A new species of microsporidian, *Septata intestinalis*, was recently recognized as an opportunistic pathogen of AIDS patients. In this study, it was cultured from the nasopharyngeal aspirate of a human immunodeficiency virus type 1-infected patient with disseminated microsporidiosis. In human embryonic lung cells exposed to *S. intestinalis*, a cytopathic effect appeared within 28 days as foci of rounded up cells. Thin-section electron microscopy showed a variety of developmental stages of the microsporidium within parasitophorous vacuoles. In monocyte-derived macrophages, evidence of infection and development of the parasite was demonstrated by light and electron microscopy. In both infected human embryonic lung cells and monocyte-derived macrophages, a network of septa separated individual spores. Partial sequencing of the RNA small-subunit gene (16S rDNA gene) confirmed the identity of this parasite as *S. intestinalis*. This is the first report of the isolation of *S. intestinalis* in vitro and provides evidence that the parasite can be disseminated by macrophages.

**MATERIALS AND METHODS**

**Origin of material.** In October 1992, a 32-year-old HIV-1-infected male presented with nasal stuffiness and clinical features of sinusitis. His CD4 cell count was 10/mm³, and *Mycobacterium avium* complex (MAC) bacteremia had occurred in September 1992 as his AIDS-defining illness. His medication included zidovudine, co-trimoxazole for *Pneumocystis carinii* pneumonia prophylaxis, and *M. avium* complex therapy. Sinus X rays revealed opacification of his right and left maxillary sinuses, and he was treated with amoxicillin-clavulanate (500 mg three times a day) and decongestants. One week later, he reported that his nasal stuffiness had improved. In February 1993, the patient presented with a 2-week history of nasal stuffiness and green nasal discharge. He was afebrile and did not have tenderness over the facial sinuses. He was treated with amoxicillin-clavulanate (500 mg three times a day) for 7 days. A swab of the nasal discharge was taken for culture, and trichrome blue staining for microsporidia was also performed (17). Bacterial and viral cultures were negative, but microsporidia were detected. An NPA was obtained, and staining of a smear by the trichrome blue method (17) revealed microsporidial spores. Subsequent examination of feces and urine by the same procedure showed the presence of spores of similar size and staining characteristics. One month later, the patient reported ongoing nasal stuffiness, epigastric pain, and a 2-week history of diarrhea. A gastroscopy was performed and it revealed candida esophagitis. A biopsy of the duodenum revealed intracytoplasmic microsporidia. *Clostridium difficile* toxin was isolated from the stool. The patient received a 2-week course of albendazole, 400 mg twice a day, for treatment of the microsporidial infection and commenced fluconazole (200 mg daily) and metronidazole (400 mg three times a day for 10 days) therapy. Four weeks later, the patient reported that his symptoms of nasal stuffiness and sinus discomfort had resolved, and subsequently he experienced only occasional and mild nasal symptoms. Long-term oral vancomycin therapy was subsequently required to control relapsing *C. difficile* diarrhea. Following the course of albendazole, microsporidial spores were not seen again in the stools. The patient developed pancreatitis in July 1993 secondary to didanosine (ddI) treatment and died in November 1993 following a gradual deterioration in health.

**In vitro growth in HEL cells.** NPA was inoculated into 6-day-old confluent HEL cells in 75-cm² tissue culture flasks (Costar, Cambridge, Mass.). The specimen was allowed to absorb for 2 h at 37°C, and the monolayer was then rinsed twice with 30 ml of basal Eagle medium (Flow ICN, Irvine, Scotland) supplemented with 5% fetal calf serum (Commonwealth Serum Laboratories Ltd., Parkville, Victoria, Australia), 100 IU of penicillin per ml, and 100 μg of streptomycin (Commonwealth Serum Laboratories Ltd.) per ml. The cells were incubated in this medium for 2 days at 37°C and then maintained in 30 ml of basal Eagle medium–2% fetal calf serum with the penicillin and streptomycin, also at 37°C. The medium was changed weekly; every 3 to 4 weeks, the medium was removed and infected cells were resuspended with approximately 1 ml of 0.25% trypsin–EDTA (disodium salt) and added to a suspension of fresh uninfected HEL cells. Uninfected HEL cells were similarly cultured as a control.

