Isolation and Characterization of a New Human Microsporidian, *Encephalitozoon hellem* (n. sp.), from Three AIDS Patients with Keratoconjunctivitis

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A new human microsporidian was isolated from cultures of Madin-Darby canine kidney cells incubated with conjunctival scrapings or corneal tissues from three AIDS patients with keratoconjunctivitis. The three isolates were morphologically similar to *Encephalitozoon cuniculi*. The spores averaged 1 × 1.5–2.0 μm, had six to eight polar filament coils, displayed monokaryotic nuclei, and possessed relatively thick endospores with irregularly shaped exospores. Organisms developed within a parasitophorous vacuole. By SDS-PAGE analysis, the three isolates appeared to be identical but were different from *E. cuniculi*. Identical banding patterns on Western blots of the three isolates were expressed by each patient's serum. By Western immunoblotting, murine antisera to *E. cuniculi* reacted to several antigens of the new AIDS-related microsporidian, whereas murine antisera bound weakly to *Nosema corneum*. The name *Encephalitozoon hellem* (n. sp.) is proposed to identify this new human microsporidian.

Microsporidia are ubiquitous obligate intracellular protozoan parasites that infect all animal phyla [1]. Microsporidia are unique enough to be classified in a separate phylum, Microspora [2, 3], and are characterized by the polar filament, which is used to inject sporoplasm into the host cell. Species of microsporidia that infect mammals are unicellular, Gram-positive organisms with mature spores 0.5–2 × 1–4 μm in diameter [1]. Classification is based on size, nuclear arrangement (mono- or diplokaryotic), mode of division, and association of proliferative forms within the host cell (development within a parasitophorous vacuole or direct association with host cytoplasm).

Microsporidia are found commonly in laboratory animals such as mice, rabbits, and hamsters. Published reports of clinically significant microsporidiosis in humans are increasing in association with the increase in patients with AIDS. Four genera of microsporidia are known to infect humans. *Enterocytozoon bieneusi*, the most common microsporidian observed in AIDS patients, infects enterocytes of the bowel and causes diarrhea [4–8]. The estimated prevalence may be as high as 10% in AIDS patients [9]. A *Pleistophora* species was observed in an immunocompromised (HIV-seronegative) individual with myositis [10, 11]. *Encephalitozoon cuniculi*, most commonly observed in laboratory animals, has been reported in several immunocompromised patients [12] and recently was reported in AIDS patients with peritonitis [13] and hepatitis [14]. Ocular infections with a *Nosema* species [15] and with *Nosema corneum* (n. sp.) [16] have been reported in HIV-seronegative patients, and infections with *E. cuniculi*–like organisms were reported in several AIDS patients with conjunctivitis [17, 18] (unpublished data).

Isolation of human microsporidia is becoming important for developing diagnostic procedures. Previously, *E. cuniculi* was the only available mammalian microsporidian for use in serologic tests [19]. Recently, Shadduck et al. [20] isolated the first human microsporidian, *N. corneum*, from the corneal stroma of an HIV-seronegative patient with keratitis. Here we report the first isolation and characterization of a new microsporidian from three HIV-seropositive patients with keratoconjunctivitis.

Materials and Methods

In vitro growth of microsporidia. *E. cuniculi*, *N. corneum*, and *Nosema algerae* were grown in Madin-Darby canine kidney (MDCK)

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cells (ATCC CCL 34) using RPMI 1640 culture medium (Flow, McLean, VA) supplemented with 5% heat-inactivated fetal bovine serum and antibiotics. Corneal tissues from one and conjunctival scrapings from two HIV-seropositive patients with microsporidial keratoconjunctivitis [18] (unpublished data) were minced and added to near-confluent monolayers of MDCK cells. The cultures were incubated at 37°C with 5% CO_2 except for *N. algerae* cultures, which were incubated at room temperature [21].

