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Polyclonal and Monoclonal Antibody and PCR-Amplified Small-Subunit rRNA Identification of a Microsporidian, *Encephalitozoon hellem*, Isolated from an AIDS Patient with Disseminated Infection

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Microsporidia are primitive, spore-forming, mitochondria-lacking, eukaryotic protozoa that are obligate intracellular parasites. They are known to parasitize almost every group of animals including humans. Recently, microsporidia have increasingly been found to infect patients with AIDS. Five genera (*Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Septata*, and *Pleistophora*) of microsporidia are known to infect humans. *Enterocytozoon* organisms cause gastrointestinal disease in a majority of AIDS patients with microsporidiosis. However, a smaller, but an expanding, number of patients with AIDS are being diagnosed with ocular and disseminated infection with *Encephalitozoon hellem*. Although microsporidial spores can be identified in clinical samples by a staining technique such as one with Weber's chromotrope stain, identification to the species level is dependent on cumbersome and time-consuming electron microscopy. We have recently isolated and established in continuous culture several strains of *E. hellem* from urine, bronchoalveolar lavage, and sputum samples from AIDS patients with disseminated microsporidiosis. We developed polyclonal and monoclonal antibodies and PCR primers to a strain of *E. hellem* that can be used successfully to identify *E. hellem* from other species of microsporidia either in clinical specimens or in cultures established from clinical specimens. Since patients infected with *Encephalitozoon* spp. are known to respond favorably to albendazole, identification of the parasite to the species level would be invaluable in the treatment of disseminated microsporidiosis.

Microsporidia are primitive, eukaryotic protozoa that lack mitochondria (2, 6, 18). They are obligate intracellular parasites and are known to infect a wide variety of cell types. Although they are most often known to parasitize insects and fish, they have been found to infect members of almost every major phylum of the animal kingdom (6). During the past decade microsporidia have increasingly been recognized to infect patients with AIDS (2, 3, 5, 7–10, 13, 15, 18, 27). To date microsporidia belonging to five genera, *Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Pleistophora*, and *Septata*, have been known to cause infections in humans (2, 3, 6, 8, 19). A sixth taxon, *Microsporidium*, has also been established to include insufficiently described microsporidia of undetermined taxonomic status (4, 6).

We recently reported the in vitro culture of a microsporidian parasite, *Encephalitozoon* sp. (CDC:0291:V213), isolated from the urine of a patient with AIDS (22) and subsequently identified it as *Encephalitozoon hellem* (14–16). In this report we decribe its growth properties, morphologic characteristics, antigenic profile on the basis of its reactivity with polyclonal antibodies and monoclonal antibodies (MAbs), and sequence analysis of the small-subunit rRNA (SSU-rRNA)-coding region.

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MATERIALS AND METHODS

Sources of parasites. *E. hellem* CDC:0291:V213 was isolated from the urine of an AIDS patient and was grown on monkey kidney cell (E6) and human lung fibroblast (HLF) monolayers (22). *Encephalitozoon cuniculi*, isolated from a rabbit and grown on rabbit kidney cells (17), was obtained from John Shadduck and Elizabeth Didier and was designated strain JS. *E. hellem*, isolated from the corneal button of an AIDS patient with keratoconjunctivitis and grown on MDCK cells and designated strain ED (9), and *Nosema corneum*, isolated from the cornea of a human immunodeficiency virus (HIV)-seronegative patient (19), were also obtained from Elizabeth Didier. All of these parasites were subsequently adapted to grow on the E6 cell line.

Parasite growth and harvest. To obtain maximum infection of the host cells and optimum growth of the parasites, the culture medium was replaced every 3 days. The spent culture medium from all flasks, which contained extruded spores and unattached host cells infected with developmental stages of the parasite, was centrifuged at 1,500 × *g* for 20 min at 4°C, and the supernatant was aspirated. The pellets were put back into the same culture flasks. This facilitated the infection of a maximum number of host cells (>70%), as revealed by microscopic examination, with the respective parasites. Thereafter, spores that were extruded into the culture supernatants from all parasites were harvested by centrifugation as described above. The spores were suspended in 0.25% sodium dodecyl sulfate (SDS), vortexed briefly, and incubated in a water bath for 20 min at 37°C (9). The spores were next washed three times in Hanks' balanced salt solution (HBSS), counted in a

hemacytometer, suspended in enough HBSS to obtain 10^9 spores per ml, and stored at 4°C until use (22).

Polyclonal antibody. Antibody to *E. hellem* CDC:0291:V213 was produced in female New Zealand White rabbits weighing about 2 kg. Each of several rabbits were prebled to check for spontaneous antibody to *E. cuniculi* by indirect immunofluorescence (IIF). Two rabbits with no background levels of antibody to *E. cuniculi* were selected, and 0.1 ml of spore suspension containing 10^8 spores of CDC:0291:V213 was repeatedly injected into the marginal ear vein. Blood samples were taken periodically and were tested by IIF for antibodies to CDC:0291:V213 (14).

