 5.** A binucleate meront (*Me*) lies adjacent to the membrane of the PV. *Asterisk*, Host-cell cytoplasm. ×19000

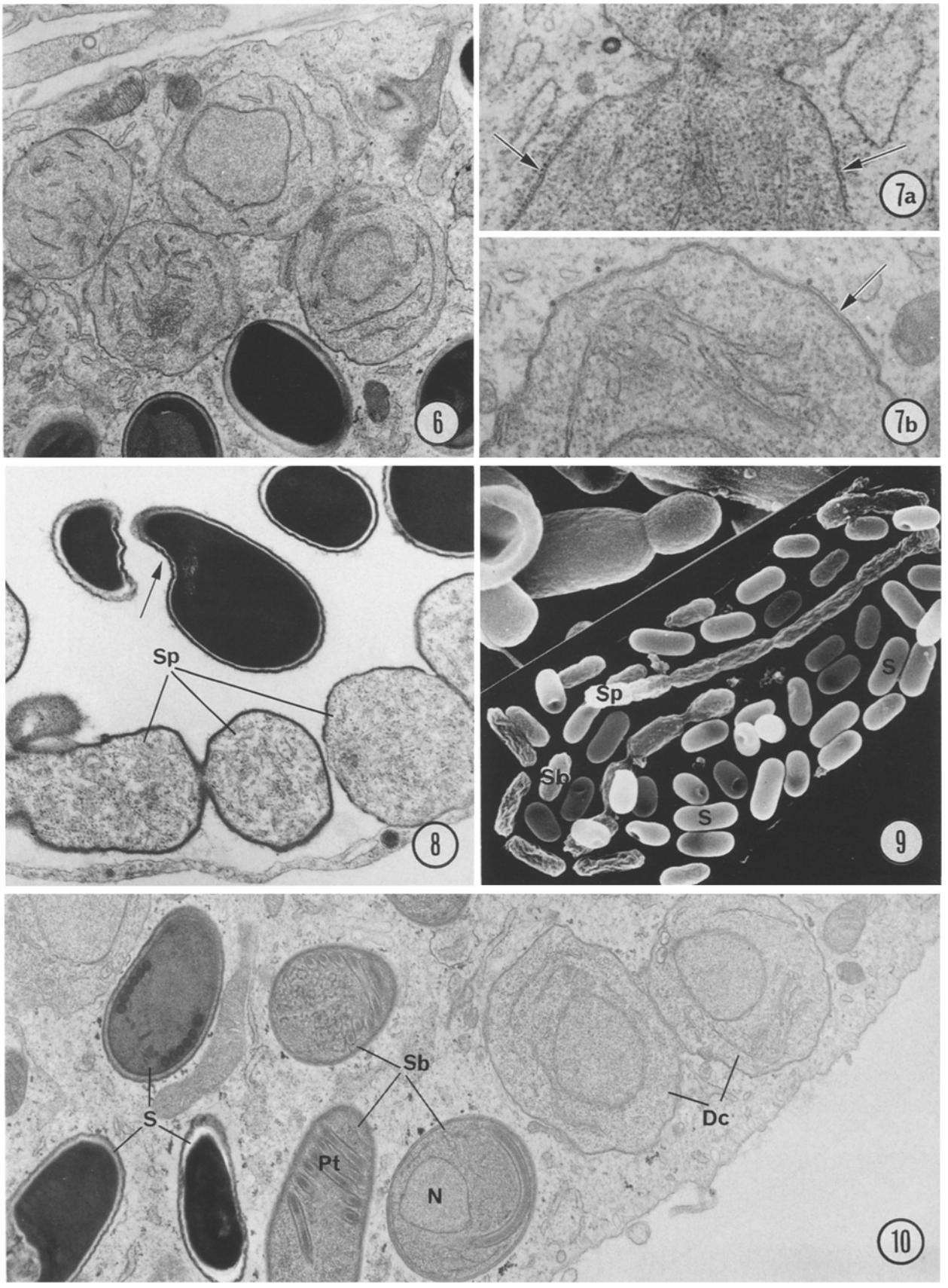
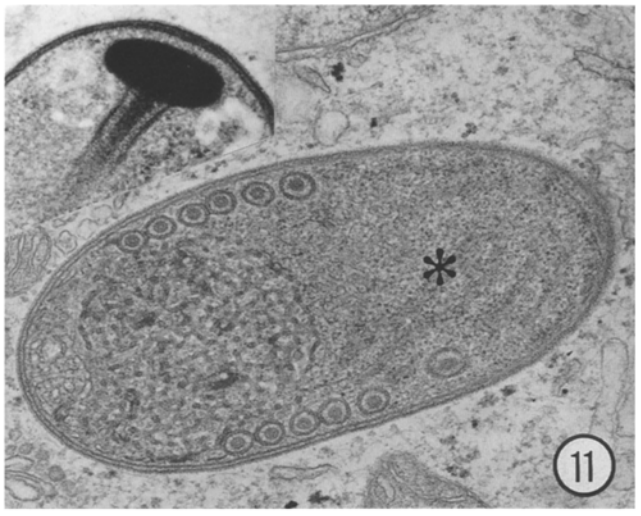


