

Original Contributions

A Microsporidian Previously Undescribed in Humans, Infecting Enterocytes and Macrophages, and Associated With Diarrhea in an Acquired Immunodeficiency Syndrome Patient

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To date, the only microsporidian that has been associated with diarrhea and weight loss in acquired immunodeficiency syndrome patients is the newly identified *Enterocytozoon bieneusi*. A second species is now described that was associated with intestinal symptoms in a 32-year-old, human immunodeficiency virus-seropositive, Native American male homosexual. Stool studies and routine light microscopy of multiple small intestinal biopsies that showed atrophy with acute and chronic inflammation were without apparent pathogens. Light microscopy of semi-thin plastic sections, cytochemical stains of paraffin sections, and ultrastructural studies revealed extensive microsporidial infection of enterocytes and submucosal macrophages. No other pathogens were identified. Unlike *E. bieneusi*, this microsporidian appeared to develop within septated parasitophorous vacuoles, and lacked polar disks and clear clefts. It most closely resembled, but was distinguishable from, members of the genus *Encephalitozoon*. Awareness of the microsporidia as potential opportunists in acquired immunodeficiency syndrome patients is increasing the incidence of identification of these organisms. HUM PATHOL 23:722-728. Copyright © 1992 by W.B. Saunders Company

Chronic diarrhea is one of the most common and debilitating complications of human immunodeficiency virus (HIV) infection worldwide.¹⁻¹⁰ No etiology is ascertained from stool studies and routine histologic evaluation of endoscopic biopsies in up to 50% of all diarrhea cases.^{1,2,4} A new genus of the phylum Microspora, *Enterocytozoon bieneusi*, has been demonstrated as the etiology of the enteritis in over 75 acquired immunodeficiency syndrome (AIDS) patients with chronic diarrhea and weight loss in North America, Western Europe, and Africa.¹¹⁻²⁵ The diagnosis has been made by identifying parasites in small intestinal biopsies, predomi-

nantly by ultrastructural studies, but recently by light microscopy of semi-thin plastic sections¹⁶ and Giemsa-stained smears of fresh biopsies.^{18,22} Very recently, spores have been identified in stool^{23,24} and duodenal fluid²⁴ specimens by light and transmission electron microscopy. The actual incidence of *E. bieneusi* intestinal microsporidiosis in HIV-infected patients is unknown. However, preliminary studies indicate that it may be the cause of diarrhea in over 25% of patients with negative stool studies.^{11,16,21-23,25,26}

Encephalitozoon cuniculi, a microsporidian with a wide range of vertebrate targets,²⁷⁻³² has been diagnosed as the cause of hepatitis³³ and, more recently, peritonitis in individual AIDS cases.³⁴ The first human species of *Encephalitozoon*, *Encephalitozoon hellem*, has recently been isolated from the conjunctiva of several AIDS patients with superficial keratoconjunctivitis.³⁵⁻³⁹

This report describes an AIDS patient with severe diarrhea and an intestinal infection with a microsporidian that is ultrastructurally distinct from *E. bieneusi*,⁴⁰ *E. cuniculi*,³⁰ and *E. hellem*.³⁵⁻³⁹

MATERIALS AND METHODS

Forty-four cases of *E. bieneusi* microsporidiosis, from a group of over 200 patients, have been diagnosed by transmission electron microscopy (TEM) and plastic-section light microscopy (LM) in our laboratory. This represents a 20% incidence in a population of AIDS patients with diarrhea and no pathogens identified in stool specimens and paraffin sections prepared from small-bowel biopsies. To date, we have not identified *E. bieneusi* in large-bowel biopsies even from the same patients with biopsy-proven small-bowel disease. When comparing jejunal to duodenal biopsy specimens from the same patients, the jejunal biopsy specimens contained at least as many, and often more, organisms than the duodenal biopsy specimens (Orenstein J, unpublished observation).

