In Vitro Culture and Serologic and Molecular Identification of *Septata intestinalis* Isolated from Urine of a Patient with AIDS

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Microsporidian spores were identified, on the basis of Weber's staining, in urine, stool, nasal, and saliva samples of an AIDS patient with diarrhea, hematuria, dysuria, and dementia. Urine and stool samples contained numerous spores, whereas few spores were seen in the nasal and saliva samples. Spores were concentrated from urine samples and inoculated into monkey kidney cell (E6) monolayers. After 6 to 8 weeks of culture, infected E6 cells filled with spores as well as spores free in the culture supernatants were seen daily. Transmission electron microscopy revealed that all stages of the parasite (CDC:V297) developed within septated, honeycomb-shaped parasitophorous vacuoles. Indirect immunofluorescence and immunoblotting studies using rabbit anti-*Encephalitozoon cuniculi*, anti-*Encephalitozoon hellem*, and anti-CDC:V297 sera revealed that CDC:V297 reacted intensely with the homologous serum but minimally with the heterologous sera. DNA isolated from CDC:V297, when PCR amplified with *E. hellem* and *E. cuniculi* primers, did not produce the diagnostic bands of ~547 and ~549 bp characteristic of *E. hellem* and *E. cuniculi*, respectively. On the basis of these studies, we concluded that CDC:V297 fits the description of *Septata intestinalis* (A. Cali, D. P. Kotler, and J. M. Orenstein, J. Eukaryot. Microbiol. 40:101-112, 1993).

Members of the phylum Microspora are primitive eukaryotic protozoa that lack mitochondria (5, 16). Nearly 1,000 species belonging to more than 100 genera are included in this phylum (13). Microspora are obligate intracellular parasites known to infect primarily insects, fish, and common rodents (5). However, microsporidia have also been found to infect members of almost every major animal group, including primates (2, 5, 13). The first case of human microsporidial infection in an immunologically healthy person was described in 1959 (9), and since then nine additional cases in otherwise healthy persons have been described (19). However, with the advent of AIDS, more than 400 cases of microsporidiosis in patients with AIDS have been documented (3). Microsporidia belonging to five genera (*Encephalitozoon*, *Enterocytozoon*, *Nosema*, *Pleistophora*, and *Septata*) are known to cause disease in humans (2-4). Although as many as 25 documented cases of infection caused by *Encephalitozoon* spp. (e.g., *E. hellem* and *E. cuniculi*) involving corneal, respiratory, peritoneal, hepatic, and urogenital organs have occurred in patients with AIDS, most of the 400 cases are believed to be caused by *Enterocytozoon bienewisi* (3), which is associated with gastrointestinal infection. Recently, however, another new microsporidian parasite, classified as belonging to *Septata intestinalis* gen. nov., sp. nov., has been identified as the causal agent of intestinal as well as disseminated microsporidiosis (4, 10, 17). Although several human-infecting microsporidia (e.g., *E. hellem*, *E. cuniculi*, and *Nosema corneum*) have been established in culture (3, 6, 11, 12, 15), neither *E. bienewisi* nor *S. intestinalis* has been cultured in vitro. Albendazole and

metronidazole are potentially effective against *S. intestinalis* and *E. bienewisi* (1, 7, 17). However, *S. intestinalis* is known to be strikingly more susceptible than *E. bienewisi* to albendazole, making possible complete resolution of symptoms in patients (17). An early diagnosis leading to the identification of the species involved would be helpful in instituting therapy. In vitro culture of the parasite would therefore be invaluable for the development of serologic and/or molecular diagnostic methods that would identify particular microsporidia.

