

# Histological diagnosis of intestinal microsporidiosis in patients with AIDS

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## Abstract

Fifty nine patients seropositive for human immunodeficiency virus (HIV) and diarrhoea and 20 with weight loss were investigated for microsporidiosis using light and electron microscopical examination of duodenal and jejunal biopsy specimens. Eight cases of microsporidiosis were found, in five of whom it was the sole pathogen. In all eight cases the organism was identified at light microscopy without prior knowledge of the electron microscopical findings. All stages of the life cycle are best seen in resin sections cut at 1  $\mu\text{m}$  and stained with Giemsa, but spores could easily be identified in paraffin sections cut at 5  $\mu\text{m}$  and stained with haematoxylin and eosin. In all cases the parasite was identified both in duodenal pinch and jejunal "Crosby" capsule biopsy specimens. All cases of microsporidiosis occurred in patients with diarrhoea. Both electron and light microscopical examination suggested that the pathogenic mechanism involves the shedding of infected enterocytes containing large numbers of spores.

It is suggested that the optimal way to diagnose microsporidiosis is by light microscopical examination of duodenal pinch biopsy specimens.

Microsporidial infection may cause diarrhoea in up to 30% of patients positive for human immunodeficiency virus (HIV) in whom no other pathogen is found.<sup>1</sup> The organism was first discovered in the small intestine of a Haitian patient in 1980,<sup>2</sup> and infection has been reported ever since with increasing prevalence in patients from Europe,<sup>3</sup> North America<sup>1</sup> and Africa.<sup>4</sup> Four genera of microsporidia have been identified by electron microscopy in man,<sup>5</sup> three as rare isolated infections but occurring in many animal hosts. The fourth genus, *Enterocytozoon*, has only one recognised species, *E. bienersi*, and has only been found in HIV positive patients with malabsorption and diarrhoea.<sup>6</sup>

At present the diagnosis of microsporidial infection depends on the examination of sections, imprints, or smears of small bowel biopsy specimens as oocysts have only recently been shown in human faeces<sup>7</sup> and no serological tests have yet been developed. Although the organisms can be seen with the light microscope using fresh impression

smears of jejunal biopsy specimens stained with Giemsa,<sup>8</sup> this technique is not routinely performed and the recommended method of detection has been by electron microscopy.<sup>1-11</sup> This is time-consuming, expensive, restricted in its availability and inappropriate for large numbers of samples.

The aim of this investigation was to determine the prevalence of microsporidial infection in groups of HIV antibody positive patients and to assess whether such infection can be diagnosed reliably at light microscopy. Both jejunal biopsy specimens obtained by a Watson capsule and duodenal pinch biopsy specimens were examined as the latter would be more convenient to use for routine diagnosis.

## Methods

Group A comprised 59 HIV positive patients with diarrhoea (defined as three or more liquid motions a day for more than a month). Forty five had pathogens other than microsporidia (19 *Cryptosporidium*, eight *Cytomegalovirus* (CMV), five *Salmonella* species, four *Campylobacter* species, three *Mycobacterium avium intracellulare*, six *Giardia lamblia*, two Herpes simplex virus). In 14 patients no other cause of diarrhoea was found despite microbiological analysis of multiple stool samples, duodenal aspiration, and rigid sigmoidoscopy and rectal biopsy. One patient had cytomegalovirus duodenitis, but a rectal biopsy specimen could not be obtained because of a painful anal fissure.

Group B comprised 20 HIV positive patients with weight loss (greater than 10% ideal body weight lost) but no diarrhoea, who were being investigated for causes of malabsorption.

In all the patients the HIV antibody test was confirmed by two methods. All patients had duodenal and jejunal biopsy specimens obtained during the course of fibre optic endoscopy. Spiked, fenestrated biopsy forceps were used to take six randomly sited pinch biopsy specimens from the second part of the duodenum and a Watson capsule was positioned in the distal duodenum endoscopically and then advanced into the jejunum to obtain jejunal biopsy specimens.

Rectal biopsy specimens were obtained in all patients during rigid sigmoidoscopy.