In the course of evaluating bowel biopsy specimens from AIDS patients with diarrhea and no LM diagnosis, six endoscopic biopsy specimens were obtained from the AIDS patient reported here. All specimens had been embedded in paraffin for LM and four of them were also fixed in glutaraldehyde and embedded in Spurr's epoxy for semi-thin plastic section

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LM and TEM. Plastic sections were stained with toluidine blue or the combined methylene blue-azure II, basic fuchsin stain. Paraffin sections were stained with hematoxylin-eosin, Giemsa, Gram, acid fast, periodic acid-Schiff, and Grocott methenamine silver stains.¹⁶

CASE REPORT

A 32-year-old, HIV-seropositive, Native American male homosexual was evaluated for diarrhea, a 20-lb weight loss over an 8-month period, fever, vomiting, oral candidiasis, night sweats, and sinusitis. There was moderate wasting and mild normochromic anemia and hypoproteinemia. Serum antibodies to hepatitis A and B and abnormal liver function tests were documented.

Stool studies for enteric pathogens and parasites were negative on two occasions. Because of progressive wasting, upper intestinal endoscopy with jejunal biopsy was performed using a pediatric colonoscope (PCF 10; Olympus Corp, Lake Success, NY). Severe small intestinal injury with ulceration was observed. Light microscopy of paraffin sections revealed atrophy, acute and chronic inflammation with submucosal collections of macrophages, and focal granulomatous reaction, but no apparent pathogens. Jejunal tissue was fixed in glutaraldehyde for TEM. Mycobacterial, fungal, and viral cultures were negative and trimethaprin sulfa (Bactrim DS; Roche Laboratories, Nutley, NJ) treatment was begun. Two weeks later, the patient was admitted to St Luke's-Roosevelt Hospital Center for further evaluation of the persistent symptoms. A repeat jejunal biopsy specimen was identical, by LM, to the first biopsy specimen (no tissue submitted for TEM) and a colorectal biopsy specimen was also found to be negative (submitted for TEM). X-ray films of the small bowel revealed a nodular ulcerating jejunitis. A biopsy specimen of the frontal sinus was diagnosed as chronic sinusitis. The patient was continued on Bactrim DS and begun on zidovudine (AZT), acyclovir, and nasal decongestants. The patient responded with a 25-lb weight gain, cessation of diarrhea and vomiting, loss of fever, improved energy, and a sense of well being.

Ten months later, the patient began to experience right facial pain that was attributed to maxillary sinusitis that was refractory to antibiotic therapy. A biopsy revealed an aspergilloma and amphotericin B therapy was begun. Meanwhile, the diarrhea and rapid weight loss had reappeared, accompanied by fever spikes, nausea, and vomiting. A repeat colonic biopsy was negative by LM (submitted for TEM). Upper gastrointestinal endoscopy (14 months after the initial studies) revealed white plaques and edema in the distal duodenum. Light microscopy of this biopsy specimen disclosed acute and chronic inflammation, signs of repair, and "intracytoplasmic inclusions highly suspicious of protozoa" (submitted for TEM). No collections of macrophages were seen as in the previous specimens. Two weeks later, pseudomembranous colitis was diagnosed on colonic biopsy (not submitted for TEM) following an episode of bloody diarrhea, and oral vancomycin was begun.

The terminal illness involved high fevers, disorientation, diffuse pulmonary infiltrates, and evidence of peritonitis on physical examination. Despite intravenous Bactrim, the patient died (15 months after the first biopsy) and a request for autopsy was denied by the family.