Fig. 6. Paired daughter cells bounded by a bi-membraned wall are visible in the host-cell cytoplasm. × 18800. **Fig. 7. a** Dividing meront. Note the accumulation of dense particles (*arrows*) external to the limiting membrane of the developing daughter cells. × 51000. **b** The boundary layer of the daughter cells is composed of two closely apposed membranes (*arrow*). × 51750. **Fig. 8.** Sporont chain with an electron-dense wall situated within the PV. A distinct indentation (*arrow*) can be seen in a mature spore. × 22800. **Fig. 9.** SEM illustrating an elongate, segmented and wrinkled sporont (*Sp*) chain, sporoblasts (*Sb*), and mature spores (*S*). × 4000. *Inset:* Young sporont undergoing division. × 11000. **Fig. 10.** Paired daughter cells (*Dc*), developing sporoblasts (*Sb*), and mature spores (*S*) lie free in the host-cell cytoplasm. Note the nucleus (*N*) and polar tubules (*Pt*) in the sporoblasts. × 19200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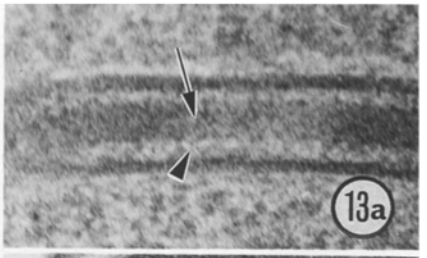