MAb. MAb to CDC:0291:V213 was produced in female BALB/c mice by published methods (11, 23). Each of four 8-week-old mice were injected intraperitoneally with 0.1 ml of washed parasite suspension containing 10^6 spores and unattached E6 cells containing developing stages on days 0, 7, 14, 21, 28, 35, and 42. Spleen cells from two of the mice that had high titers of antibody (>4,096 by IIF) were fused with SP2/0 myeloma cells by the addition of polyethylene glycol. Stable hybrids were selected by growth in RPMI 1640 medium containing 10% fetal bovine serum, hypoxanthine, aminopterin, and thymidine as described earlier (23). Supernatant culture medium was tested for antibody activity against *E. hellem* CDC:0291:V213 and *E. cuniculi* JS by the IIF test (22, 23). Six of the 24 hybrids showing antibody activity were selected for further cloning and expansion. The MAbs were also tested for their isotypes by immunodiffusion by using mouse immunoglobulin isotype-specific antisera (immunoglobulin M [IgM], IgA, IgG1, IgG2a, IgG2b, and IgG3). One of the clones (ED4H10B11/B12), which showed high levels of antibody activity, was also injected into BALB/c mice after pristane priming for ascites production (23).

Scanning and transmission electron microscopy. For scanning electron microscopy E6 monolayers were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), processed as described previously (22), and examined with a JEOL JSM 820 scanning electron microscope. For transmission electron microscopy, the glutaraldehyde-fixed samples were postfixed in a solution of 1% OsO_4, processed, and examined with a JEOL 1200 EX transmission electron microscope as described previously (22).

IIF. The IIF test was performed as described earlier (22). Briefly, washed spores of *E. cuniculi* JS and *E. hellem* ED and CDC:0291:V213 were suspended in enough 1% buffered Formalin to obtain 10^7 spores per ml. Antigen slides were prepared by depositing 20 μl of the spore suspension onto each well of several 12-place slides as described previously (22). Rabbit anti-*E. cuniculi* and rabbit anti-*E. hellem* or the MAb were serially diluted with phosphate-buffered saline (PBS; 0.01 M; pH 7.6) beginning at 1:2 in U-type microtitration plates (Linbro Scientific Co., Inc., Hamden, Conn.). The serially diluted reagents were transferred to the 12-place slides (15 μl of each dilution per well), and the slides were incubated at 37°C for 30 min in a moist chamber. The slides were then washed three times in PBS (10 min per wash), and then each well was covered with 20 μl of fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG (Cappel Laboratories, Westchester, Pa.) at a dilution of 1:2,000 containing Evans blue as the counterstain or FITC-conjugated anti-mouse IgG. The slides were incubated at 37°C for 30 min and washed three times as described above, the specimens were mounted with buffered glycerin (pH 9.0) and covered with a coverslip, and the slides were examined with an Olympus BH2 fluorescence microscope equipped with epifluorescence illumination.

Thin smears of culture-derived *N. corneum* and Formalin-fixed stool samples obtained from patients with diarrhea identified as being caused by *Enterocytozoon bieneusi* were prepared, and the IIF test was performed.

IIF was also performed on tissue sections of lungs, kidneys, and prostate obtained from our patient at the time of autopsy. The tissues were fixed in 10% neutral buffered Formalin, and 5 to 6-μm-thick sections were cut and stained with hematoxylin-eosin and Brown and Hops stains. Additional sections were cut from the blocks that were positive for microsporidia, and the sections were deparaffinized, hydrated through a graded series of ethanol solutions, rinsed in water and PBS, and then covered with a 1:100 dilution of the rabbit anti-*E. hellem* or the MAb and processed as described above. The sections were next covered with a 1:500 dilution of the FITC-conjugated goat anti-rabbit IgG or FITC-conjugated goat anti-mouse Ig and were incubated at 37°C and processed as described above. The slides were examined and photographed with an Olympus microscope (14–16). Since no fluorescence was observed in the tissue sections when they were reacted with the MAb by this protocol, the sections were next pretreated for 30 min with a solution containing 0.2% $CaCl_2$ and 0.2% trypsin, rinsed with water, and then allowed to react overnight at 4°C with the MAb (12).

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting. Proteins were extracted from purified spores of each parasite by suspending them in sample buffer containing 2.5% SDS and 2.25 M urea and heating the mixture at 65°C for 15 min (9, 22). Proteins extracted from approximately 5×10^6 spores were loaded onto each lane of a 1.0-mm 2.5 to 27% gradient acrylamide gel (Isolab Inc. Akron, Ohio), and the gels were subjected to electrophoresis (22). The separated proteins were transferred onto Immobilon membranes. The membranes were reacted with either a 1:500 dilution of a rabbit anti-*E. cuniculi* serum (courtesy of John Shadduck), a 1:500 dilution of rabbit anti-*E. hellem* CDC:0291:V213 serum, or a 1:100 dilution of the MAb ED4H10B11/B12. After appropriate washes, the membranes were next reacted with a 1:5,500 dilution of peroxidase-conjugated goat anti-rabbit IgG (Cappel Laboratories) or a 1:1,000 dilution of peroxidase-conjugated anti-mouse IgG. Hydrogen peroxide (3%) and diaminobenzidene (0.005%) were used as substrate and chromogen, respectively (22).

PCR amplification, cloning, and sequencing of SSU-rRNA coding region. (i) Extraction of DNA. DNA was extracted from the following four different cultures: (i) uninfected (E6) cell culture (control), (ii) E6 culture infected with *E. hellem* ED, (iii) E6 culture infected with *E. hellem* CDC:0291:V213, and (iv) E6 culture infected with *E. cuniculi* JS. All cultures were grown on 75-cm^2 plastic flasks (Corning, Ithaca, N.Y.). Cells from each of the four cultures were scraped separately and were transferred to 15-ml tubes. After centrifugation at 1,000 × *g* for 20 min at 4°C, the supernatant was aspirated and the sediment was suspended in 0.8 N NaCl (saline) and washed three times by centrifugation as described above. After the third wash, the supernatant was aspirated again and the DNA was extracted from the pellet by using the ONCOR nonorganic DNA extraction kit (Oncor, Gaithersburg, Md.) according to the manufacturer's instructions. Nucleic acids from each sample were resuspended in 50 μl of distilled water and were amplified by PCR.