RESULTS

Light Microscopy of Semi-thin Plastic Sections

The first material examined was the plastic sections on which a presumptive diagnosis of microsporidiosis

was made. The initial jejunal biopsy specimen and the distal duodenal biopsy specimen taken 14 months later showed striking numbers of densely staining bodies concentrated in the supranuclear cytoplasm (often indenting the nucleus) of the enterocytes (both absorptive and goblet cells) and increasing toward the tips of the villi (Fig 1, top). In the jejunal biopsy specimen, they were also seen within macrophages that extended from the lamina propria through the muscularis mucosae into

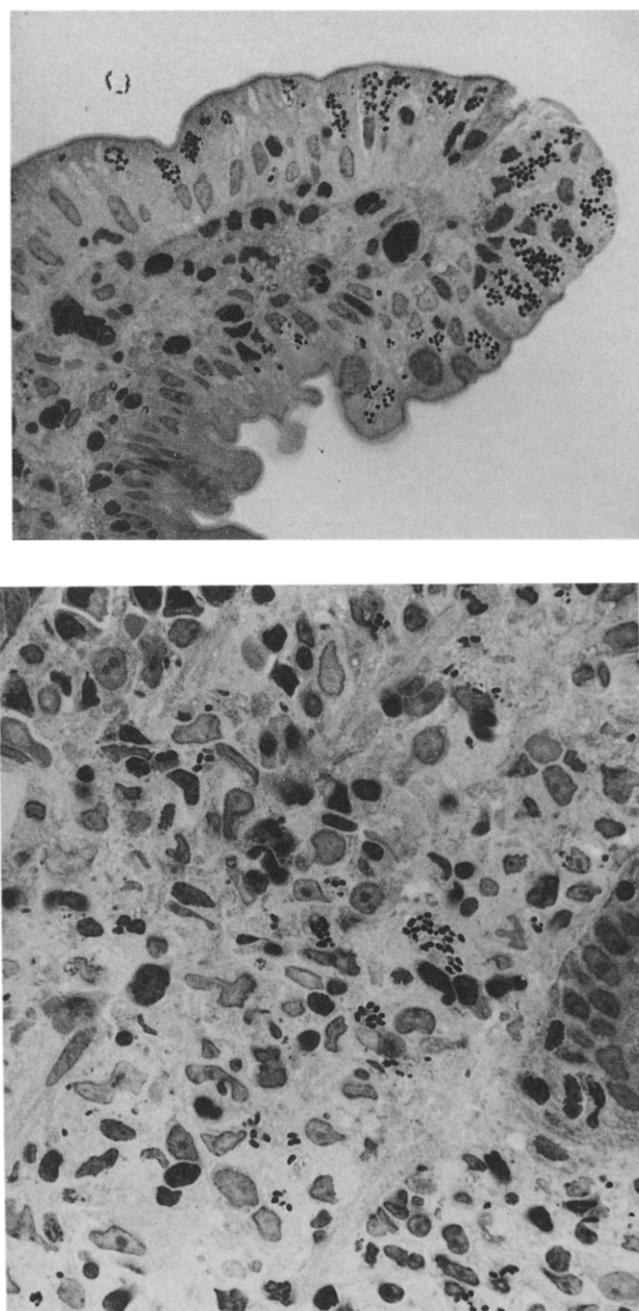


FIGURE 1. Light micrographs. (Top) Densely-staining spores are concentrated in the cytoplasm of enterocytes at the tip of a distorted duodenal villus. (Semi-thin plastic section, toluidine blue stain; magnification $\times 40$.) (Bottom) Semi-thin plastic section showing densely-staining elliptical spores within the cytoplasm of lamina propria macrophages. (Methylene blue-azure II, basic fuchsin stain; magnification $\times 686$.)