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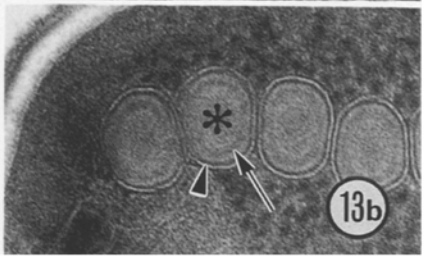
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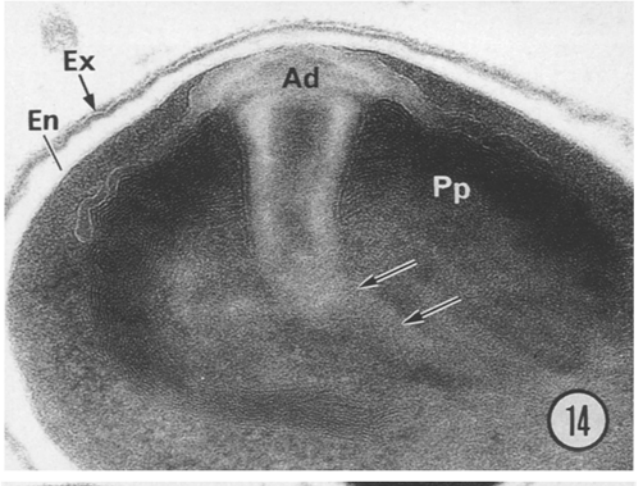
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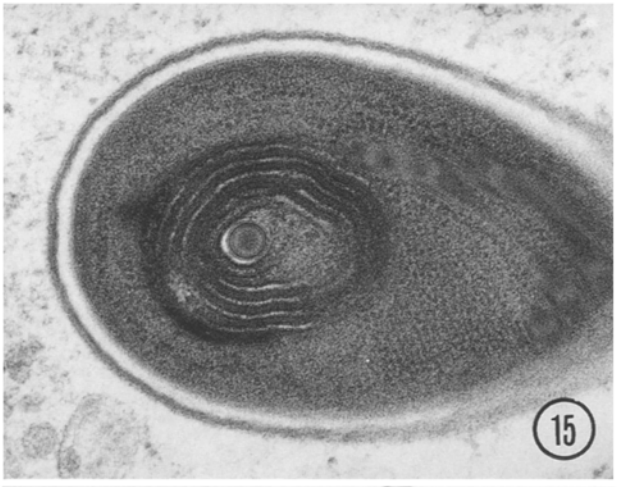
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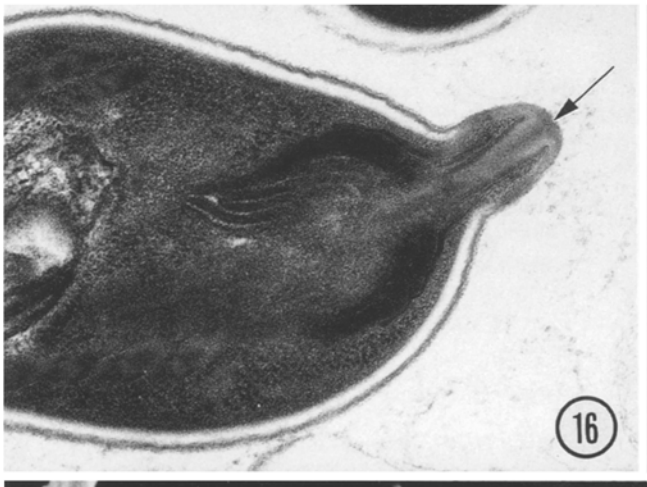
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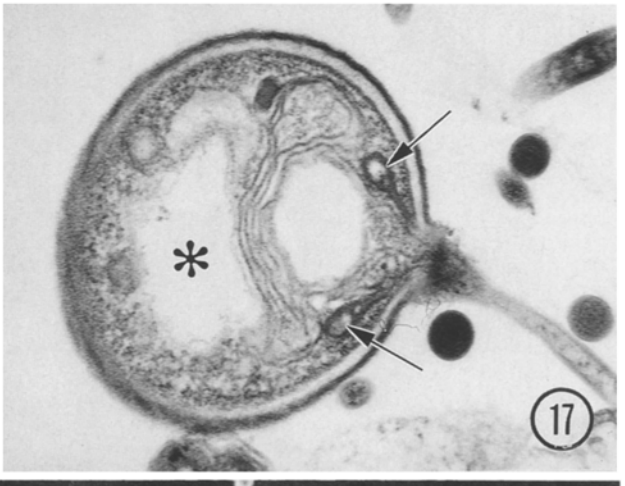
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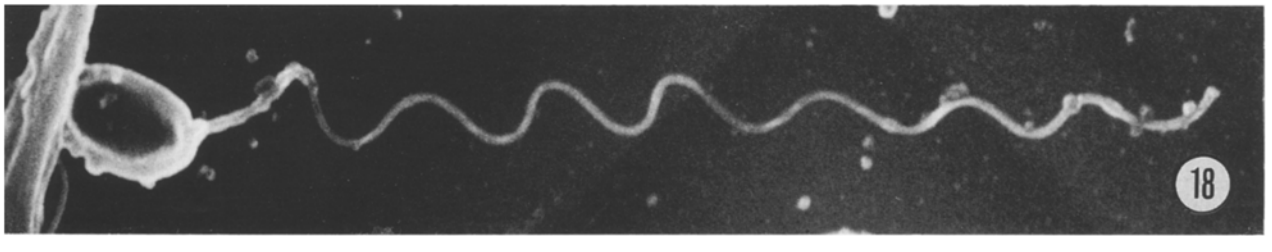
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lay in direct contact with the host-cell cytoplasm (Figs. 1, 4, 6, 7, 10); in others, the parasites were situated within a clearly defined parasitophorous vacuole (PV) (Figs. 2, 5, 8).