(ii) PCR. Primers for amplification of the microsporidial SSU-rRNA-coding region were designed by using the published sequence of *Vairimorpha necatrix* SSU-rRNA (24, 25). The forward primer was complementary to bases 1 to 21 of the *V. necatrix* sequence; the reverse primer was complementary to bases 1224 to 1244. In addition, PCR was performed by using

two pairs of primers: the first pair, specific for *E. hellem*, amplified a 547-bp diagnostic fragment from the *E. hellem* CDC:0291:V231 SSU-rRNA sequence (N. J. Pieniazek et al., GenBank accession number L19070). The forward primer (5′-TGAGAAGTAAGATGTTTAGCA-3′) was designed on positions 358 to 378 and the reverse primer (5′-GTAAAAA CACTCTCACACTCA-3′) was designed on positions 884 to 904 of this sequence. The second pair, specific for *E. cuniculi*, amplified a 549-bp fragment diagnostic for the *E. cuniculi* SSU-rRNA (N. J. Pieniazek et al., GenBank accession number L17072). The forward primer (5′-ATGAGAAGTGATGTGT GTGCG-3′) was designed on positions 344 to 364 of this sequence, and the reverse primer (5′-TGCCATGCACTCA CAGGCATC-3′) was designed on positions 872 to 892 of this sequence. PCR was performed by using a Perkin-Elmer Cetus (Norwalk, Conn.) GenAmp kit according to the manufacturer's instructions. Denaturation, annealing, and elongation temperatures of 94, 55, and 72°C, respectively, were used for a total of 30 cycles in all reactions. The products of the amplifications were electrophoretically resolved on a 2% agarose gel (Seakem GTG; FMC Bioproducts, Rockland, Maine) and were visualized for analysis by staining with ethidium bromide.

After amplification with the *V. necatrix* primers, the PCR products were analyzed on an agarose gel, and only when a template from an infected cell was used was a band of about 1.3 kb seen. PCR products were then purified, cloned, and sequenced. For all PCR products, sequencing was done for three independent clones for both strands. Sequences were deposited in the GenBank database under the following accession numbers: L17072 for *E. cuniculi* and L19070 for *E. hellem* CDC:0291:V213 and ED.

RESULTS

Growth of parasites. Foci of infection were noticed in the monolayers about a month after the inoculation of the spores. At first, the developmental stages of the parasites (*E. hellem* ED and CDC:0291:V213 and *E. cuniculi*) began to appear as bluish dots within the E6 cells, which increased in number with time; finally, the cells became distended with spores (Fig. 1a and 1b), leading to the rupture of cells and the liberation of the spores into the culture medium. All of the three parasites readily infected the E6 monolayers and released spores into the culture medium. The patterns of growth of the three parasites were similar. In older cultures, especially those of *E. hellem* CDC:0291:V213, when almost all host cells had been destroyed and masses of spores were seen attached to the cytoskeletal remains of the host cells, many of the spores were seen to have extruded their polar tubes (Fig. 2a to d).

Only *E. hellem* CDC:0291:V213 was evaluated ultrastructurally. Developmental or proliferative forms were seen attached to the parasitophorous vacuole membrane. Many early and late sporogonial developmental stages suggested that they are disporous. In the early stages, the sporont membrane thickened and the sporont detached from the parasitophorous vacuole membrane. In the maturing sporonts, the polar tubules in various stages of development were seen (Fig. 3). The formation of the polaroplast as well as the anchoring disc was also clearly discernible.

The spores of CDC:0291:V213 were characterized by their oval shape, birefringence, and gram-positive staining. When stained with the chromotrope-based stain of Weber et al. (26), they stained pinkish to red. The spores appeared smooth walled when examined by phase-contrast microscopy and scanning electron microscopy. They measured 2.25 to 2.8 μm in length and 1.25 to 1.85 μm in breadth. When examined by transmission electron microscopy the spores were seen to be characterized by a thin, electron-dense outer wall, the exospore, an inner, thick electron-lucent endospore, and a thin cell membrane that surrounded the spore contents. Other prominent features seen in the transmission electron micrographs were cross sections of five to seven coils of the polar tubule on either side of the spore, the anchoring disc at the anterior end from which the polar tubule emanated, the lamellar polaroplast, also at the anterior end of the spore, and the vacuole prominently located at the posterior end (Fig. 3).