**In vitro growth in MDM.** Monocytes were isolated from HIV-seronegative bulky effusates (purchased from the Red Cross Blood Bank, Melbourne, Australia) by Ficol-Hypeaque (Pharmacia, Uppsala, Sweden) density gradient centrifuga-
tion and plastic adherence, as described previously (6). Monocytes were cultured in Iscove’s medium (Cytosystems, Castle Hill, New South Wales, Australia) supplemented with 10% heat-inactivated human AB-positive serum (generously donated by Red Cross Blood Bank, Sydney, New South Wales, Australia), 2 mM L-glutamine (Flow ICN), 100 IU of penicillin (Flow ICN) per ml, and 100 μg of streptomycin (Flow ICN) per ml. Monocytes were cultured in suspension, at 10^6/ml, using Teflon-coated (polytetrafluorethylene) jars (Savillex). The medium was changed weekly.

Three days postisolation, 2 × 10^7 monocytes were incubated with 200 μl of NPA which contained approximately 5 × 10^5 microsporidia per ml. After 2 h of incubation at 37°C, macrophage medium was added to maintain a cell concentration of 10^6/ml. Monocytes from the same donor not exposed to microsporidia were similarly cultured as a control.

Light microscopy. Ten milliliters of the culture fluid harvested from the infected and uninfected HEL cells (day 29 postinfection) and macrophages (days 3, 7, 14, and 21 postinfection) was centrifuged at 4,000 × g for 10 min, and 8.5 ml of the supernatant was removed. Slides were prepared from the deposit by cytocentrifugation at 1,000 × g for 10 min (Shandon Cytospin 3), air dried, fixed in methanol for 10 min, and then stained by the trichrome blue method (17).

Electron microscopy of HEL cells. Fourteen days after the first subculture (approximately 6 weeks postinfection), infected and uninfected HEL cellswere harvested for thin-section electron microscopy (TSEM). Cells were washed in Hanks’ basal salt solution and incubated in 0.25% trypsin–EDTA for 5 min at 37°C. The resulting cell suspension was centrifuged for 10 min at 2,400 × g. The supernatant was discarded, and the pellet was resuspended in basal Eagle medium–2% fetal calf serum and then centrifuged at 10,700 × g for 2 min. The pellets were processed for TSEM as described by Lee et al. (12) except that cells were embedded in Spurr resin (Ladd Industries, Inc., Burlington, Vt.). Ultrathin sections were stained with uranyl acetate (21) and lead citrate (16). Sections were examined with a Philips CM12 electron microscope.

Electron microscopy of MDM. On days 3, 7, 14, and 21 postinfection, 10^6 MDM were removed from both the infected and uninfected cultures. The cells were washed with phosphate-buffered saline and centrifuged at 5,500 × g for 3 min. The pellets were then fixed and processed for electron microscopy as described above.

16S rDNA gene sequencing. Purified DNA was extracted from a washed pellet of infected HEL cell supernatant by cell lysis in 1 mg of proteinase K per ml–1% sodium dodecyl sulfate followed by two extractions with phenol-chloroform (50:50) and ethanol precipitation. The DNA pellet was washed with 70% ethanol, dried, and stored at −20°C until reconstitution with sterile water (1). Near-full-length amplification of the RNA small-subunit gene (16S rDNA gene) was performed as described previously, using primers that are conserved in microsporidial species (26): P1, 5′-CAC CAGGT GAT TCT GCC TGA C-3′; P2, 5′-GTT TTA CCT TCT TAC GAC TT-3′. An amplification product of approximately 1,450 bp was obtained and purified by gel extraction (Wizard PCR Preps purification; Promega, Melbourne, Victoria, Australia). Partial sequencing of the amplified 16S rDNA gene with the P1 and P2 primers (fmol DNA Sequencing System; Promega) obtained 394 of the 1,450 bases for comparison. Analysis of the sequence data was performed by using MacVector software and the February 1994 Entrez database.