The earliest developmental stage seen, the meronts, either occurred randomly in the host-cell cytoplasm (Figs. 1, 4, 6, 10) or lay in direct contact with the PV membrane (Fig. 5). Meronts were irregular in outline and bounded by a plasma membrane. They had spherical nuclei, numerous free ribosomes, scattered cisternae of endoplasmic reticulum (ER), and a few vesicular membranous inclusions (Figs. 4–6). The meronts divided by binary fission and the resultant daughter cells were often seen in pairs (Fig. 6). The boundary layer of daughter cells appeared to be thickened with dense particles external to the limiting membrane (Fig. 7a). Mature cells were bounded by two closely apposed membranes (Fig. 7b).

The daughter cells transformed into sporonts, which in more advanced stages were easily recognized by their elongated, link-sausage-like appearance and their wrinkled surface. In some cells, sporonts were found adjacent to the PV membrane (Figs. 8, 9), and in others, they were observed in direct contact with the cytoplasm. The boundary layer of sporonts was thickened and electron-dense and appeared to have been formed by the deposition of dense material between the two membranes seen in the daughter cells (Fig. 8). Sporonts contained a nucleus and numerous free ribosomes. Chains of sporonts appeared to form by repeated fission (Figs. 8, 9, inset), later separating to form sporoblasts.

Sporoblasts were bounded by a distinct wall composed of two membranes, the outermost of which was covered by a flocculent dense layer (Figs. 10–12). The surface of sporoblasts was wrinkled, and mature spores had a distinct subapical "dimple" that was apparent both by TEM (Figs. 1, 2, 8) and by SEM (Figs. 3, 9). Each sporoblast had a nucleus, ribosomes, cisternae of rough ER, and accumulations of dense, membranous Golgi-like material, the latter of which appeared to contribute to the formation of the polar tubule (Figs. 11, inset; 12). Different stages of the formation of the polar tubule were seen in developing spores. The earliest stage consisted of a central dense tubular structure, the inner portion of the polar tubule, surrounded by an electron-lucent halo; the latter was enclosed by an electron-dense layer (Figs. 11, 12). The tubular nature of the dense core was less apparent in more advanced stages (Fig. 13a). The dilated terminal portion of the outer polar tubule was attached to an oval dense body near the anterior end of the spore (Fig. 11, inset). In mature spores, the polar tubule was surrounded by a trilaminar layer forming the cytoplasmic sleeve (Fig. 13b). The inner membrane-like layer formed the wall of the outer polar tubule. Within the matrix of transectioned tubules, an internal tubular structure with a dense core was seen.

The polar tubule was arranged into six coils in the posterior region of the spore (Figs. 10, 11). The broad end of the external polar tubule in mature spores was attached to the anchoring disc, a canopy-like structure (Fig. 14). The anterior end of the internal tubule abutted against the central region of the anchoring disc. The peripheral flange of the anchoring disc was draped over the electron-dense, closely packed lamellae of the polaroplast. More posterior to the lamellar polaroplast, the tubular polaroplast membranes were concentrically arranged around the transversely sectioned polar tubule (Fig. 15). The lamellar polaroplast surrounded the expanded regions of the polar tubule that connected to the anchoring disc (Figs. 14, 15). Closely packed ribosomes imparted a dense appearance to the cytoplasm of mature spores (Figs. 14–16). The wall of mature spores was thickened. External to the plasma membrane of the parasite's cytoplasm was a broad, electron-lucent layer, the endospore. This layer was overlain by the outer layer of the spore wall, the exospore, which was covered with a dense flocculent material (Figs. 13b, 14–17). The surface of mature spores was smooth (Figs. 3, 9).

The anterior end of the polar tubule extruded through the attenuated spore wall adjacent to the anchoring disc, the central portion of which appeared to rupture (Fig. 16). Subsequently, the outer tubule was everted, its dilated terminal portion remaining attached to the remnants of the anchoring disc, which formed a dense sleeve near the exit point of the tubule in the discharged spore (Fig. 17). Mature spores measured $1.15 \pm 0.08 \times 2.40 \pm 0.33$ µm. Extruded polar tubules measured 19.26 ± 3.35 µm in length (Fig. 18).

Fig. 11. Longitudinal section of a sporoblast with transected polar tubules. Note the dense, membranous Golgi-like material (*asterisk*) × 36000. *Inset:* Terminus of the outer polar tubule attached to an oval dense body in the subapical end of the sporoblast. × 48000. **Fig. 12.** Dense, membranous Golgi-like material in the sporoblasts appears to contribute to polar tubule formation (*arrow*). The sporoblast wall is composed of two closely apposed membranes, the outermost of which is covered by a dense flocculent layer. × 80000. **Fig. 13. a** Longitudinal section through an early stage of the polar tubule in a sporoblast. Note the central dense material (*arrow*) surrounded by an electron-lucent layer (*arrowhead*). × 49500. **b** Polar tubules in a mature spore. Note the cytoplasmic sleeve (*arrowhead*), inner tubule (*arrow*), and dense core (*asterisk*). × 58500. **Fig. 14.** The dilated terminal portion of the external tubule is attached to the anchoring disc (*Ad*). The tubule is surrounded by dense, closely packed lamellae of the polaroplast (*Pp*). Posterior to this, the polaroplast membranes assume a tubular form (*small arrows*). Note the exospore (*Ex*) and endospore (*En*). × 105000. **Fig. 15.** Concentrically arranged whorls of tubular polaroplast membranes surround the transversely sectioned polar tubule below the level of the anchoring disc. Note the densely packed ribosomes characteristic of mature spores. × 54000. **Fig. 16.** Early stage of extrusion of the internal component of the polar tubule (*arrow*). × 56000. **Fig. 17.** Discharged spore with remnants of the anchoring disc (*arrows*) and posterior vacuole (*asterisk*). × 52000. **Fig. 18.** SEM of a discharged spore with a fully extruded polar tubule. × 8400