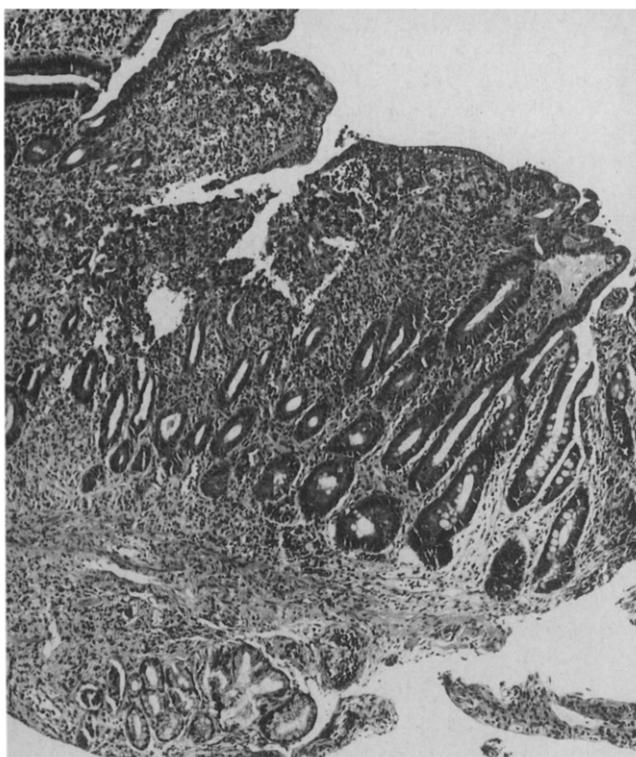


FIGURE 2. Light micrographs. (Top) Jejunal biopsy showing: severe villus atrophy and blunting, an epithelialized erosion, a fragmented epithelium, and cells filling the lamina propria and submucosa. (Hematoxylin-eosin stain; magnification $\times 69$.) (Bottom) Duodenal biopsy showing severely atrophic and disrupted mucosa with a central defect that at higher magnification was rich in neutrophils. Note Brunner's glands in lower field. (Hematoxylin-eosin stain; magnification $\times 69$.)

the submucosa (Fig 1, bottom). Neutrophils contained organisms, but not the plasma cells or eosinophils that were scattered among the macrophages. In the duodenal biopsy specimen, the lamina propria was rich in plasma cells with only scattered macrophages containing dense bodies. The duodenum contained small foci of lamina propria necrosis that appeared to contain free organisms. Only an occasional infected enterocyte was seen in either of the colorectal biopsy specimens. Generally, the bodies stained slightly better with the combined

methylene blue-azure II, basic fuchsin stain than with toluidine blue.

Light Microscopy of Paraffin Sections

Since several biopsy specimens were embedded together in a single paraffin block, a better appreciation of the pathologic changes and the pattern of infection could be obtained than from the single biopsy specimen embedded in plastic. The bodies stained a prominent

deep blue with both the Brown-Brenn and Brown-Hopps gram stains, which were far superior to all other stains tested, including hematoxylin-eosin, periodic acid-Schiff, Grocott methenamine silver, Giemsa, Wolbach Giemsa Rosin, and Weigert hematoxylin, all of which stained variably, if at all. The bodies were birefringent in paraffin, but not in plastic sections.⁴¹

The three small-bowel biopsies showed striking villus atrophy and blunting, as well as flattening of individual enterocytes (Fig 2). While the degree of epithelial cell involvement was more striking in the duodenal biopsy specimen (Fig 1, top) than in the two jejunal biopsy specimens (essentially identical), the reverse was true for the degree of macrophage involvement (Fig 2, top). Enterocytes were infected at all levels of the distorted duodenal crypts, a feature that was not seen in either of the jejunal biopsy specimens. Foci of mucosal necrosis, containing neutrophils, were more prominent in the duodenal biopsy. The organisms concentrated in enterocytes of the tips of the villi, which also displayed the greatest histopathology, eg, lipid vacuolization, pyknosis, focal necrosis, and cell sloughing. The degree of damage appeared to parallel the intensity of infection.

Gram stain of sections from the first and last colorectal biopsy specimens revealed organisms in scattered enterocytes and macrophages, but not within the pseudomembranes. The colorectal biopsy specimens showed focal mucosal necrosis and edema.