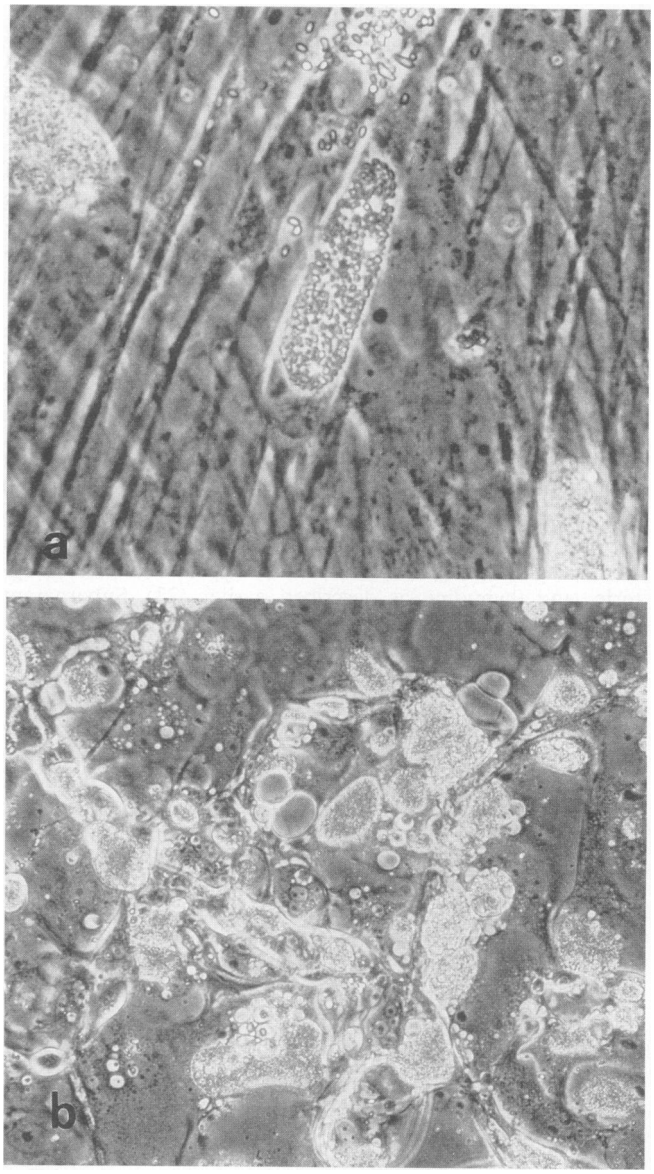

FIG. 1. Monolayers of HLF (a) and E6 (b) cell cultures infected with *E. hellem* CDC:0291:V213. Note that the cells in both monolayers are distended with spores of *E. hellem*. Phase-contrast microscopy was used. Magnification, ×600.

IIF assay. In the IIF test, both the CDC:0291:V213 and ED strains of *E. hellem* reacted with the anti-CDC:0291:V213 serum almost to the same extent and gave a titer of >4,096. *E. cuniculi*, however, reacted less strongly and produced a titer of 256. Dramatic differences were, however, noted when *E. cuniculi* was reacted with the anti-CDC:0291:V213 serum

