Isolation of a Microsporidian from a Human Patient

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Several genera of microsporidia have been identified morphologically in human tissue but none has yet been propagated in vitro. These primitive, obligate intracellular parasitic protozoa are poorly understood pathogens of a wide variety of vertebrates and invertebrates. In humans they are especially important as opportunistic pathogens in AIDS patients. A microsporidian was recovered from a human patient and propagated in vitro. The organism has diplokarya, divides by binary fission, and often is found free in the host cell cytoplasm. The name *Nosema corneum* is suggested.

Organisms of the phylum Microspora [1] are primitive [2] eukaryote obligate intracellular parasites that are widely distributed in nature [3]. They occur as hyperparasites in nematodes, are ubiquitous among insects, and have been detected morphologically in all major groups of vertebrates [1]. The only mammalian organism isolated to date, *Encephalitozoon cuniculi*, has been recovered from rabbits, mice, hamsters, rats, dogs, and foxes. All available evidence suggests that these isolates are identical. Infection is associated with clinical illness, severe lesions, and death in athymic mice, young carnivores, neonatal squirrel monkeys, and some birds. Infection is persistent but usually clinically silent in euthymic mice, rabbits, adult squirrel monkeys, and adult carnivores. Persistent infections are detected by a variety of serologic tests. Both vertical and horizontal transmission occur in animals [1, 3].

Four genera of microsporidia have been seen in tissue from human patients but none has been isolated or propagated in vitro. *Enterocytozoon bieneusi* has been reported a number of times in intestinal biopsy samples from patients with severe diarrhea associated with AIDS [4–6]. Organisms resembling members of the genus *Encephalitozoon* [7, 8] have been reported in tissues from other AIDS patients, and another immunodeficient but human immunodeficiency virus (HIV)-negative patient had organisms resembling the genus *Pleistophora* [9, 10]. A child with severe combined immunodeficiency was found at autopsy to be heavily infected with a microsporidian of the genus *Nosema*—*Nosema connori* [11]. There are two published reports of microsporidial infections of human corneas [12, 13], which were subsequently placed in the genus *Microsporidium* [1]. Several human infections were detected by serologic methods in which the mammalian parasite *Encephalitozoon cuniculi* was used as antigen [14–16]. The source(s) of human infections is unknown. There is no treatment for either animal or human infections, although an antibiotic (fumagillin) is effective against nosematosis of honeybees and is parasitostatic against *Encephalitozoon cuniculi* in vitro [17].

Subject and Methods

We report the first in vitro isolation and propagation of a microsporidian from a human patient, an otherwise healthy 45-year-old man with no history of previous ocular trauma, disease or contact lens wear [18]. He had recently traveled to the Caribbean and Central America and had lived near a large recreational lake before the onset of his ocular disease. He had an 18-month course of persistent central disciform keratitis, recurrent patchy infiltration of the anterior stroma, and iritis. The patient was treated with topical steroids and broad-spectrum antibiotics but ultimately required a corneal transplant. No viral or bacterial pathogens were recovered. No evidence of recurrent keratitis has been noted since transplantation. The patient is HIV-seronegative.

After corneal biopsy, organisms compatible with microsporidia were seen in the corneal stroma by light and electron microscopy. At the time of corneal transplant surgery, part of the keratoplasty specimen was placed in Hanks' balanced salt solution and shipped overnight to the laboratory. Two established cell lines and one primary cell culture were used. SIRC (ATTC 60) is an established cell line originally derived from rabbit corneal epithelium and MDCK (ATTC 34) was derived from canine kidney. Both are epithelioid and anchorage-dependent. Primary rabbit embryo fibroblast cells were obtained from 14- to 16-day-old rabbit fetuses and grown as anchorage-dependent fibroblastoid cultures. Pieces of minced corneas, with or without treatment for 75 min at 37°C with 0.1% trypsin and 0.25% collagenase, were placed on 25-cm^2 flasks on partly confluent cell culture monolayers. Some bits of cornea were explanted directly into 24-well plates. Eagle's minimum essential medium supplemented with 5% fetal bovine serum and 0.1% gentamicin was used and the cultures were incubated at 37°C in 5% CO_2 in air.