RESULTS

In vitro growth of S. intestinalis in HEL cells. (i) Light microscopy. A cytopathic effect (CPE) was first detected at day 28 in HEL cells inoculated with microsporidia but not in uninfected control cells. The CPE appeared as foci of rounded, nonrefractile cells within the matrix of elongated fibroblastic HEL cells (Fig. 1A and B). As the CPE progressed, the foci of small round cells became progressively larger.

Trichrome blue staining indicated that spores were plentiful in the supernatant fluid when the CPE was well advanced. Spores appeared as red-staining ovoid structures (2 to 2.5 by 1.0 μm) and showed a characteristic vacuole (Fig. 2). Occasionally, a disrupted cell disgorging large quantities of spores was noted.

(ii) TSEM. Replication of the parasite in infected HEL cells was confirmed by the observation of a variety of developmental
stages in parasitophorous vacuoles in the host cell cytoplasm. Microsporidia were not detected in uninfected cells.

Mature spores generally appeared in a longitudinal section as ovoid structures measuring about 1.8 µm in length and about 0.9 µm in width. They were marked by a distinct internal coiled tube (polar tubule) about 100 nm in diameter. The coil occupied the periphery of the spore interior and commonly showed four to five turns (Fig. 3A). In a transverse section, the polar tubule was made up of three bands: an inner fuzzy band and two outer membranes (Fig. 3B). Depending on the plane of section, the spore interior could be seen to contain ribosomes often in regular arrays (Fig. 3C), an occasional vacuole (Fig. 3D), and a nucleus (Fig. 3A). The spore was surrounded by a unit membrane (plasmalemma), which in turn was surrounded by an outer coat. The outer coat was made up of two layers: a lighter inner endospore and a darker, sometimes corrugated, multilayered outer exospore (Fig. 3E). On some occasions spores with extruded polar tubules were noted. The tubule arose from a bulbous protrusion (anchoring disk) at one end of the spore (Fig. 3F).

Single nucleated cells in parasitophorous vacuoles in the cytoplasm of the host cell were the earliest recognized stages in the development of the parasite. These forms had a large nucleus/cytoplasm ratio and were in contact with the membrane of the surrounding parasitophorous vacuole. The cytoplasm of these forms showed occasional strands of rough endoplasmic reticulum (Fig. 3A). The next stage of morphogenesis appeared to involve the formation of a sporont from the early stage. The sporont was characterized by a thickened surrounding plasmalemma and had moved away from the periphery of the enclosing parasitophorous vacuole (Fig. 4A). Sporonts with more than one nucleus were also seen (Fig. 4B). In some cases the cytoplasm between the two nuclei was apparently pinching off to form two uninucleate sporonts (Fig. 4B). Occasionally, maturation of spores took place without complete division of the sporonts so that connected spores were formed (Fig. 4A).

A striking feature of the parasitophorous vacuole was the presence of zones of material separating individual spores. This septate material was granular in appearance and similar in texture to the cytoplasm of the host cell (Fig. 5). A distinctive clear area separated the spore from the septate material.

**In vitro growth of** *S. intestinalis* **in MDM.** (i) Light microscopy. No CPE was evident in the infected MDM culture. A systematic scan of the trichrome blue-stained supernatant from *S. intestinalis*-infected HEL cells with advanced CPE. Bar = 10 µm.
of the species in this study were not seen to abut each other. Cali et al. (5) reported bi- and tetranucleated forms of the sporont stage; in this study, bi- and trinucleated forms of the parasite were noted. Although a tetranucleated form was not seen, this may simply relate to the low probability of having all four nuclei in the one plane of section. On the basis of this evidence, it is reasonable to conclude that the species in this report is *S. intestinalis*.

The species responsible for disseminated microsporidiosis may also be separated at a genetic level. Nucleotide sequencing of the well-conserved 16S rDNA gene of *E. cuniculi* and *E. hellem* has revealed high sequence similarity, although there