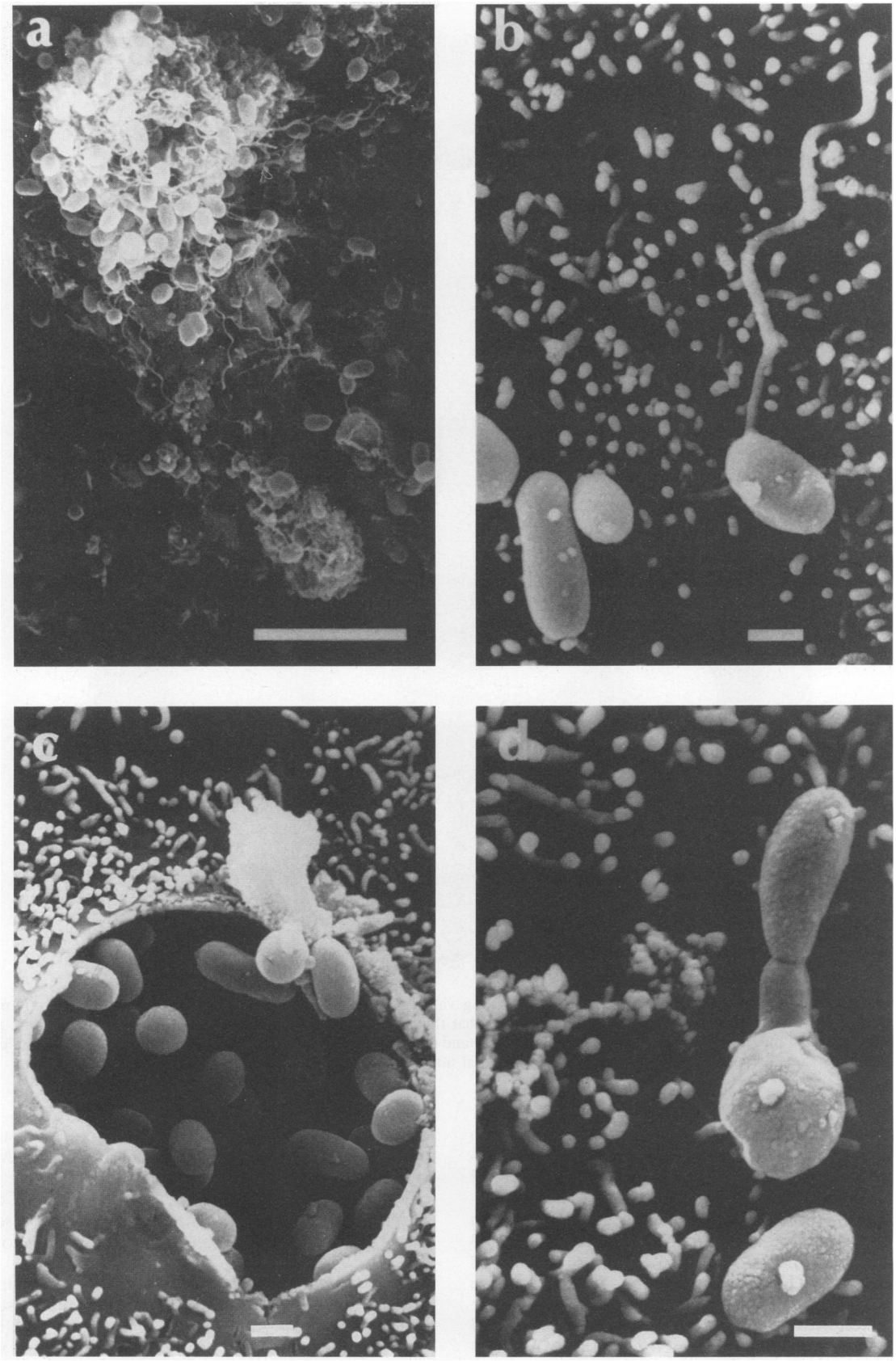

FIG. 2. Scanning electron micrographs depicting masses of spores of *E. hellem* with extruded polar tubules and developing stages. (a) HLF monolayer with masses of spores, many with extruded polar tubes; (b) a spore with extruded polar tubule that is probably entering an E6 cell; (c) a distended E6 cell that has just burst revealing a parasitophorous vacuole lined with spores; (d) chain-like developmental form often seen in the monolayers along with a mature spore. Bars, 1 μm.

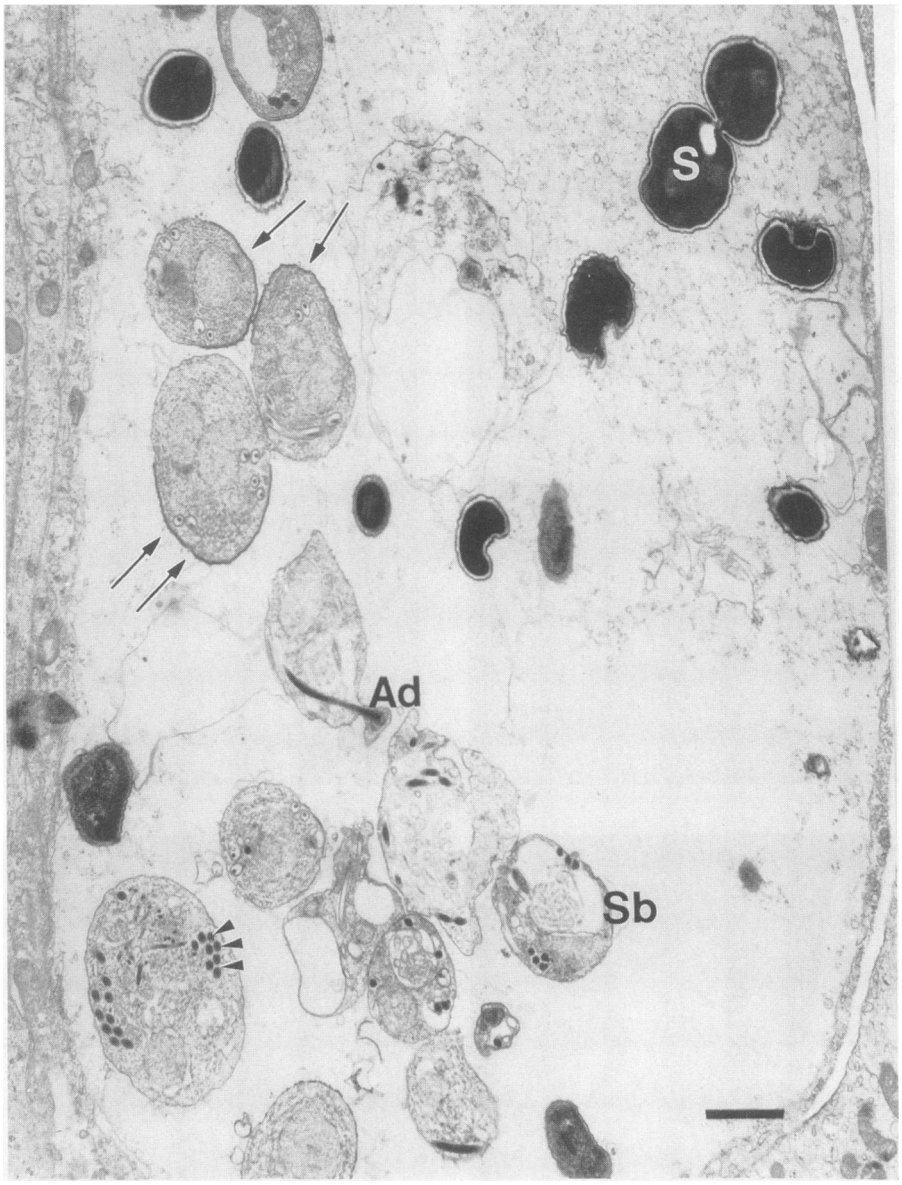

FIG. 3. Transmission electron micrograph of an E6 cell showing various stages of sporogonial development. The sporont membrane is beginning to thicken (arrows) and some are binucleated, indicating that they are disporous. Cross sections of the polar tubules are already seen in some of the late stages of the sporonts. Also seen is a cluster of early and late stages of sporoblasts (Sb) showing developing organelles, anchoring disc (Ad) and cross sections of the polar tubule (arrowheads). Several mature electron-dense spores (S) are also seen. Bar, 1 μm.

absorbed with *E. cuniculi* spores. *E. cuniculi* showed no reactivity with the absorbed serum, indicating that all cross-reacting antigens were absorbed out. Both the CDC:0291:V213 and ED strains of *E. hellem*, however, showed some fluorescence at the 128 dilution, indicating specific antigens common to both of these parasites. When either ED or CDC:0291:V213 spores were reacted with anti-CDC:0291:V213 serum absorbed with ED spores or CDC:0291:V213 spores, no fluorescence was seen.