Discussion

The presence of a single nucleus, the number of coils (6) of the polar tubule, and the dimensions of mature spores of the parasite described in this report indicate that it is a species of *Encephalitozoon*. Although the par-

Table 1. Comparative data on ocular microsporidiosis in humans

	Nosema corneum	*Encephalitozoon cuniculi*	*E. hellem*	Present study
Mono/diplokaryotic	Diplo	Mono	Mono	Mono
PV	No	Yes	Yes	Yes/no
Spore size (μm)	1.5–2.5 × 3–5	Up to 1.1 × 2.5	1.0–1.5 × 2–2.5	1.15 × 2.40
Number of coils of polar tubule	11–13	Up to 7	6–8	6
Source of organisms	Deep ulcers in corneal stroma (biopsy material)	Corneal kerato-conjunctival scrapings (biopsy material)	Kerato-conjunctival scrapings cultured in Madin-Darby canine kidney cells	Corneal scrapings, swabs cultured in MRC-5 (human lung fibroblast) cells
References	Davis et al. 1990; Cali et al. 1991a, b	Friedberg et al. 1990	P.J. Didier et al. 1991	

asite shares many ultrastructural features with *E. cuniculi* and *E. hellem*, it differs from them in that stages from meronts to mature spores occurred both in the cytoplasm and within a parasitophorous vacuole (see Table 1 for comparison with other microsporidian parasites described from the human eye).

These discrepancies may have been due to differences in the processing of infected cultures. In the present study, cells were fixed on plastic coverslips and parasites were observed in situ. In the other studies in which cultured cells were used (Shadduck et al. 1990; E.S. Didier et al. 1991; P.J. Didier et al. 1991), MDCK cells were scraped off their substrate and centrifuged – procedures that might lead to additional artifacts. Although possible, it seems unlikely that two morphologically similar species of *Encephalitozoon* were present. Because of these uncertainties, we are reluctant to assign a specific epithet for the parasites described herein. The prolific growth of the microsporidian parasite in MRC-5 cells facilitated a detailed understanding of the events leading to spore formation, and these are depicted diagrammatically in Fig. 19.

Elongate, segmented sporonts like those reported herein have also been recorded for *E. hellem* by E.S. Didier et al. (1991) and P.J. Didier et al. (1991), who describe them as late meronts. In contrast, sporonts of *E. cuniculi* appear to undergo a single division, giving rise to two sporoblasts (Sprague and Vernick 1971; Pakes et al. 1975). The polar tubule (a tubule within a tubule), which is typical of other microsporidian species (Weidner 1976, 1982a, b), was formed from a reticulated membrane network in the sporoblast cytoplasm, with the broad terminal end of the external tubule remaining attached to the anchoring disc in mature spores. The outer surface of the mature spores was smooth, its walls being thickened due to an accumulation of electron-lucent material,