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**FIG. 3.** Electron micrograph of “early stages” and mature spores of *S. intestinalis* in HEL cells. (A) Mature spore (S) and two early stages (ES) in a parasitophorous vacuole in the HEL cell cytoplasm (C). Note the polar tubule (PT), nucleus (N), anchoring disk (AD), and lamellar polaroplast (LP) in the spore. The two early stages are in contact with the periphery of the parasitophorous vacuole and have an undifferentiated appearance. Bar = 0.5 μm. (B) Transverse section of a polar tubule in a mature spore showing the inner fuzzy band (dark arrow) and the outer membranes (open arrow). Bar = 100 nm. (C) Mature spore with an array of ribosomes (R). Bar = 0.5 μm. (D) Mature spore showing a characteristic vacuole (V). Bar = 0.5 μm. (E) High-power image of the outer coat of a mature spore. Note the lighter inner endospore (open arrow), the dark outer exospore (dark arrow), and the unit membrane plasmalemma surrounding a parasite (triangle). Bar = 100 nm. (F) Spore with an extruded polar tubule (PT) arising from an anchoring disk (AD). Bar = 0.5 μm.
FIG. 4. Electron micrograph of sporonts and mature spores of *S. intestinalis* in HEL cells. (A) Sporont (arrow) showing the thickened plasmalemma. Two connected mature spores (S) are also present in the parasitophorous vacuole. Bar = 0.5 μm. (B) Sporont with two nuclei (N) apparently pinching off (arrows) to form two sporonts. Bar = 0.5 μm.
are significant differences to allow a distinction between these closely related organisms (23). The subsequently published sequence data for *S. intestinalis* (26) show high similarity but features distinct from those of *E. cuniculi* and *E. hellem*. Comparison of the sequence of 16S rDNA of the isolate described in this study with previously published sequences (23, 26) confirms the morphological observation that the parasite grown is *S. intestinalis*.

At present the mechanism of dissemination of microsporidia in persons with advanced HIV disease is unclear. This study, taken with the findings of Cali et al. (5) and Ryan et al. (17), supports the view that dispersion of the parasite occurs via macrophages. Cali et al. (5) have previously noted the presence of *S. intestinalis* in macrophages of the lamina propria of infected gut. The presence of the parasite in cells from NPA which morphologically resemble macrophages has also been demonstrated (17). In this study microsporidia causing disseminated disease in a patient with advanced HIV infection were clearly shown to replicate within macrophages in vitro. These findings suggest that microsporidia can become disseminated through infection of macrophages which migrate to a variety of tissues in the host.

The process by which macrophages disseminate microsporidia in individuals with advanced HIV disease is unclear. It is well established that macrophage function is altered by infection with HIV (3, 9), and this is probably an important factor in the process of dissemination. Baldwin et al. (2) have shown that HIV-infected macrophages are less capable of killing *Candida pseudotropicalis* than uninfected macrophages. It is possible that microsporidia are also less likely to be killed in HIV-infected macrophages than in uninfected macrophages.

The definitive diagnosis of disseminated microsporidiosis requires the classification of microsporidia to species level in a number of sites in the infected host. In this study the microsporidia isolated from NPA were shown to be *S. intestinalis* after culture in HEL cells and MDM. TSEM of a duodenum biopsy taken about 1 month after the collection of the NPA from the patient revealed identical parasitophorous vacuoles with spores separated by distinct septa (unpublished observation). Thus, at the very least, *S. intestinalis* has been shown to be present in the gut and the NPA of this patient. Taken with the light microscopy findings of spores in the NPA, feces, and urine of this individual (see Materials and Methods), this report provides compelling evidence in support of a case of disseminated microsporidiosis.

The successful in vitro growth of *S. intestinalis* will provide antigen for the development of immunological assays for diagnostic and epidemiological studies and also permit better evaluation of drug strategies. In addition, the growth of the parasite in macrophages will enable a better understanding of the nature of disseminated microsporidiosis.

**FIG. 5.** Electron micrograph of *S. intestinalis* in a parasitophorous vacuole in a HEL cell. Note the septate material (arrows) separating the spores. Bar = 0.5 μm.
FIG. 6. Electron micrographs of various developmental stages of *S. intestinalis* in macrophages. (A) Sporont with three nuclei (N). Bar = 0.5 µm. (B) Sporoblast with polar tubule exhibiting a dense core (arrows). A nucleus (N) and anchoring disk (AD) are also evident. Bar = 0.3 µm. (C) Parasitophorous vacuole containing spores separated by distinct septa (arrows). Bar = 0.5 µm.
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