MATERIALS AND METHODS

Case report. Urine, fecal, nasal swab, saliva, and serum samples from a 26-year-old man with AIDS were obtained in November 1993. His earlier history was as follows. He was diagnosed as positive for the human immunodeficiency virus in 1986. He was treated for *Pneumocystis carinii* pneumonia in 1992 and was found to have progressive human immunodeficiency virus-related encephalopathy at that time; he had a CD4 count of 40/mm³ and weighed 132 lb (ca. 59.9 kg) in February 1993; and a clinical evaluation in July 1993 revealed that he had dysuria, frank hematuria, and intermittent diarrhea. His urine sample was negative for bacteria, and his stool sample was negative for *Salmonella*, *Shigella*, and *Campylobacter* spp. as well as for ova and parasites, including *Cryptosporidium* and *Isospora* spp. He received loperamide HCl for symptomatic control of diarrhea and doxycycline for the empirical control of dysuria. In August 1993, he experienced recurrent hematuria, dysuria, and diarrhea and an exacerbation of herpes zoster of T12. Large doses of acyclovir relieved his herpes zoster. A repeat urine and stool examination revealed microsporidial infection. He was admitted to the hospital in September 1993, complaining of shortness of breath and diarrhea. An evaluation revealed a pleural effusion and cytomegalovirus retinitis, and he was treated with ganciclovir IV. His mental status continued to deteriorate. He also experienced intermittent diarrhea and dysuria, leading to intermittent bowel and bladder incontinence. His weight decreased to 109 lb (ca. 49.4 kg), and he died in January 1994. An autopsy was not performed.

Laboratory investigations. (i) **Smears.** At least three smears each were made from unconcentrated fecal, urine, nasal swab, and saliva samples. The smears were stained with Gram and Weber's stains (18). A small aliquot of Formalin-fixed fecal sample was also processed for electron microscopy after it was fixed with 2.5% glutaraldehyde.

(ii) **Urine.** The urine sample was washed by centrifugation (1,500 × g for 20 min) three times in distilled water, the sediment containing spores was inocu-

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FIG. 1. (a) Formalin-fixed smear of a fecal sample from our patient stained with Weber's stain. Magnification, $\times 1,250$. (b) A second fecal smear made from the same preparation as for panel a, reacted with a 1:100 dilution of the anti-CDC:V297 serum. Note the bright fluorescence of the spores. Magnification, $\times 1,250$. (c) Transmission electron micrograph of a fecal smear. Note the large numbers of electron-dense spores. Magnification, $\times 5,000$.

lated onto human lung fibroblasts or monkey kidney (E6) cell cultures as described previously (14, 15), and the cultures were incubated at 37°C. The culture supernatants were decanted once every 3 days, the supernatants were centrifuged and the sediment was reinoculated into the original culture flask, fresh medium containing antibiotics was added, and the mixture was incubated as before. The resultant culture was designated CDC:V297.

(iii) **Microorganisms.** *E. cuniculi*, courtesy of J. Shaddock and E. S. Didier and designated JS, and *E. hellem* CDC:0291:V213 were maintained on E6 monolayers (14, 15).

(iv) **Electron microscopy.** Scanning electron microscopy was performed with a JEOL JSM 820 scanning electron microscope as described previously (14, 15). For transmission electron microscopy, E6 monolayers infected with CDC:V297 microsporidia were covered with a solution of trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) until cells were detached (about 3 to 5 min), and the cells were washed in Hanks' balanced salt solution (HBSS). The cells were next fixed in 2.5% glutaraldehyde buffered with cacodylate, postfixed in a solution of 1%

OsO₄, dehydrated in ethanol, embedded in Epon 812, and processed as described previously (14, 15).

(v) **Generation of rabbit anti-CDC:V297 serum.** Rabbits were repeatedly injected intravenously with spores of CDC:V297 as described elsewhere (14, 15). Serum samples were taken 10 days after the last injection, and the serum was separated into 1-ml aliquots and stored at -20°C until used.

(vi) **IIF test.** Spores of *E. cuniculi* JS, *E. hellem* CDC:0291:V213, and the new isolate (CDC:V297) were harvested from culture supernatants by centrifugation at $1,500 \times g$ for 20 min at 4°C and washed three times in HBSS, counted in a hemacytometer, suspended in HBSS containing 1% Formalin to obtain 10^7 spores per ml, and processed for the indirect immunofluorescence (IIF) test as described previously (14, 15). IIF was also performed on smears of fecal samples from our patient as well as on smears that were known to contain *E. bienersi*.