Transmission Electron Microscopy

The organism was identified as a member of the phylum Microspora by the presence of the characteristic injection apparatus in the spores (Fig 3). As many as 50 spores could be seen in the supranuclear cytoplasm in a single plane of cell section (Fig 4). The earliest stage of development was a meront containing a single nucleus, surrounded by abundant ribosomes and profiles of rough endoplasmic reticulum, and in intimate contact with the host cytoplasm. Next, several meronts were seen arranged around the edge (petal-like) of the enlarging cluster, while the densely staining spores tended to be more centrally located. The vacuole often had a scalloped edge formed by invaginations of the surrounding cell cytoplasm. The vacuoles were further divided into compartments, giving them a honeycomb or septated appearance, each chamber containing a developing uninucleate spore. Binucleate meronts were seen; however, they appeared to be in a state of division. Uninucleate meronts and sporonts measured approximately $3.5 \times 1.5 \mu\text{m}$, while the more regular elliptical spores measured approximately $1.2 \times 2.2 \mu\text{m}$. The spores had an endospore of approximately 100 nm in thickness and six coils of their polar tubule or filament (Fig 3, top).

Single meronts up to the multichambered vacuoles were also seen within the jejunal macrophages, which also contained degenerating organisms that stained progressively lighter and appeared collapsed (Fig 5, top left and top right). At times, spores could be identified within membrane-bound structures consistent with lysosomes (Fig 5, bottom). Only degenerating organisms were seen in neutrophils. The foci of duodenal necrosis

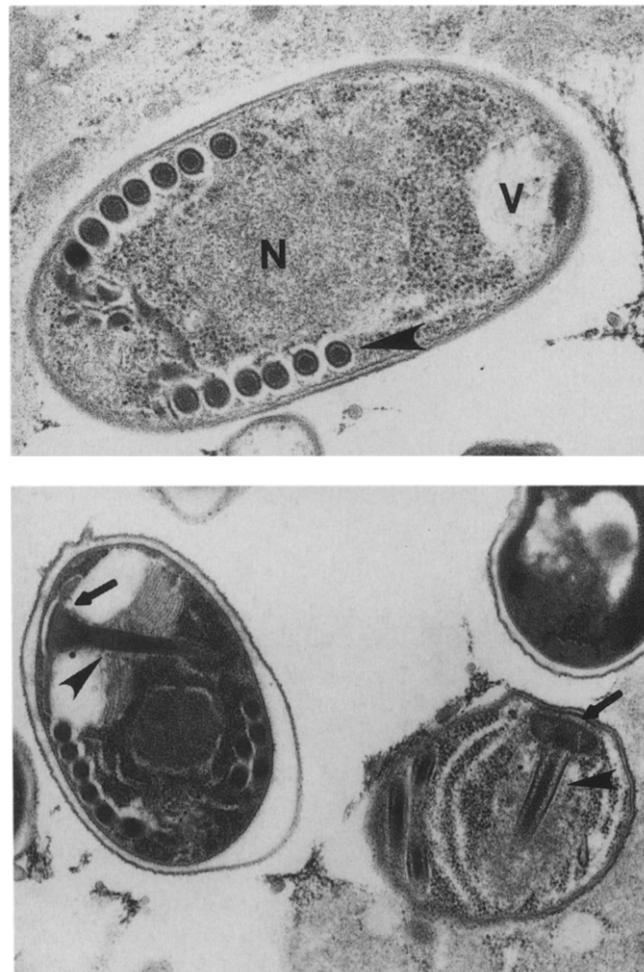


FIGURE 3. Transmission electron micrograph. (Top) A typical sporont with an injection apparatus having six coils (arrow), one nucleus (N), and a small vacuole (V). (Magnification $\times 34,650$.) (Bottom) The anchoring plates (arrows) and straight (arrowheads) and coiled portions of the injection apparatus can be seen in two spores. (Magnification $\times 26,235$.)

observed by LM contained developing and degenerating spores intermixed with cellular debris. No other potential pathogens were identified in the biopsy material by TEM or LM.