Parasite harvest. Tissue culture supernatants containing parasites were centrifuged at 400 *g* for 30 min, resuspended in PBS with 0.1% Tween 20, and centrifuged over a 50% Percoll (Pharmacia, Piscataway, NJ) cushion (500 *g* for 30 min) to remove host cell debris. The parasite pellets were washed several times in Hanks' buffered saline solution (HBSS).

Murine antisera. Antisera to *E. cuniculi, N. corneum,* and *N. algerae* were raised in BALB/c mice (five per group) given two intraperitoneal injections of 5×10^7 organisms at 1-month intervals. Sera were collected 1 week after the second injection and stored frozen at −70°C until used.

SDS-PAGE. Parasites were dissolved in electrophoresis sample buffer containing β-mercaptoethanol [22]. Proteins from 1×10^6 parasites per lane (~ 70 μg) or 10^8 parasites (~7 mg) per preparative gel were separated by electrophoresis in 12% acrylamide-DATD (*N,N*'diallyltartardiamide) minigels (Bio-Rad, Richmond, CA). Either the gels were double-stained with Coomassie brilliant blue (R250) and silver stain (Bio-Rad), or proteins were electrophoretically transferred onto Immobilon-p membranes (Millipore, Bedford, MA) for Western blot immunodetection [23]. Molecular weight protein standards were from Bio-Rad.

Western blot immunodetection. All incubations were done with agitation using a rotator platform. The blots were first incubated in 5% (wt/vol) instant nonfat milk (Carnation) in PBS for 1 h, followed by overnight incubation with test sera diluted 1:200. After several washes with 0.3% Tween 20 in PBS, the blots were incubated with alkaline phosphatase–conjugated goat anti-human or anti-murine IgG (1:1000; Sigma, St. Louis) for 2 h at room temperature. The blots were then washed several times with 0.3% Tween 20 in PBS followed by a wash in PBS. To detect the antibody-binding sites, the blots were rinsed with developing buffer (50 m*M* Tris, 5 m*M* $MgCl_2$, pH 10.0), followed by incubation with tetrazolium salts and bromochloroindolyl phosphate for 30 min [24]. The blots were rinsed in distilled water and stored in the dark until photographed.

Transmission electron microscopy (TEM). Tissue culture supernatants or trypsinized host cells were fixed in Karnovsky's fixative, post-fixed with 2% osmium tetroxide, embedded in Spurr's resin, and processed for TEM. Thin sections stained with lead citrate were examined using a JEOL (Japan Electronic Optics Laboratory) 1200EX electron microscope.

Results

Isolation of conjunctival/corneal microsporidia. Parasites isolated from the three AIDS patients with keratoconjunctivitis appeared morphologically similar. An electron micrograph of parasites from one patient, which were grown in MDCK cells, is shown in figure 1. The proliferative stages appeared similar to *E. cuniculi*. The larger proliferative stages (meronts) were attached to a parasitophorous vacuole membrane, and all proliferative organisms were monokaryotic. The smaller, electron-dense mature spores were detached in the vacuole lumen and appeared similar in size (1.0–1.5 × 2.0–2.5 μm) and morphology to *E. cuniculi*. The spores displayed relatively thick electron-lucent endospore and irregular, electron-dense exospore structures. In addition the spores contained about six to eight turns of the polar filament and monokaryotic nuclei. The tissue culture–derived spores isolated from MDCK cells were morphologically identical to the spores seen in biopsy material from each patient [18] (unpublished data).