(vii) **SDS-polyacrylamide gel electrophoresis and immunoblotting.** Spores of *E. cuniculi*, *E. hellem*, and CDC:V297 were harvested from cell cultures and washed in HBSS. Proteins were extracted from approximately 5×10^6 spores by

FIG. 2. (a) Growth pattern of *S. intestinalis* (CDC:V297) within monolayers of a human lung fibroblast cell culture. Note that the cells are distended with spores of *S. intestinalis*. Phase-contrast microscopy was used. Magnification, $\times 750$. (b) Scanning electron micrograph of *S. intestinalis* (CDC:V297) growing on E6 cells. Note the spores within a still-intact parasitophorous vacuole. Magnification, $\times 7,000$.

being heated at 65°C in a buffer containing 2.5% sodium dodecyl sulfate (SDS) and 2.25 M urea, loaded onto each lane of a 3 to 27% gradient acrylamide gel, and electrophoretically separated as described elsewhere (14, 15). The separated proteins were either silver stained or electrophoretically transferred to nitrocellulose membranes by using a Trans-blot cell and a model 200/2.0 power supply (Bio-Rad) at a constant voltage of 30 V overnight. After appropriate washes, the membranes were reacted overnight at 4°C on a rocker platform with the rabbit sera made against *E. hellem*, *E. cuniculi*, and CDC:V297 or with sera obtained from our patient as well as from a patient infected with *E. hellem* and processed as described previously (14, 15).

(viii) **PCR amplification, cloning, and sequencing of the small subunit RNA (SSU-rRNA) coding region.** DNA was extracted from the following four cultures: an uninfected E6 cell culture (control), an E6 culture infected with *E. hellem*

CDC:0291:V213, an E6 culture infected with *E. cuniculi* JS, and an E6 culture infected with CDC:V297. All cultures were grown in 75-cm^2 plastic flasks (Corning, Ithaca, N.Y.). Cells from each of the four cultures were scraped separately and transferred to separate 15-ml tubes. After centrifugation at $1,000 \times g$ for 20 min at 4°C , the supernatant was aspirated and the sediment was suspended in 1 ml of 0.001 M phosphate-buffered saline (PBS), pH 7.6. The material was transferred to 1.5-ml siliconized microcentrifuge tubes, centrifuged for 5 min at $14,000 \times g$, and resuspended in 1 ml of PBS. This last step was repeated twice more, and the supernatant was aspirated. DNA was extracted from the pellet by using a nonorganic DNA extraction kit (Oncor, Gaithersburg, Md.) according to the manufacturer's instructions. Nucleic acid from each sample was resuspended in $50\text{ }\mu\text{l}$ of distilled water and amplified by PCR.

(ix) **DNA amplification.** Microsporidial SSU-rRNA coding regions were am-

FIG. 3. Transmission electron micrograph of *S. intestinalis* (CDC:V297) within an E6 cell. Note the spores and developing forms within a septated honeycomb-like parasitophorous vacuole. S, spore; ST, sporont. Bar = 1 μ m. Cross sections of four to seven coils of the polar tube (arrowheads) are indicated.

plified by using a forward primer (5'-CACCAGGTTGATTCTGCCTGA-3') and a reverse primer (5'-TTATGATCCTGCTAATGGTTCTCCAAC-3') based on nucleotides 1 to 21 (Micro-F) and 1244 to 1218 (Micro-R), respectively, of *Vairimorpha necatrix* SSU-rRNA (14, 16). These primers were designed to amplify a fragment of approximately 1,300 bp. Two additional pairs of primers were used for PCR amplification. The first pair, specific for *E. hellem*, was designed for amplification of a 547-bp fragment. The forward primer (5'-TGAGAAGTAA GATGTTTAGCA-3') was designed on the basis of positions 358 to 378, and the reverse primer (5'-GTAAAAAACTCTCACACTCA-3') was based on positions 884 to 904 of the *E. hellem* SSU-rRNA sequence (10a). The second pair specifically amplified a 549-bp diagnostic fragment of *E. cuniculi* DNA. The forward primer (5'-ATGAGAAGTGATGTGTGTGCG-3') was designed on the basis of positions 344 to 364, and the reverse primer (5'-TGCCATGCACTCA CAGGCATC-3') was based on positions 872 to 892 of SSU-rRNA sequence (10b). 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 amplification were electrophoretically resolved on a 2% agarose gel (SeaKem GTG; FMC Bioproducts, Rockland, Maine) and visualized for analysis after being stained with ethidium bromide.