Each jejunal biopsy specimen was bisected. One piece was processed for light and the other for electron microscopical examination. Specimens for light microscopy were fixed for

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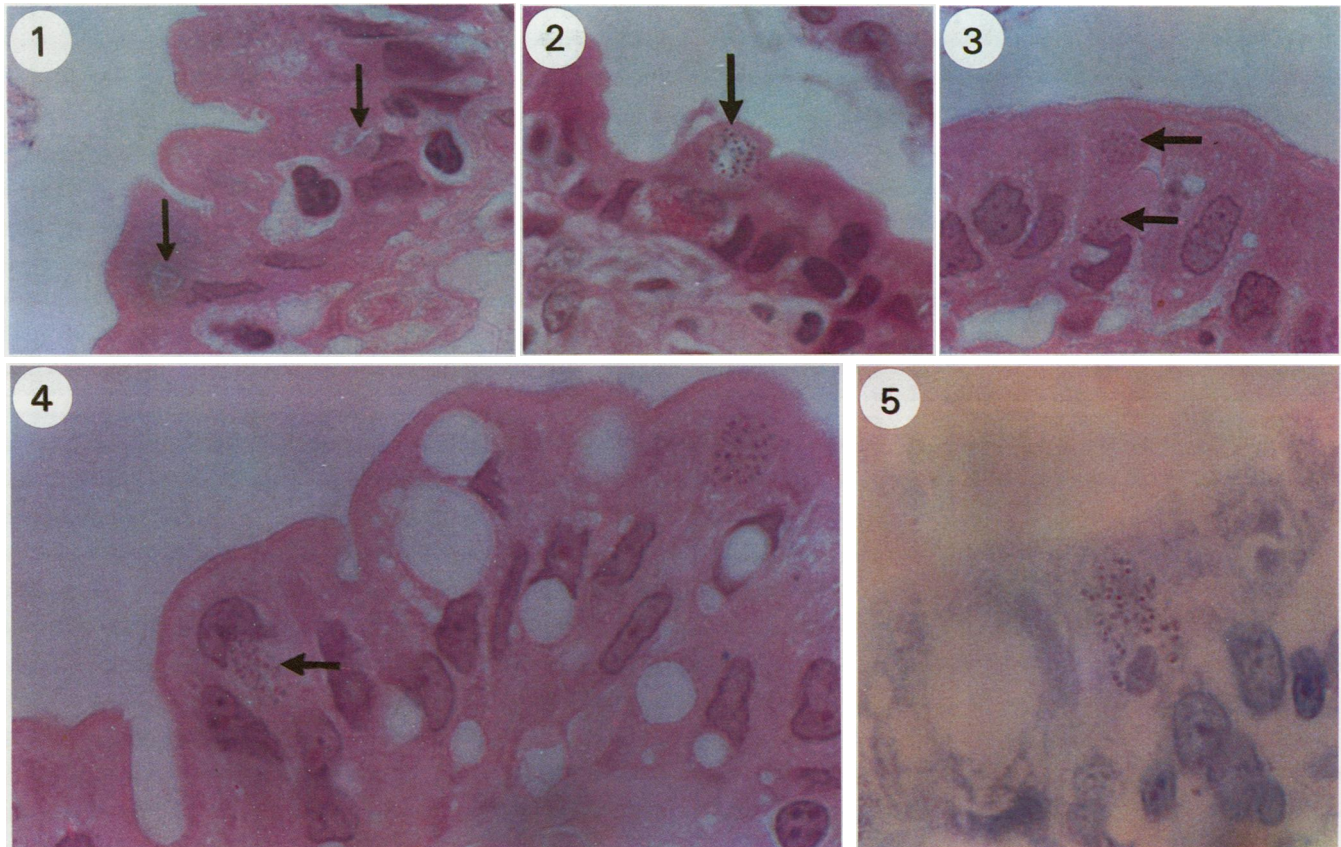


Figure 1 An early meront with a single cleft (small arrow) but no discernible nuclei yet, and another at a later stage containing several clefts (larger arrow) and several nuclei, found in enterocytes of the small intestine (a 1  $\mu$ m resin section stained with haematoxylin and eosin).

Figure 2 A multinucleate sporont, or plasmodium, produced by nuclear divisions in the meront is arrowed (a 3  $\mu$ m paraffin wax section stained with haematoxylin and eosin).

Figure 3 An enterocyte containing two sporonts (arrowed), the lower one indenting the host cell nucleus; section prepared as in fig 1.

Figure 4 A sporont deforming the host cell nucleus (arrowed). Note a further sporont at the upper right region of this figure: section prepared as in fig 1.

Figure 5 Developing spores, which at this stage are freely dispersed throughout the host cell cytoplasm (a 1  $\mu$ m resin section stained with Giemsa).

a minimum time of three hours in 10% formal saline. Specimens were then processed following a routine schedule by dehydrating through graded alcohols, clearing in CitrocLEAR, and embedding in paraffin wax. Sections 2–5  $\mu$ m thick were cut using a rotary microtome, placed on albumin coated slides, and heated on a hotplate for 15 minutes. Slides from each specimen were then stained with haematoxylin and eosin, Giemsa, Gram's stain, periodic acid Schiff and Ziehl Neelsen stain.