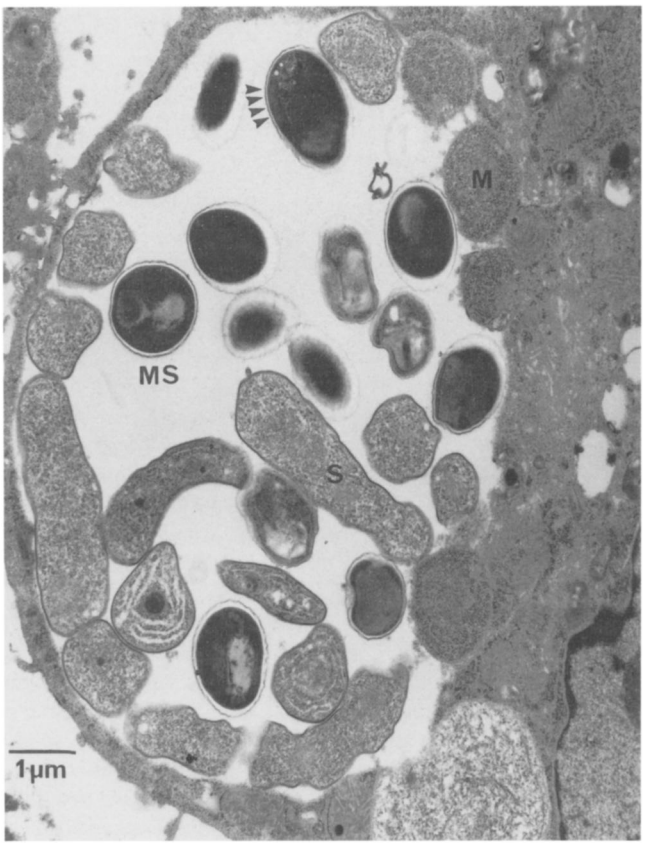

Figure 1. Electron micrograph of developing forms of AIDS-related ocular microsporidia within infected Madin-Darby canine kidney cell. Immature organisms (meronts, M) are attached to wall of parasitophorous vacuole. Intermediate stages (sporonts, S) and smaller, mature spores (MS) are seen within lumen. Mature spores have irregular electron-dense exospores, electron-lucent endospores, and polar filaments. (arrowheads).

SDS-PAGE. To further examine whether these corneal/conjunctival isolates from AIDS patients were identical to *E. cuniculi*, parasite proteins were separated by SDS-PAGE and double-stained with Coomassie blue and then silver stain (figure 2). The protein profiles of parasites isolated from patients 1–3 were identical but were different from *E. cuniculi*. *N. corneum*, a human microsporidian isolated from the corneal stroma of an HIV-seronegative individual, and *N. algerae*,

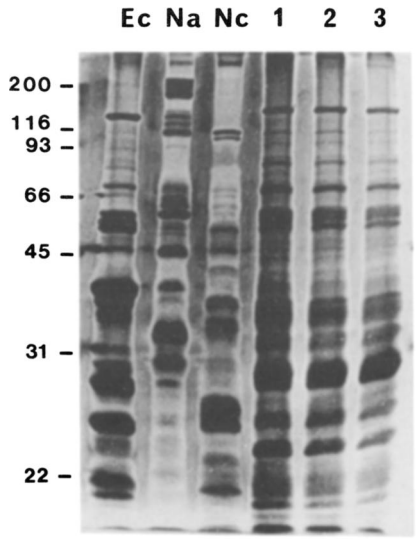

Figure 2. SDS-PAGE of microsporidia. Protein from 1 × 10^6 spores of *Encephalitozoon cuniculi* (Ec), *Nosema algerae* (Na), *Nosema corneum* (Nc), and parasites from patients 1, 2, and 3 were separated in 12% DATD-acrylamide minigel. Gels were stained with Coomassie brilliant blue (R250) followed by silver stain, and molecular masses were noted in kilodaltons.

a mosquito microsporidian to which man may be exposed, were included for comparison and produced unique protein profiles as well.