(x) **Cloning and sequencing.** The products of amplification generated by Micro-F and Micro-R primers were cloned by using the PCR-script cloning kit (Stratagene, La Jolla, Calif.) and sequenced. Sequencing was done for three independent clones, and the consensus sequence from them was aligned with other microsporidial SSU-rRNA sequences by using the IBI Pustell program, version 3.1.

Nucleotide sequence accession number. The SSU-rRNA sequence generated from CDC:V297 was deposited in GenBank under accession no. SIU09929.

RESULTS

Clinical specimens. Stained smears of feces, urine, nasal swabs, and saliva revealed oval spores characteristic of microsporidia. Fecal smears contained many spores (Fig. 1a and c), and the spores fluoresced brightly when reacted with a 1:100 dilution of the rabbit antiserum made against the cultured parasites (Fig. 1b). Transmission electron microscopy of the fecal sample revealed electron-dense spores (Fig. 1c). Urine smears also contained numerous spores, whereas few spores were seen in the nasal and saliva samples.

Culture isolation and ultrastructure of the parasites. The cell culture inoculated with the urine samples did not show any overt bacterial contamination. After 3 weeks of manipulation as described in Materials and Methods, foci of cells infected with the parasites were seen in the culture flasks (Fig. 2a) when they were examined with an Olympus IMT-2 inverted microscope equipped with phase-contrast optics. Thereafter, spores that were released into the culture supernatants were regularly

FIG. 4. Immunoblot profiles of microsporidian strains obtained by using a serum sample from a patient infected with *E. hellem* (A) and serum from our patient (B). Lanes 1, E6 cells; lanes 2, *S. intestinalis* CDC:V297; lanes 3, *E. cuculi* JS; lanes 4, *E. hellem* CDC:0291:V213. Molecular weights (MW) are shown on the left (in thousands).

seen during transfers. The spores were birefringent and variably gram positive. The spores appeared to be smooth and when examined by scanning and transmission electron microscopy were seen to lie within the parasitophorous vacuole (Fig. 2b and 3). However, all the developing stages and the spores were present within chambers of a septated honeycomb-like fibrillar network (Fig. 3) as described for *S. intestinalis* (4). Mature spores measured 1.2 to 2.0 μm by 0.9 to 1.2 μm . Both scanning and transmission electron micrographs also revealed that many of the spores had already discharged their polar tubules, which measured 15 to 22 μm in length. The spores were characterized by a thin electron-dense exospore, a thick electron-lucent endospore, and a thin cell membrane that surrounded the spore contents. Spore contents appeared to be electron dense; however, a lamellar polaroplast at one end and a vacuole at the other could be discerned in some spores. Cross sections of four to seven coils of the polar tube (Fig. 3) were also seen in some spores.

IIF assay. The spores of CDC:V297 reacted with the rabbit anti-CDC:V297 serum in the IIF test and produced a titer of $>4,096$, whereas spores of *E. cuculi* and *E. hellem* gave a titer of <128 .