Dramatic differences were also observed when the spores of *E. hellem* and *E. cuniculi* were reacted with the MAb ED4 H10B11/B12 with IgG1 specificity. *E. hellem* spores reacted with this MAb and produced a titer of >8,192, whereas *E. cuniculi* spores failed to react with this MAb at the 1:128 dilution but showed a slight reaction at the 1:64 dilution. This MAb also did not react with the Formalin-fixed stool smears positive for *E. bieneusi* as well as culture-derived *N. corneum*.

The parasites in the kidney and the lung tissue sections of our patient reacted intensely with the anti-CDC:0291:V213 serum and produced a bright apple green fluorescence indicating that the antiserum can be used successfully to identify *E. hellem* spores in tissue sections.

The MAb, on the other hand, failed to react with the spores in the Formalin-fixed, paraffin-embedded tissue sections initially. However, bright green fluorescence of the spores was evident when the sections were treated with trypsin solution for 30 min and then reacted with the MAb overnight at 4°C (Fig. 4).

SDS-PAGE and immunoblotting. Proteins extracted from *E. hellem* ED and CDC:0291:V213 after separation and silver

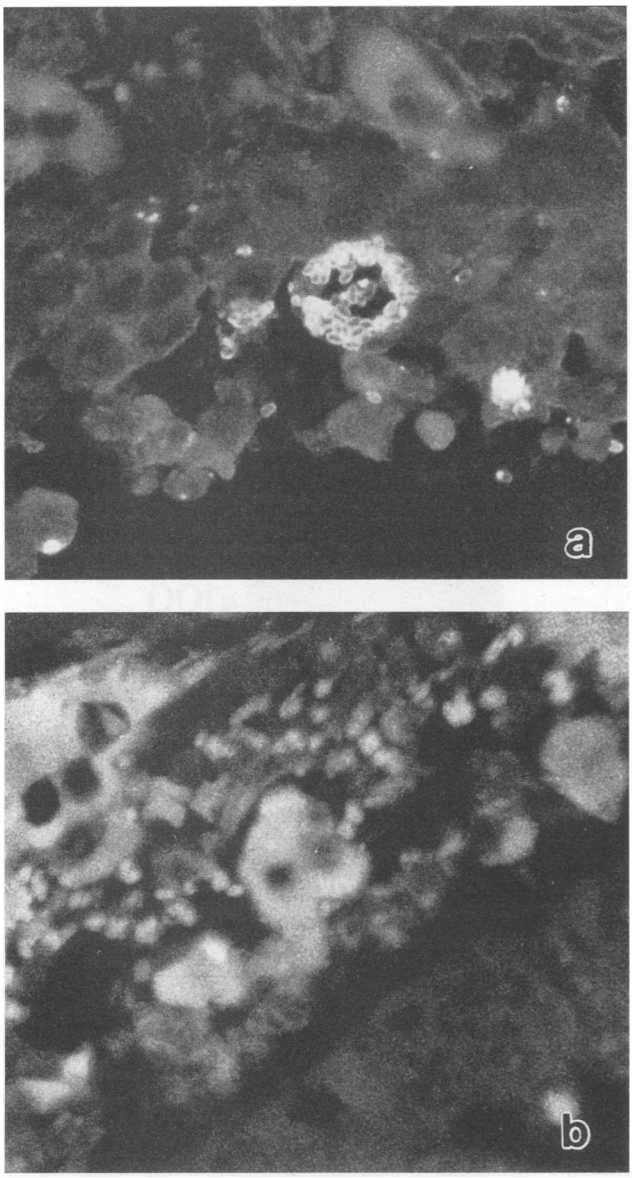

FIG. 4. Results of IIF test performed on the lung sections of our patient. (a) Positive fluorescence profiles of *E. hellem* spores in a section that was reacted with a 1:100 dilution of MAb ED4H10B11/B12. Magnification, ×400. (b) A negative reaction of spores in a section similar to that shown in panel a reacted with a 1:100 dilution of an irrelevant MAb.

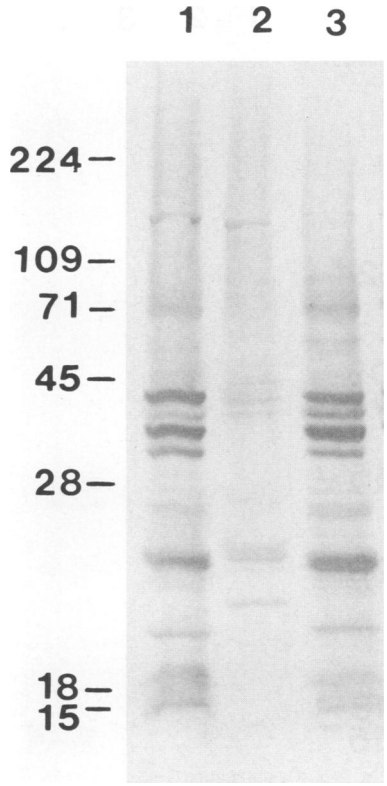

FIG. 5. Immunoblot profiles of *E. hellem* ED (lane 1) and CDC:0291:V213 (lane 3) and *E. cuniculi* JS (lane 2) after reaction with rabbit anti-CDC:0291:V213 serum.