DISCUSSION

To our knowledge, the ultrastructural features of this microsporidian have not been reported previously^{28-30,32,39} and are those of a second species of Microspora that appears to cause enteritis and diarrhea in immunocompromised HIV-infected patients. The first species, *E. bieneusi*, represents the first member of a new genus of microsporidian, *Enterocytozoon*.^{12,40} *E. bieneusi* is uniquely characterized by its development, up through late sporogony, as large multinucleate plasmodia that are in intimate contact with the cell cytoplasm and never associated with a vacuole.^{12,16,40} Development of polar filaments or tubules from disks derived from clear, cleft-like structures is also a unique feature. The spores of *E.*



FIGURE 4. Transmission electron micrograph. Separated vacuoles containing developing spores are especially concentrated in the apical cytoplasm of these duodenal enterocytes. Note the intraepithelial lymphocyte. (Magnification $\times 3,330$.)

bieneusi are relatively small for microsporidia (approximately $1 \times 1.5 \mu\text{m}$), usually having six turns (occasionally up to eight) of their polar tubule, and are always found in direct contact with the host cytoplasm. To date, *E. bieneusi* has only been observed within enterocytes, especially those at the tips of the villi.¹⁶ Organisms have not been seen in epithelial cells of the crypts or in non-epithelial cells in the lamina propria. Involvement (minimal) of colonic epithelium was noted in only two of the published cases,¹⁴ and has never been observed by these investigators.

In contrast, development of this species takes place within a vacuole of unique appearance, resembling a honeycomb of apparent parasitic origin. Compared with *E. bieneusi*, the microsporidian described here lacks large plasmodial stages containing polar disks and clear clefts and has a larger spore ($2.2 \times 1.2 \mu\text{m}$). In contrast to *E. bieneusi*, which has only been observed infecting enterocytes, this microsporidian appears to have the ability to infect and develop within macrophages. Whereas the jejunal biopsy specimens had considerably greater macrophage involvement than the distal duodenal biopsy specimen (obtained 14 months later), the latter displayed more mucosal involvement as well as infection of crypt epithelium and foci of parasites associated with lamina propria necrosis, features not seen in either of the jejunal biopsy specimens. Although this could be related to the difference in biopsy sites, in fact, the distal duodenum and proximal jejunum are adjacent to one another. More likely, it relates to the patient's clinical course and the evolution of the infection in the interval. The relatively small amount of large-bowel infection could represent "spillover" from the primary site of infection, the small bowel. Finally, the overall intensity of infection and degree of histopathology in this pa-

tient's specimens were greater than those observed in the 44 cases of *E. bieneusi* microsporidiosis studied in our laboratory.^{16,40}

The exact taxonomic classification of this microsporidian is unclear. Its morphologic features suggest the genus *Encephalitozoon*,²⁷⁻³² eg, *E. cuniculi* or the newly reported human species *E. hellem*.^{35,37,38} However, development of this microsporidian occurs within a septated parasitophorous vacuole, whereas the vacuole of *E. cuniculi*^{27,30} and *E. hellem*^{35,37,38} consists of a single membrane envelope surrounding the entire cluster of organisms, with no internal chambers. Both *E. cuniculi*³⁰ and this microsporidian have the capacity to infect macrophages. *E. cuniculi* may enter the host via the intestinal tract; however, infection of bowel mucosa and associated diarrhea with malabsorption have never been demonstrated in association with this parasite.^{29,30} After infection, *E. cuniculi* apparently homes to different sites within the host, eg, the peritoneal cavity, liver, central nervous system, and kidney. There is no evidence for this migration occurring with *E. bieneusi*. Whether the abnormal liver function tests, the peritonitis, or any other symptoms relate to disseminated microsporidiosis in our patient could not be determined because of the lack of an autopsy.