Immunoblotting. In the immunoblotting procedure, proteins extracted from spores of CDC:V297, *E. hellem* CDC:0291:V213, and *E. cuculi* JS were assayed with the serum of a patient infected with *E. hellem* as well as our patient serum. CDC:V297 reacted extensively with both sera and produced a number of darkly staining bands with molecular masses of approximately 16, 19, 22, 28, 35, 45, 70, 90, and 190 kDa. The antigens extracted from *E. hellem* and *E. cuculi* also reacted with the patient sera and produced several bands but with slightly different molecular masses (Fig. 4). Both *E. hellem* and *E. cuculi* reacted fairly intensely with the serum of the *E. hellem*-infected patient; however, they reacted only slightly with the serum of our patient.

Dramatic differences were observed, however, when the proteins extracted from *S. intestinalis*, *E. hellem*, and *E. cuculi* were assayed with rabbit antisera against these three parasites.

FIG. 5. Immunoblot profiles of microsporidian strains obtained by using immune sera made in rabbits. (A) Proteins extracted from E6 cells (lane 1), *S. intestinalis* CDC:V297 (lane 2), *E. cuculi* JS (lane 3), and *E. hellem* CDC:0291:V213 (lane 4) with anti-*E. hellem* serum. (B) Proteins extracted from E6 cells (lane 1), *S. intestinalis* CDC:V297 (lane 2), *E. hellem* CDC:0291:V213 (lane 3), and *E. cuculi* JS (lane 4) with anti-*S. intestinalis* serum. (C) Proteins extracted from *E. cuculi* JS (lane 1), *S. intestinalis* CDC:V297 (lane 2), *E. hellem* CDC:0291:V213 (lane 3), and E6 cells (lane 4) with anti-*E. cuculi* serum. Molecular weights (MW) are shown on the left (in thousands).

As shown in Fig. 5, the antigens of *E. hellem*, *S. intestinalis*, and *E. cuculi* reacted intensely with their homologous antibodies and produced a number of bands ranging from 16 to 190 kDa. Heterologous reactions were minimal.

PCR amplification. As expected, bands of approximately 1,300 bp were amplified by the Micro-F and Micro-R pair of primers when cultures infected with CDC:V297, *E. cuculi*, and *E. hellem* as well as one clone of *E. cuculi* SSU-rRNA were used as templates (Fig. 6, lanes 1, 2, 3, and 5, respectively). No such band was detected when the amplification was performed with the uninfected control cell templates (Fig. 6, lane 4). The CDC:V297 template was not amplified by either the *E. hellem*-specific primers or the *E. cuculi* primers (Fig. 6, lanes 6 and 11, respectively). However, bands of 547 and 549 bp were detected when *E. hellem* and *E. cuculi* templates were amplified by *E. hellem* and *E. cuculi* diagnostic primers, respectively (Fig. 6, lanes 10 and 12, respectively).

SSU-rRNA sequence analysis. The consensus sequence obtained from three independent clones of CDC:V297 consisted of 1,290 bp corresponding to the size of other, published microsporidian SSU-rRNA sequences. Similarity searches showed that the highest index was obtained with three different sequences in the GenBank database. Sequences under accession no. L16866 and L16867 described by Hartskeerl et al. (8) showed 99.8 and 99.6% similarity, respectively. Both are described in the GenBank database as SSU-rRNA of an *Encephalitozoon* sp. The third sequence, under accession no. L19567, described by Zhu et al. as that of *S. intestinalis* (20), showed 97.3% similarity. This confirmed the molecular identification of CDC:V297 as *S. intestinalis*.

DISCUSSION

This is the first report of the isolation and continuous in vitro cultivation of ultrastructurally, antigenically, and molecularly confirmed *S. intestinalis* from urine of an AIDS patient with