staining showed remarkably similar patterns. Although *E. hellem* shared many proteins with *E. cuniculi*, differences between the two were clearly apparent, especially in the 14- and 18-kDa and the 28- to 70-kDa regions. When these proteins were transferred to the Immobilon membranes and then treated with the rabbit anti-*E. hellem* serum, characteristic differences were seen (Fig. 5). *E. hellem* proteins (Fig. 5, lanes 1 and 3) reacted extensively with the anti-*E. hellem* serum and produced many darkly staining bands that were not present in *E. cuniculi* (Fig. 5, lane 2). These differences were most obvious, especially in the regions of 14 to 20, 22, 29 to 43, and 72 kDa. Both the CDC:0291:V213 and ED proteins also reacted with the anti-*E. cuniculi* serum and produced a number of bands (data not shown), but the reactivities of these were considerably less than those in the homologous reactions. Both CDC:0291:V213 and ED, however, reacted extensively with the anti-*E. hellem* serum and produced darkly staining bands that were unique to *E. hellem*. The banding patterns of *E. hellem* and *E. cuniculi* were, however, very different when the spore proteins were reacted with MAb ED4 H10B11/B12 (Fig. 6). Both CDC:0291:V213 and ED strains reacted well with MAb ED4H10B11/B12 and produced darkly staining bands at about 18, 28.5, 30, 36, and 40 kDa and a doublet at 109 kDa (Fig. 6, lanes 1 and 3). *E. cuniculi* JS reacted minimally with the MAb and produced relatively strong bands at 40 and 36 kDa and weak bands at 90 and 28 kDa (Fig. 6, lane 2).

SSU-rRNA sequence analysis. Species-specific PCR primers targeting SSU-rRNA-coding sequences selectively amplified *Encephalitozoon* diagnostic fragments with no background from mammalian cellular DNA. When DNA isolated from cultures infected with *E. hellem* ED and CDC:0291:V213 was PCR amplified by using *E. hellem*-specific primers, an expected diagnostic band of aproximately 547 bp was detected upon analysis on an agarose gel (Fig. 7, lane 1 and 2). No such band, however, was seen when DNA from E6 cells infected with *E. cuniculi* or uninfected cells were used (Fig. 7, lanes 3 and 4). In addition, no band was detected when *E. hellem* template was amplified with *E. cuniculi* primers (data not shown). The consensus SSU-rRNA sequences for ED and CDC:0291:V213 showed 100% similarity. The *E. cuniculi* consensus SSU-rRNA sequence showed only 86% similarity to that of *E. hellem*.

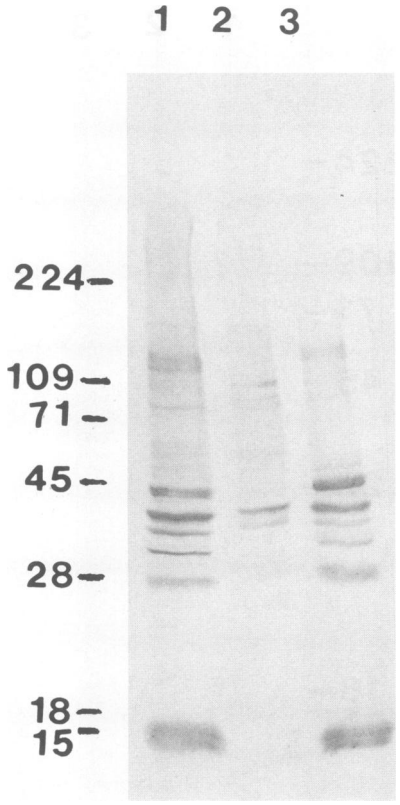

FIG. 6. Immunoblot profiles of *E. hellem* CDC:0291:V213 (lane 1) and ED (lane 3) and *E. cuniculi* JS (lane 2) after reaction with MAb ED4H10B11/B12.

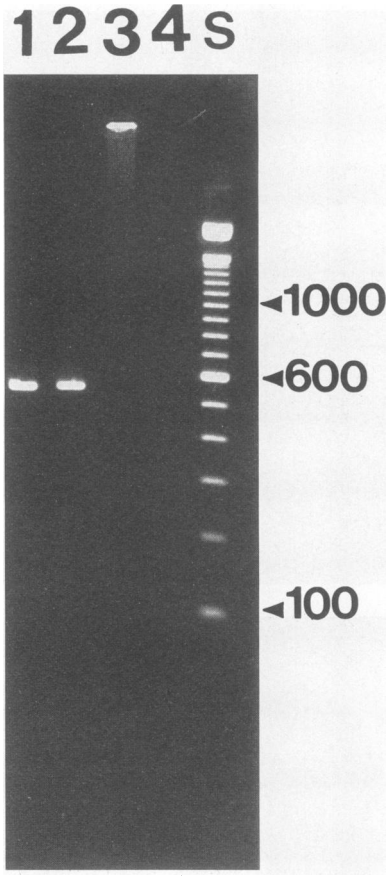

FIG. 7. Agarose gel electrophoresis of diagnostic fragments amplified by PCR by using specific primers for *E. hellem*. Lane 1, DNA from E6 cells infected with *E. hellem* ED; lane 2, DNA from E6 cells infected with CDC:0291:V213; lane 3, DNA from E6 cells infected with *E. cuniculi*; lane 4, DNA from uninfected E6 cells; lane S, 100-bp ladder standard.