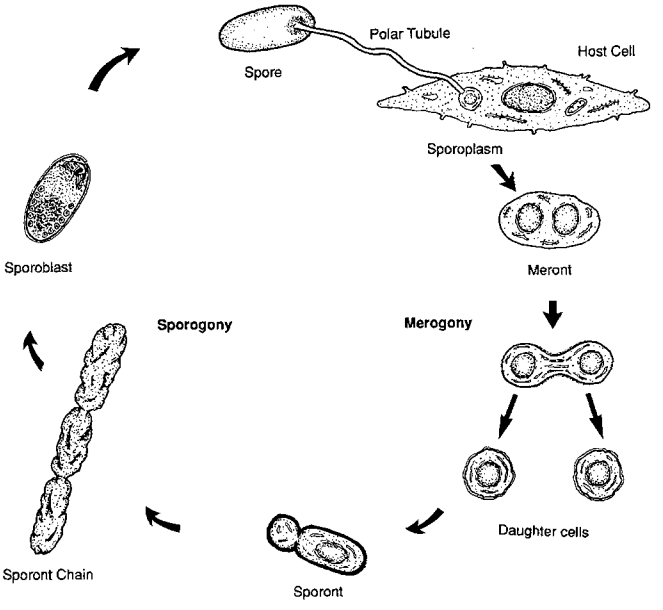

Fig. 19. Diagrammatic representation of the developmental cycle of *Encephalitozoon* sp. in human fibroblast cells: uninucleate sporoplasm injected into a host cell gives rise to a meront in the cytoplasm or in a PV; meronts undergo binary fission to form daughter cells, each of which transforms into a dense-walled sporont; sporonts elongate and undergo division to form chains; individual units separate, each becoming a sporoblast. These undergo differentiation to form mature spores; the polar tubule is discharged by a process of extrusion and eversion, and the sporoplasm is injected into the host cell

the endospore, found between the plasma membrane of the parasite and the dense surface layer, the exospore. The thinner region in the wall of mature spores overlying the anchoring disc corresponded to the "dimpling" observed by both SEM and TEM.

Much remains unknown about the number of species of *Encephalitozoon* that infect humans or about the range of tissues that are invaded by these parasites. Canning and Hollister (1991) have suggested that as many strains as possible should be isolated to establish a bank against which patient sera can be tested. Further studies on the development of these parasites are warranted and could be facilitated by using cell cultures grown on plastic coverslips, as this permits ultrastructural investigation of intact infected cells.

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References

Cali A (1991) General microsporidian features and recent finding on AIDS isolates. J Protozool 38:625–630

Cali A, Meisler DM, Rutherford I, Lowder CY, McMahon JT, Longworth DL, Bryan RT (1991a) Corneal microsporidiosis in a patient with AIDS. Am J Trop Med Hyg 44:463–468

Cali A, Meisler DM, Lowder CY, Lembach R, Ayers L, Takvorian PM, Rutherford I, Longworth DL, McMahon J, Bryan RT (1991b) Corneal microsporidioses: characterization and identification. J Protozool 38:215S–217S

Canning EU, Hollister WS (1991) In vitro and in vivo investigations of human microsporidia. J Protozool 38:631–635

Davis RM, Font RL, Keisler MS, Shadduck JA (1990) Corneal microsporidiosis: a case report including ultrastructural observations. Ophthalmology 97:953–957

Didier ES, Didier PJ, Friedberg DN, Stenson SM, Orenstein JM, Yee RW, Tio FO, Davis RM, Vossbrinck C, Millichamp N, Shadduck JA (1991) Isolation and characterization of a new human microsporidian, *Encephalitozoon hellem* (n. sp.), from three AIDS patients with keratoconjunctivitis. J Infect Dis 163:617–621

Didier PJ, Didier ES, Orenstein JM, Shadduck JA (1991) Fine structure of a new human microsporidian, *Encephalitozoon hellem* in culture. J Protozool 38:502–507

Friedberg DN, Stenson SM, Orenstein JM, Tierno PM, Charles NC (1990) Microsporidial keratoconjunctivitis in acquired immunodeficiency syndrome. Arch Ophthalmol 108:504–508

Orenstein JM (1991) Microsporidiosis in the acquired immunodeficiency syndrome. J Parasitol 77:843–864

Pakes SP, Shadduck JA, Cali A (1975) Fine structure of *Encephalitozoon cuniculi* from rabbits, mice and hamsters. J Protozool 22:481–488

Shadduck JA (1989) Human microsporidiosis and AIDS. Rev Infect Dis 11:203–207

Shadduck JA, Meccoli RA, Davis R, Font RL (1990) Isolation of a microsporidian from a human patient. J Infect Dis 162:773–776

Sprague V, Vernick SH (1971) The ultrastructure of *Encephalitozoon cuniculi* (Microsporida, Nosematidae) and its taxonomic significance. J Protozool 18:560–569

Weidner E (1976) The microsporidian spore invasion tube: I. The ultrastructure, isolation, and characterization of the protein comprising the tube. J Cell Biol 71:23–34

Weidner E (1982a) The microsporidian spore invasion tube: II. Role of calcium in the activation of invasion tube discharge. J Cell Biol 93:970–975

Weidner E (1982b) The microsporidian spore invasion tube: III. Tube extrusion and assembly. J Cell Biol 93:976–979