FIG. 6. Agarose gel analysis of PCR-amplified products. Lanes 1 to 5, *V. necatrix* primers (Micro-F and -R); lanes 6 to 10, *E. hellem* diagnostic primers; lanes 11 to 15, *E. cuniculi* diagnostic primers. Lanes 1, 6, and 11 show results of reactions with E6 cells infected with CDC:V297. An amplified fragment of 1,300 bp is observed in lane 1, in which generic primers were used for amplification, but no diagnostic fragment is seen in lanes 6 and 11, which contained specific diagnostic primers for *E. hellem* and *E. cuniculi*, respectively. Lanes 2, 7, and 12 show amplification with E6 cells infected with *E. cuniculi*, and lanes 3, 8, and 13 show amplification of E6 cells infected with *E. hellem*. In lanes 2 and 3, fragments of approximately the same size amplified from CDC:V297-infected cells can be seen with the two microsporidian species; however, amplification of the diagnostic fragment at about 500 bp was observed only when specific primers were used with their respective templates (lane 8 for *E. hellem* and lane 12 for *E. cuniculi*). Lanes 5 and 15 show amplification with SSU-rRNA cloned from *E. cuniculi* as a template, and lane 10 shows amplification with the *E. hellem* SSU-rRNA cloned sequences. These clones were used as standard positive controls for PCR. Lanes 4, 9, and 14 show results of reactions with uninfected E6 cells which were used as negative controls. Lane S contains the 100-bp ladder standard. Molecular sizes (in base pairs) are shown on the left.

intestinal and disseminated microsporidian infection. Orenstein et al. (10) were the first to recognize this syndrome caused by an *Encephalitozoon*-like microsporidian parasite in patients with AIDS. Subsequently, this microsporidian was established as a new genus and new species, *S. intestinalis*, by Cali et al. (4). Unlike *E. cuniculi* and *E. hellem*, the two microsporidian parasites that are known to cause disseminated infection not involving the gastrointestinal tract in humans, *S. intestinalis* causes gastrointestinal infection as well as disseminated infection affecting the urinary tract, nasal mucosa, and other areas.

Our patient had diarrhea, frank and microscopic hematuria, pleural effusion, and dementia. Unfortunately, an autopsy was not performed and various organs of the body, including the brain, could not be examined for possible etiologic agents. However, the stool samples from this patient showed innumerable microsporidian spores that fluoresced brightly with the rabbit anti-CDC:V297 serum made against the culture-de-

rived organisms isolated from his urine. We were also able to demonstrate *S. intestinalis* in the saliva, nasal mucosa, and urine both by chromotrope staining and by immunofluorescence.

Orenstein et al. (10) have also demonstrated the presence of spores in renal tubular transitional cells, lamina propria macrophages of the intestinal biopsies, gallbladders, and parenchymal cells of the liver and bronchial epithelium of patients infected with *S. intestinalis* (*Encephalitozoon*-like microsporidian organism). According to Orenstein et al., *S. intestinalis* infects the intestine and also disseminates and causes renal pathology. They also state that dissemination from the intestinal tract is not remarkable since spores, both free and within infected cells, were identified in an intrahepatic portal vein, which is consistent with a vascular route of dissemination from the bowel. It is well known that spores of *E. cuniculi* that can disseminate widely, including to the brain, have been found in mucosal macrophages in infected animals such as foxes, dogs, and rabbits (5).

Didier et al. (6) have shown that AIDS patients do indeed mount an immune response to microsporidian infection, and these researchers speculate that a serologic diagnosis for microsporidian infection is possible. Our patient, despite having an advanced stage of AIDS, mounted an antibody response to the infecting agent. His serum also cross-reacted with *E. hellem* and *E. cuniculi*. Furthermore, antigens extracted from *S. intestinalis* isolated from our patient as well as those of *E. hellem* and *E. cuniculi* also reacted strongly with a serum sample from a patient infected with *E. hellem*, indicating that a serologic diagnosis is possible for microsporidiosis, as suggested by Didier et al. (6).

Recent studies (17, 19) indicate that *S. intestinalis* is susceptible to the anthelmintic albendazole. Since the rabbit anti-*S. intestinalis* serum generated in our laboratory can specifically identify *S. intestinalis* spores in Formalin-fixed fecal specimens, infections with this microsporidian can be readily diagnosed so that treatment with albendazole can be initiated and the infection can be controlled in a timely fashion. Further tests are in progress to ascertain the sensitivity and specificity of the anti-*S. intestinalis* serum.

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