DISCUSSION

Although microsporidia have been known since 1930 to infect insects, fish, and common laboratory animals such as mice, hamsters, and rabbits, only recently have they been found to infect humans. The first case of human microsporidiosis was diagnosed in 1959 in a presumably immunocompetent 9-year-old Japanese boy who developed fever, headache, seizures, and loss of consciousness. Organisms identified as *Encephalitozoon* spp. were demonstrated in his urine and cerebrospinal fluid (2). Since then, seven more cases in non-HIV-infected patients have been described: a second case of seizure disorder associated with *Encephalitozoon* sp. in a child (2); one case of disseminated infection with *Nosema connori* in an immunocompromised but presumably HIV-negative infant (20); four cases of corneal infection each caused by a different organism (*Microsporidium africanum* [6], *M. ceylonensis* [6], *N. corneum* [19], and *N. ocularum* [4]), and one case of myositis caused by a *Pleistophora* sp. in an immunodeficient but HIV-negative person (2).

Until today, five genera (*Encephalitozoon*, *Nosema*, *Pleistophora*, *Enterocytozoon*, and *Septata*) and the collective genus *Microsporidium* have been associated with human microsporidiosis. Since 1985, however, microsporidiosis has become a burgeoning problem in HIV-infected and AIDS patients with chronic diarrhea (2, 5, 13, 18). *E. bieneusi* is the most commonly identified etiologic agent in up to 30% of AIDS patients with chronic diarrhea (2, 5, 13, 18). In addition to *E. bieneusi*, *Septata intestinalis* has recently been described in a few patients with HIV-related chronic diarrhea (3), and *Pleistophora*-associated myositis has also been reported in one AIDS patient (8). Additionally, a smaller, but expanding, number of AIDS patients are being diagnosed with ocular and disseminated microsporidioses caused by *Encephalitozoon* sp. and *E. hellem* (7, 9, 10, 14–16, 27). *Encephalitozoon* spp. have been associated with keratoconjunctivitis, sinusitis, tracheobronchitis, nephritis, cystitis, and hepatitis in AIDS patients (14–16). Our patient developed disseminated infection with a microsporidian, which we isolated into culture and identified initially as *Encephalitozoon* sp. Subsequently, on the basis of IIF data, we established the species as *E. hellem* (14). We showed in the present study that both ED and CDC:0291:V213 isolates of *E. hellem* react almost identically with the rabbit anti-CDC:0291:V213 serum as well as MAb ED4H10B11/B12 in the immunoblot and IIF analyses. Furthermore, both ED and CDC:0190:V213 also share identical SSU-rRNA sequences. Both *E. hellem* and *E. cuniculi* are similar ultrastructurally but differ from one another in their antigenic profiles (9, 14). This phenomenon has been used advantageously for identifying human infections with *E. hellem* in specimens originating from conjunctiva, cornea, sputum, urine, bronchoalveolar lavage fluid, and nasal epithelium (9, 14–16, 27). The cross-reactivity of anti-*E. hellem* serum with other microsporidia, especially *E. bieneusi*, has also been demonstrated by others (1, 9, 28, 29). A fluorescence test

that recognizes microsporidial spores as well as fungi and certain bacteria has also been developed (21). Aldras et al. (1) have recently produced MAbs with IgM specificities that identified Formalin-fixed and tissue culture-derived *E. cuniculi* and *E. hellem* as well as *E. bieneusi* in Formalin-fixed stool samples. None of these tests, therefore, specifically identifies a particular species of microsporidia.

We showed in the present study that in the IIF test, the MAb ED4H10B11/B12 with IgG1 specificity reacts well with *E. hellem* but reacts minimally (<1:128) with *E. cuniculi* and not at all with *E. bieneusi* in Formalin-fixed stool smears and culture-derived *N. corneum*. Furthermore, this MAb also reacts with spores in the Formalin-fixed, paraffin-embedded tissue sections, provided that the sections were pretreated with trypsin and the incubation with the MAb was carried out overnight at 4°C. Hence, this MAb (ED4H10B11/B12) can be used specifically to identify *E. hellem* in human specimens premortem so that specific therapy can be instituted without delay and at postmortem for species identification.

According to Vossbrinck et al. (25) rRNA sequence analysis suggests that microsporidia are ancient eukaryotes. In a recent study, Vossbrinck et al. (24) have also shown that three isolates of *E. hellem* had identical sequences in the highly variable intervening spacer region of the amplified DNA segment. Using those sequence data, they have also shown that *E. hellem* and *E. cuniculi* have relatively high degrees of sequence similarity, which permits them to be included in the same genus. However, they state that "sequence data indicate that *E. hellem* and *E. cuniculi* are separate species, with greater difference in sequence than is seen between the two species of *Vairimorpha* presented for comparison." Our sequence data for the complete SSU-rRNA-coding region also indicate that the consensus SSU-rRNA sequences for ED and CDC:0291:V213 are 100% similar. However, the *E. cuniculi* consensus SSU-rRNA sequence showed only 86% similarity to that of *E. hellem*. This score is surprisingly small for two species classified within the same genus.

In conclusion the data presented here indicate that *E. hellem* and *E. cuniculi* cannot be distinguished from each other by growth characteristics or ultrastructural morphologies. The two species can be specifically identified, however, by using MAb ED4H10B11/B12 or SSU-rRNA sequences.

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