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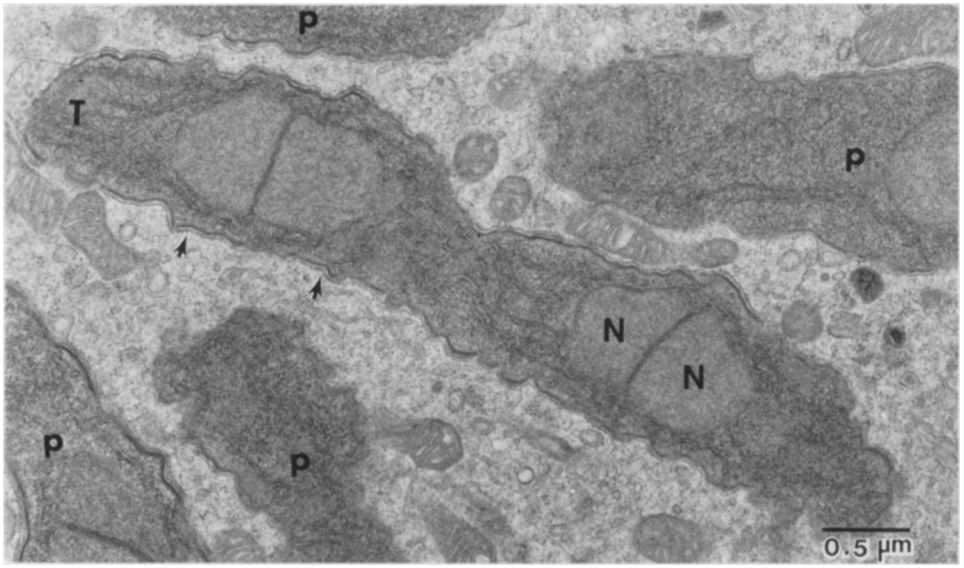

Figure 1. Dividing sporont with two diplokarya. Each subunit of one diplokaryon is marked N. There are intracellular tubular arrays (T) and a parasite cell wall (arrows) composed of a thick electron-dense inner layer separated from a thick outer electron-lucent layer by a thin parasite plasma membrane. Host endoplasmic reticulum may be present immediately adjacent to the outer electron-lucent parasite coat. Parts of four other proliferative forms (p) are seen.

Medium for primary cornea explants was further supplemented with 10 ng/ml epidermal growth factor, 5 μg/ml insulin, and 0.1 μg/ml cholera toxin.

For electron microscopy, infected monolayers were transferred to new dishes, fixed for 20 min in 2.5% glutaraldehyde in cacodylate buffer, and embedded in situ. Infected sites (previously marked) were trimmed from the disk of embedded cells and reembedded in capsules for thin sectioning.

Results

After ~30 days in culture, SIRC and MDCK cells with trypsin- and collagenase-treated corneal tissue had foci of granulated cells, which gradually enlarged over the next 14 days. Large numbers of small rod-shaped structures were seen floating in the medium beginning 60–70 days after culture. Neither the rabbit embryo fibroblast cells appeared to become infected nor did primary corneal explants appear to produce organisms, although small rod-shaped structures, compatible in size and shape to those seen in the SIRC and MDCK cultures, were seen in the stroma of the pieces of cornea adhered to the culture dishes.

Ultrastructurally, organisms in all stages of development were seen. Replication took place directly in the cytoplasm of host cells without interposition of a membrane between host and parasite. Organisms were rod-shaped and measured 3.7 × 1.0 μm (spores) to 5.6 × 0.8 μm (meronts). Division was by binary fission. The least mature forms (meronts) were characterized by thin, electron-dense outer membranes and poorly developed organelles. Recognizable nuclear envelopes surrounding two nucleus-like structures were tightly abutted along one side (a diplokaryon). Dividing meronts were markedly elongated with a diplokaryon in each future daughter cell.

Sporonts were more mature and had thicker cell walls with a distinct electron-lucent outer zone and a dark, thick inner amorphous layer on a plasma membrane. The thinner, fainter outer electron-dense layer may have been host endoplasmic reticulum with a few ribosomes attached. The diplokaryon was distinct and present in both future daughter cells of dividing organisms (figure 1). Membranous arrays suggestive of a polaroplast and polar tubule were seen. Sporoblasts had a distinct, thick electron-lucent wall separating the inner and outer electron-dense layers. Each cell had a single diplokaryon, a well-developed polaroplast, and a polar tubule coiled in five or six turns at one pole of the cell (figure 2). Spores were characterized by their thick walls and well-developed polaroplasts and polar tubules. Many mature spores contained only pale amorphous material and appeared to have lost their contents. Identical intact and degenerate spores were seen ultrastructurally in the patient's original biopsy tissue.

Even though host cells were heavily infected and produced numerous organisms, they appeared relatively normal ultrastructurally. Large numbers of host cell mitochondria clustered near the parasites (which are devoid of mitochondria). Organisms were not present in host cell lysosomes nor did lysosomes fuse with the parasite—even those that appeared to be empty spores. Parasites were released from host cells upon lysis of the cell, apparently as a result of distension of the cells by parasites.