Western blot immunodetection. The patient sera were tested against *E. cuniculi, N. algerae, N. corneum*, and the three AIDS-related corneal/conjunctival isolates to confirm that these isolates were the same organism, to determine if AIDS patients infected with microsporidia produced antibodies, and to show that these isolates were different immunologically from *E. cuniculi, N. algerae,* and *N. corneum* (figure 3). Patients 1 and 2 were in the later stages of group IV AIDS as classified by the Centers for Disease Control (CDC; Atlanta), and their sera were obtained ~1 month before death. Several bands of reactivity were detected against their homologous isolates on Western immunoblots. Identical patterns were seen when the other two AIDS-related ocular isolates were used as the antigens. Bands of reactivity to proteins of different molecular weights also were noted against *N. corneum, N. algerae*, and *E. cuniculi*. Serum from the third patient, who was also HIV-positive but was clinically healthier than patients 1 and 2 when serum was collected (late group III or early group IV CDC classification), displayed a strong response to the homologous isolate with nearly identical banding patterns to the other two AIDS-related ocular isolates. Bands of different molecular weights were observed against *E. cuniculi*, and a few weaker bands were noted against *N. algerae* and *N. corneum*. These results suggested that the three new AIDS-related ocular isolates represented one organism and were immunologically different from *E. cuniculi, N. algerae*, and *N. corneum*.

Murine antisera to *E. cuniculi, N. corneum*, and *N. algerae* also were assayed against Western blots of the microsporidia to further examine immunologic relationships among *E. cuniculi, N. corneum, N. algerae*, and the three AIDS-related ocular isolates (figure 4). The antiserum to *E. cuniculi* displayed 15–20 bands of reactivity to its own proteins. In addition, the antiserum to *E. cuniculi* cross-reacted to one major protein of *N. corneum* and about five proteins of *N. algerae* and expressed stronger bands of cross-reactivity to proteins of different molecular weights of the new ocular isolates. The murine antiserum raised against *N. algerae* reacted strongly to its own proteins and displayed several different weak bands of cross-reactivity to the other organisms. The antisera raised against *N. corneum* reacted strongly to its own proteins but did not strongly cross-react to the new ocular isolates.

Discussion

This study reports the isolation of a new human microsporidian from the corneal and conjunctival epithelium of three pa-

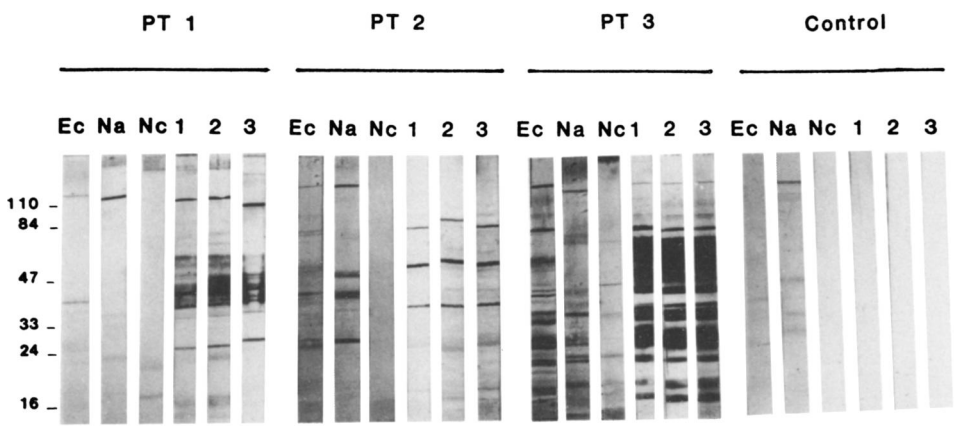

Figure 3. Western blot immunodetection of patient sera and microsporidia-negative control serum assayed against preparative minigel protein blots of microsporidia proteins. Sera were tested at 1:200 dilutions as described in text. Antibody binding was detected by incubation of blots with alkaline phosphatase–conjugated goat anti-human IgG (1:1000) and developed by incubation with tetrazolium salts and bromochloroindolyl phosphate. Approximate molecular masses are noted at left in kilodaltons. PT, patient; Ec, *Encephalitozoon cuniculi*, Na, *Nosema algerae*; Nc, Nosema corneum.