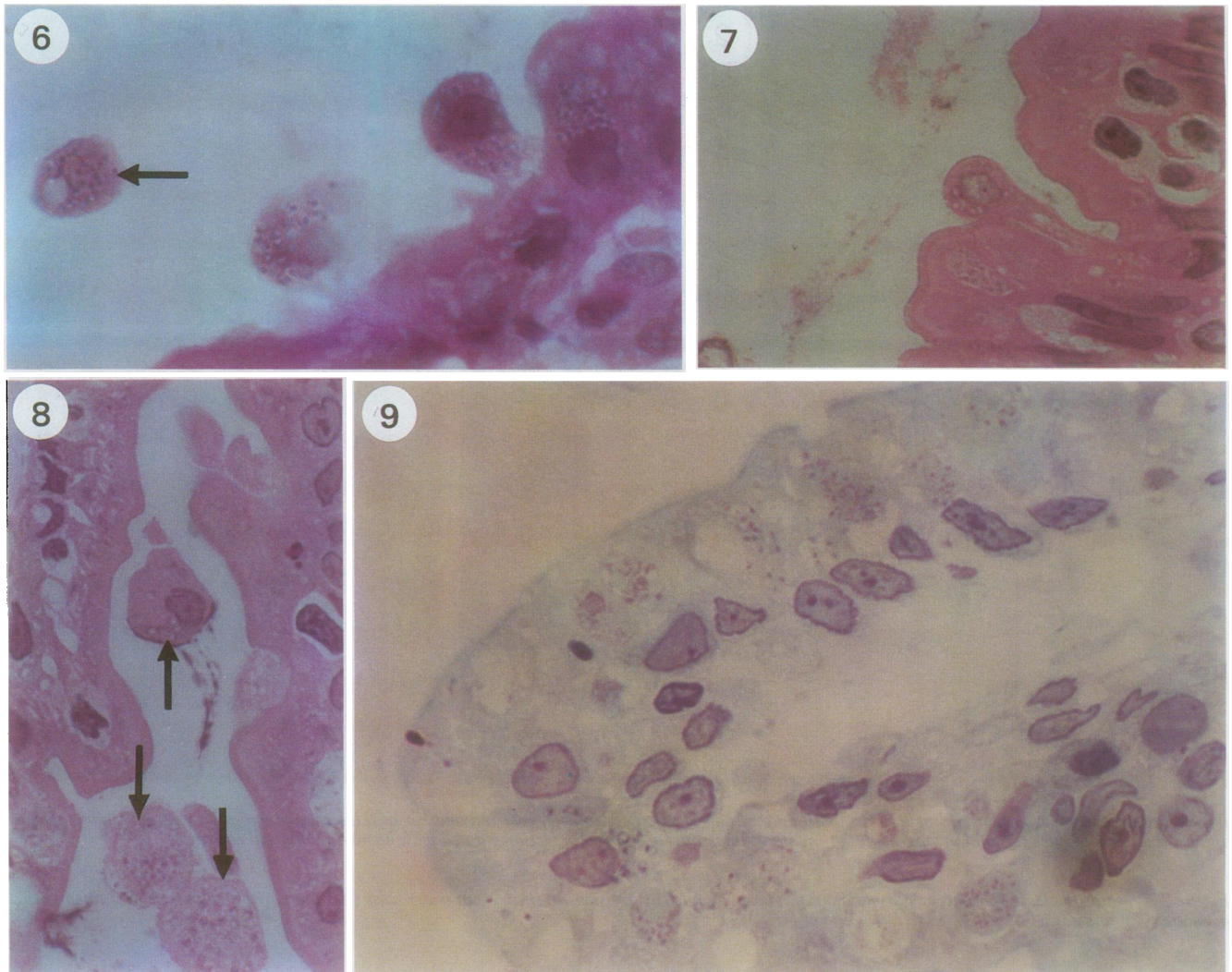
In the eight patients in whom microsporidial infection was found, further biopsy material was processed through graded alcohol and embedded in glycolmethacrylate resin. Sections of 1  $\mu$ m were cut and stained with haematoxylin and eosin and Giemsa stains.

Specimens for electron microscopical examination were fixed in 3% glutaraldehyde in 0.1 molar cacodylate buffer, pH 7.3, post-fixed in 1% osmium tetroxide, dehydrated through graded methanols, impregnated via propylene oxide and embedded in Taab resin.