There are now two species of microsporidia that have been associated with diarrhea in immunocompromised AIDS patients. The increasing awareness and familiarity with the diagnostic features of this phylum of parasites can be expected to lead to an increase in incidence of diagnosis in the bowel and, perhaps, in other sites. The actual source of the microsporidian reported here is unknown. Did it derive from the patient's Native American background, his travels, or his homosexual contacts? It is unclear when our patient acquired the

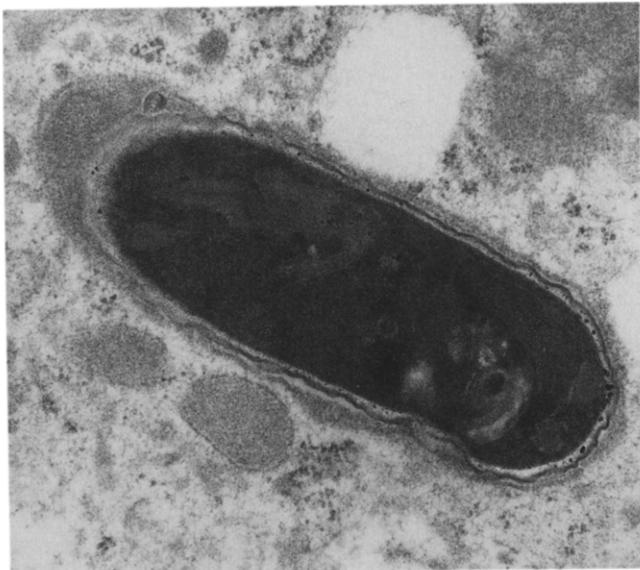
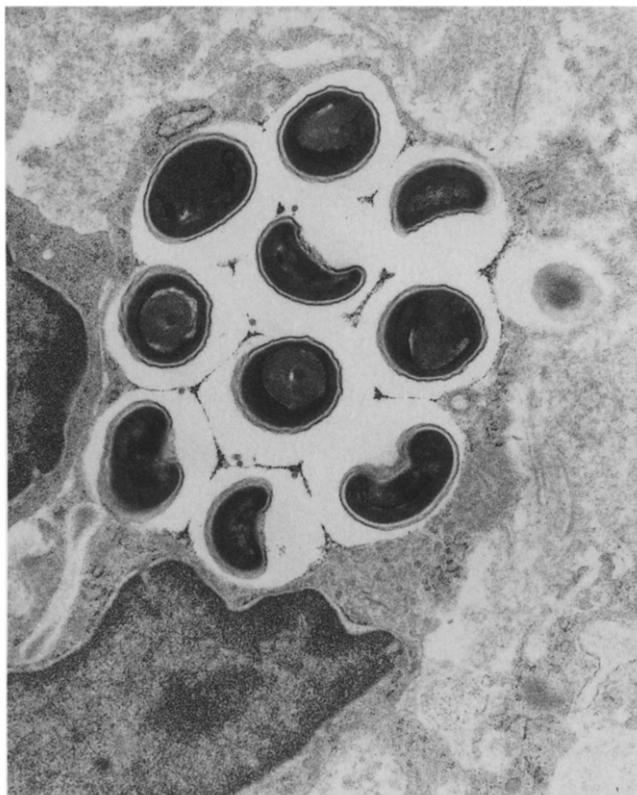


FIGURE 5. Transmission electron micrographs. (Top left) A macrophage in the lamina propria of the jejunum contains a multichambered vacuole filled with spores. (Magnification $\times 14,000$.) (Top right) As the spores in this macrophage degenerate they lose their electron density. (Magnification $\times 12,000$.) (Bottom) An apparently degenerating spore is present in an apparent lysosome within a macrophage. (Magnification $\times 33,600$.)

infection. Alternatively, it could be a reactivation of a parasite that only became a pathogen as a result of the immunocompromised state, a common scenario in AIDS.

NOTE ADDED IN PROOF

Since submission of this manuscript, three more cases have been diagnosed in our laboratory. In addition to intestinal biopsy specimens, organisms were also

identified in urine, duodenal fluid, and stool specimens from two of the patients.

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