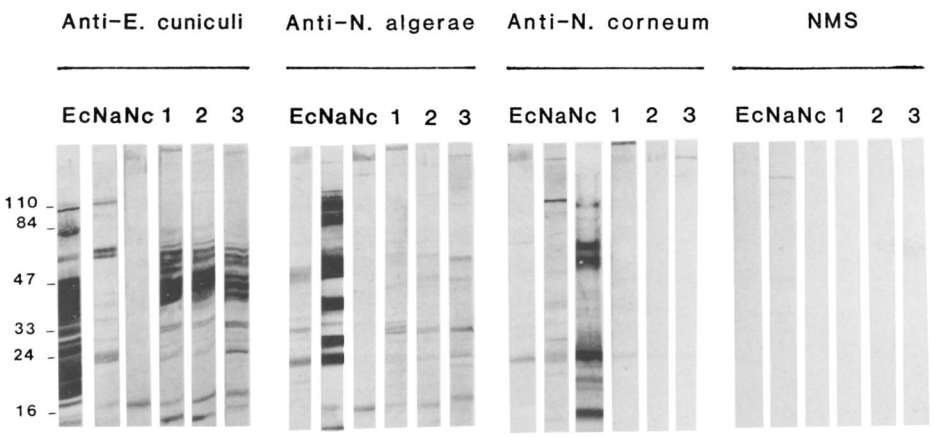

Figure 4. Western blot immunodetection of microsporidia with murine antisera raised against *Encephalitozoon cuniculi* (Ec), *Nosema algerae* (Na), or *Nosema corneum* (Nc), with normal mouse serum (NMS) as control. Parasite proteins were separated by electrophoresis and blotted as described in text. Antisera were diluted 1:500, and antibody binding was detected by incubation with alkaline phosphatase–conjugated goat anti-mouse IgG (1:1000) and developed by incubation with tetrazolium salts and bromochloroindolyl phosphate. Approximate molecular masses are noted at left in kilodaltons.

tients with AIDS who also had keratoconjunctivitis [18] (unpublished data). Electron micrographs of the mature spores from biopsy material and the spores isolated in tissue culture were morphologically identical to *E. cuniculi*. The proliferative stages of the AIDS-related microsporidia in tissue culture developed within a parasitophorous membrane-bound vacuole, as also reported for *E. cuniculi* [25], rather than in direct contact with the host cell cytoplasm as described for *N. corneum* [20].

The three AIDS-related ocular isolates appeared identical on the basis of SDS-PAGE protein profiles. In addition, serum from each of the three AIDS patients with microsporidiosis reacted to the patient's own isolate in a pattern nearly identical to that of the other two isolates. These new isolates, however, are clearly different from *E. cuniculi* on the basis of the different SDS-PAGE and Western blot immunodetection profiles.

The new ocular microsporidia, while different from *E. cuniculi*, appear more closely related to *E. cuniculi* than to *N. corneum*. Morphologically, *E. cuniculi* and the new ocular isolates are monokaryotic, whereas *N. corneum* displays a diplokaryotic nucleus. The AIDS-related ocular isolates reported here also are morphologically different from *Ent. bieneusi*. *Ent. bieneusi* is characterized by the premature development of polar filament before segmentation of the individual sporonts, which was not seen during development of the new ocular microsporidia. In addition, *Ent. bieneusi* develops in direct contact with the cytoplasm, not within a parasitophorous vacuole, unlike these new isolates [4]. It is also unlikely that these AIDS-related ocular microsporidia are a *Pleistophora* species, whose spores are larger (2.5 × 3.5 μm) and display ~11 coils of the polar filament. Immunologically, murine antiserum to *E. cuniculi* reacts to several proteins of the three AIDS-related ocular isolates, whereas the murine antiserum raised against *N. corneum* reacts weakly to the new isolates or to *E. cuniculi*. In addition, serum from patient 3, which reacted strongest to the new isolates, also reacted to numerous bands of *E. cuniculi*.