Sections 70 nm thick were stained with uranyl acetate and lead citrate and examined using a JEOL 100 CX microscope.

## Results

Eight patients with microsporidiosis were identified; in five it was the sole pathogen (one had *Mycobacterium avium intracellulare*; one had *Mycobacterium avium intracellulare* and *Campylobacter*; one had cytomegalovirus (CMV). Microsporidiosis was not diagnosed in any patient who did not have diarrhoea. In all eight cases the organism was identified by light microscopy and electron microscopy. In all the cases the microsporidia were seen independently at light microscopy using standard cut sections stained with haematoxylin and eosin without knowledge of the electron microscopical findings. None of the rectal biopsy specimens examined either by light or electron microscopy had evidence of established microsporidiosis, although in some specimens dead cysts suggestive of attempted infestation were found.



**Figure 6** Spores at various stages of maturation. An infected enterocyte (arrowed) has already broken away into the gut lumen: section prepared as in fig 2.

**Figure 7** A host cell containing infective spores being extruded into the gut lumen (arrowed). The enterocyte microvilli have been lost and the cytoplasm become vacuolated: section prepared as in fig 1.

**Figure 8** Three infected host cells (arrowed) containing mature spores lying free in the lumen of the gut. All these cells have lost their microvilli and have severely vacuolated cytoplasm: section prepared as in fig 1.

**Figure 9** A 1  $\mu\text{m}$  resin section stained with Giemsa showing a very heavy microsporidiosis of enterocytes. (This type of preparation probably gave the clearest results, but proved unnecessary for normal diagnostic purposes.)

#### LIFE CYCLE AT LIGHT MICROSCOPY

The various stages in the life cycle of the microsporidium were identified in the small intestinal biopsy specimens of all eight patients, except the initial infective sporoplasm which is propelled into the cell from the spore.

The next stage, the meront, proved the most difficult to identify with certainty. In the earliest recognised forms it appeared as a weakly basophilic body, about 3–5  $\mu\text{m}$  in diameter, often with a clear line or slash through it (fig 1). In later stages more individual nuclei were contained within the structure and more clear lines became visible (fig 1).

A multinucleate structure, the sporont, or plasmodium was identified, which was presumed to occur as a result of nuclear divi-

sion (merogony) of the meront (figs 2 and 3). This structure was seen to be in direct contact with the host cytoplasm at electron microscopical examination. Individual units were not refractile but were strongly basophilic. The plasmodia varied in size between 3–8  $\mu\text{m}$  in diameter, and more than one parasite could be found in infected host cells, giving rise to up to 40 nuclei. Sporont and meront stages were found apposed to the host cell nucleus in many different orientations, identifying the latter in some instances (figs 3 and 4).

The plasmodia were presumed to enlarge by cell division (sporogony) and to differentiate to produce spores 2–3  $\mu\text{m}$  in diameter (fig 5) which were distributed between the nucleus and the apex of the luminal surface of the cell. Unlike