The ultrastructural appearance of these organisms is characteristic of the phylum Microspora and resembles other organisms classified in the genus *Nosema* even though size, the apparent development of some stages of the parasite within cisternae of host endoplasmic reticulum, and the number of coils (5–6) of the polar tubule are more like *Encephalitozoon cuniculi*. Based on the morphologic evidence, we suggest the

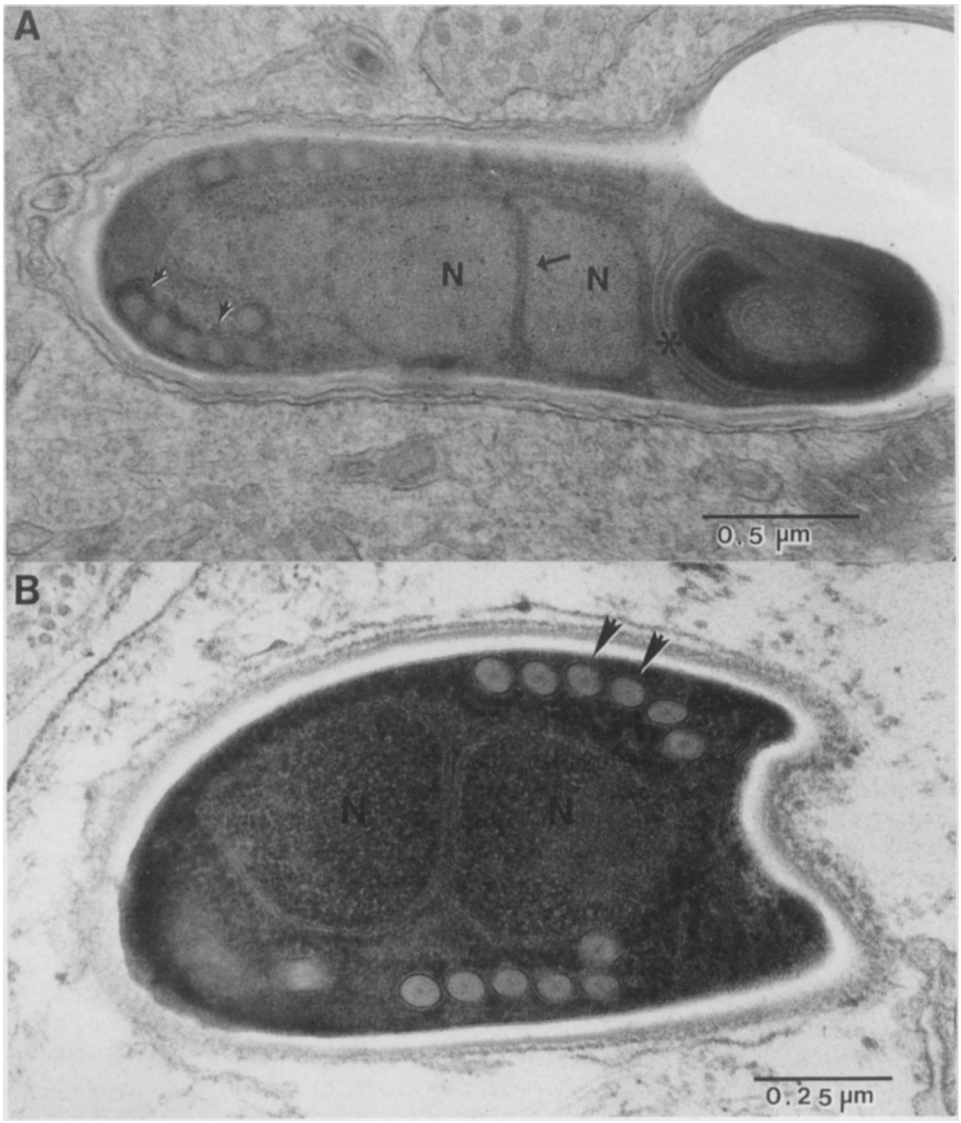

Figure 2. Late sporoblasts or early spores with a diplokarya (each subunit marked N) with abutted nuclear envelopes (arrow), polaroplast (*), and a polar tubule with six coils (arrows). **A**, Tissue culture isolate. **B**, Patient's corneal biopsy.

name *Nosema corneum*. Our isolate probably is not *Nosema connori* [11] because that organism has 10–11 coils of the polar tubule. Our isolate probably differs from those previously described in corneas of patients and named *Microsporidium ceylonensis* and *Microsporidium africanum* [11] since both had 10–11 coils of the polar tubule [12, 13] (unpublished data).

The successful in vitro isolation of a human *Nosema* species suggests that the technique may prove useful in recovering other, more commonly occurring human microsporidia. Indeed, recent preliminary findings in our laboratory suggest that human *Encephalitozoon*-like organisms also can be grown in vitro. The current availability of large quantities of *Nosema corneum* and the potential that other genera of human microsporidia will grow in vitro provide the basis for a number of important experiments. Studies on subjects ranging from genetic and immunologic comparisons of human and other isolates, drug screens, pathogenesis, epidemiology in relationship to AIDS, and a search for sources of infection now become possible.

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