Since the new ocular isolates displayed immunologic reactivities and morphologic similarities to *E. cuniculi*, we propose to name the organism from patient 1 *Encephalitozoon hellem* (n. sp.) and to refer to the organisms originally isolated from patients 2 and 3 as *E. hellem*–like. Verification of the taxonomic classification of *E. hellem* (n. sp.) and the two *E. hellem*–like organisms will be carried out by rRNA sequencing. Vossbrinck et al. [26, 27] have shown that microsporidia, while being true eukaryotes, possess a highly divergent rRNA. Sequencing the highly variable "580" region of *E. hellem* (n. sp.) for comparison with other microsporidia, including *E. cuniculi* and *N. corneum*, will provide further information about the relationship between these organisms taxonomically. In addition, such information will be useful for determining whether *E. hellem* (n. sp.) infects other animals to which humans may be exposed.

Both *E. hellem* (n. sp.) and *N. corneum* were recovered and isolated from ocular tissues, but the clinical presentations of the patients differed. *N. corneum* was isolated from deep ulcers in the corneal stroma of an HIV-seronegative patient [16, 20], whereas *E. hellem* (n. sp.) was recovered from the superficial conjunctival and corneal epithelium of three AIDS patients with keratoconjunctivitis [18] (unpublished data). This suggests that these microsporidia may display tissue-site tropism or may require different growth conditions in vivo.

The results of this study also suggest that serologic diagnoses of microsporidiosis in AIDS patients should be possible because in all three cases, positive antibody binding to *E. hellem* (n. sp.) was observed. The progression of immunodeficiency in AIDS patients and the time at which a patients becomes infected with microsporidia may influence the reliability of such tests. For example, sera from patients 1 and 2, who were near death when sera were obtained, displayed weaker responses than sera from patient 3, as measured by Western blot immunodetection. The sera from patient 3 reacted to 15–20 proteins of *E. hellem* (n. sp.) and also cross-reacted to *E. cuniculi*. Patient 3 was clinically healthier when sera were collected and is currently under treatment with zidovudine (AZT) and itraconazole (unpublished data).

Exposure to microsporidia in foods or via insect stings may

also account for this strong reactivity. Equal amounts of parasite proteins were loaded on each preparative gel, so it was unlikely that excess antigen loading would have produced this result. Coinfection of the patient 3 with *E. cuniculi* and *E. hellem* (n. sp.) seems unlikely because the intensity of antibody binding bands was less against *E. cuniculi* than against *E. hellem* (n. sp.). The most likely explanation for the reactivity of serum from patients 3 against *E. cuniculi* was due to common proteins of structures shared by microsporidia (e.g., polar filaments, spore coat components) and particularly by *E. cuniculi* and *E. hellem* (n. sp.), suggesting that these two organisms are closely related microsporidia. Additional serologic studies are necessary to define when, during the course of AIDS, antibody responses in microsporidia-infected individuals can be observed and to define common compared with unique microsporidial antigens. Such information will be useful for determining whether a patient is infected with the identical or a different microsporidial species used in the Western blot immunodetection.

The availability of a new human isolate provides antigen for testing patients sera and increases the likelihood of detecting positive sera. Until now, *E. cuniculi* was the only available mammalian microsporidian for use as antigen for serologic studies [19]. Hollister and Canning [28] reported that humans with parasitic infections who had traveled in the tropics displayed antibody responses to *E. cuniculi* as detected by Western immunoblotting and ELISA. They also reported variable banding patterns among their serum samples and suggested that this variability may have been due to infection with microsporidia other than *E. cuniculi*. The recent availability of *N. corneum* [20] and the isolation of a new AIDS-related ocular microsporidian, *E. hellem* (n. sp.), will provide increased capabilities for serologic detection of microsporidiosis.

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