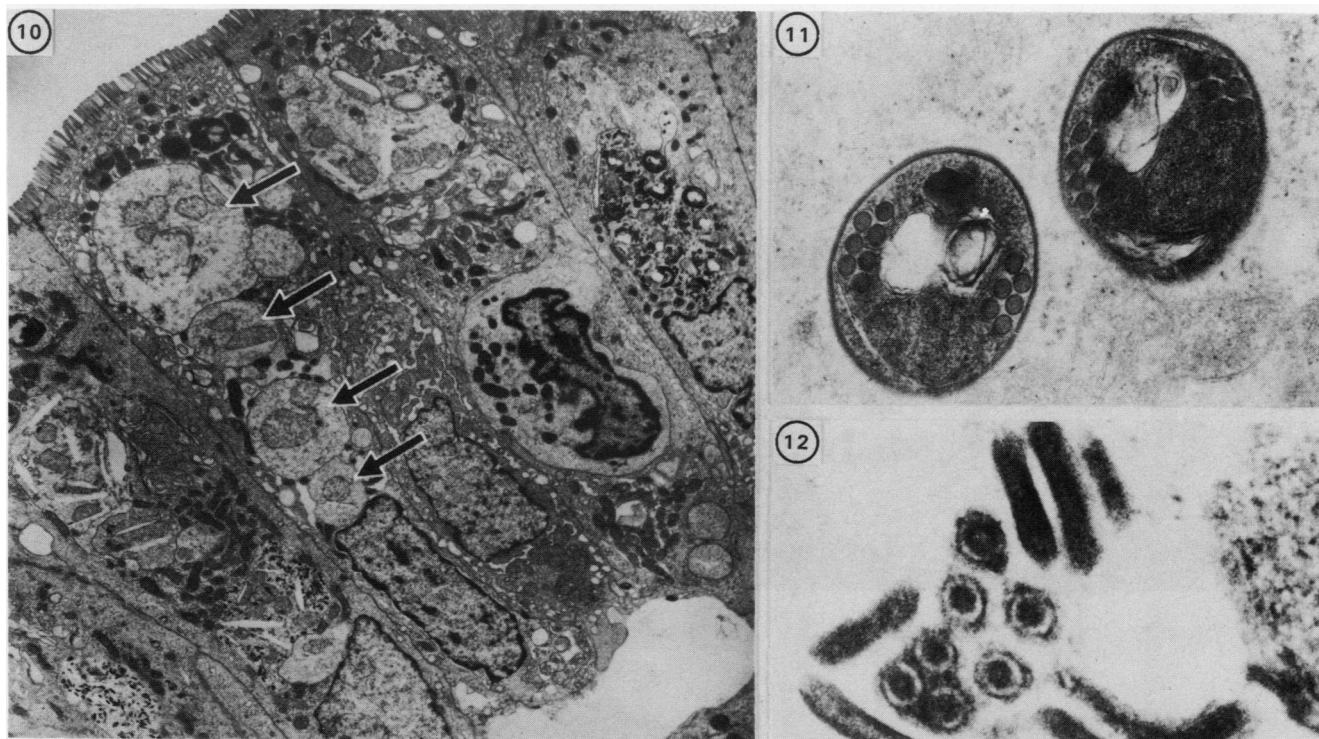


Figure 10 Heavily infected enterocytes in a patient with AIDS. Four parasites are arrowed in the central cell, the adjacent ones containing three and five meronts, respectively. Note the clefts in meronts and indented host cell nuclei.

Figure 11 Spores containing six filament coils, typical of *Enterocytozoon bienewsi*.

Figure 12 Enlarged view of the filaments, aggregated as in fig 11, showing their internal structure.

the plasmodia, the individual units were often dissociated from one another and not present in a tight group (fig 6).

Enterocytes containing mature spores appeared to be degenerate in all eight patients (fig 7), with vacuolated cytoplasm and the disappearance of the microvilli. The cells containing mature spores appeared to be actively extruded from the mucosa (fig 8). This process did not resemble normal cell replacement and adjacent uninfected cells were normal in appearance.

#### COMPARISON OF DIFFERENT STAINING METHODS AND DEPTH OF SECTIONS

Spores were the most readily identified forms of microsporidia. When the condenser on the microscope was lowered or removed, their refractile nature became more apparent making identification much easier. The spores could readily be identified on paraffin wax sections stained with haematoxylin and eosin, even in sections cut at 5  $\mu\text{m}$ . The sporont and meront stages could be identified in sections cut at 3  $\mu\text{m}$  or less, although all stages of the life cycle were best seen in resin sections cut at 1  $\mu\text{m}$ . The optimal stain for light microscopy was haematoxylin and eosin for wax embedded (fig 2) and Giemsa (fig 9) for resin embedded material. These stains yielded a clear contrast between parasite and background, allowing diagnosis at lower magnification, which was particularly striking using Giemsa. In all cases the parasite was identified on both duodenal pinch biopsy specimens and "Crosby" capsule samples.

#### ELECTRON MICROSCOPY

All stages other than the sporoplast were readily identified on electron microscopy. Infection in each case was due to *E. bienewsi* because there were six coils of the polar filament of the spores present (figs 11 and 12) and there was no parasitophorous vacuole. The early meront stages (fig 10) were easily identified because of their pale appearance within the darker staining host cytoplasm and the presence within them, even when mononucleate, of transparent clefts which seemed to become more numerous as development proceeded. The polar filaments were first seen as dark osmiophilic aggregations from which one individual filament became allocated later as it became formed, still lying within the intact parasite plasmalemma—that is, formation by endodyogeny (fig 13). The outer walls of these spores stained intensely (fig 14) and were found to be the easiest features to identify at electron microscopy when scanning the infected biopsy specimens at low magnification.

There was evidence of progressive damage to the microvilli of the infected cells (fig 15) and the mature spores seemed to be liberated into the gut lumen following the breakdown and extrusion of the latter (figs 14–17).

#### Discussion

This study seems to show that microsporidiosis is more common in selected groups of patients in the United Kingdom than in the previously published unselected series where infestation was only found in one of more than 100 HIV antibody positive patients with diarrhoea.<sup>12</sup>

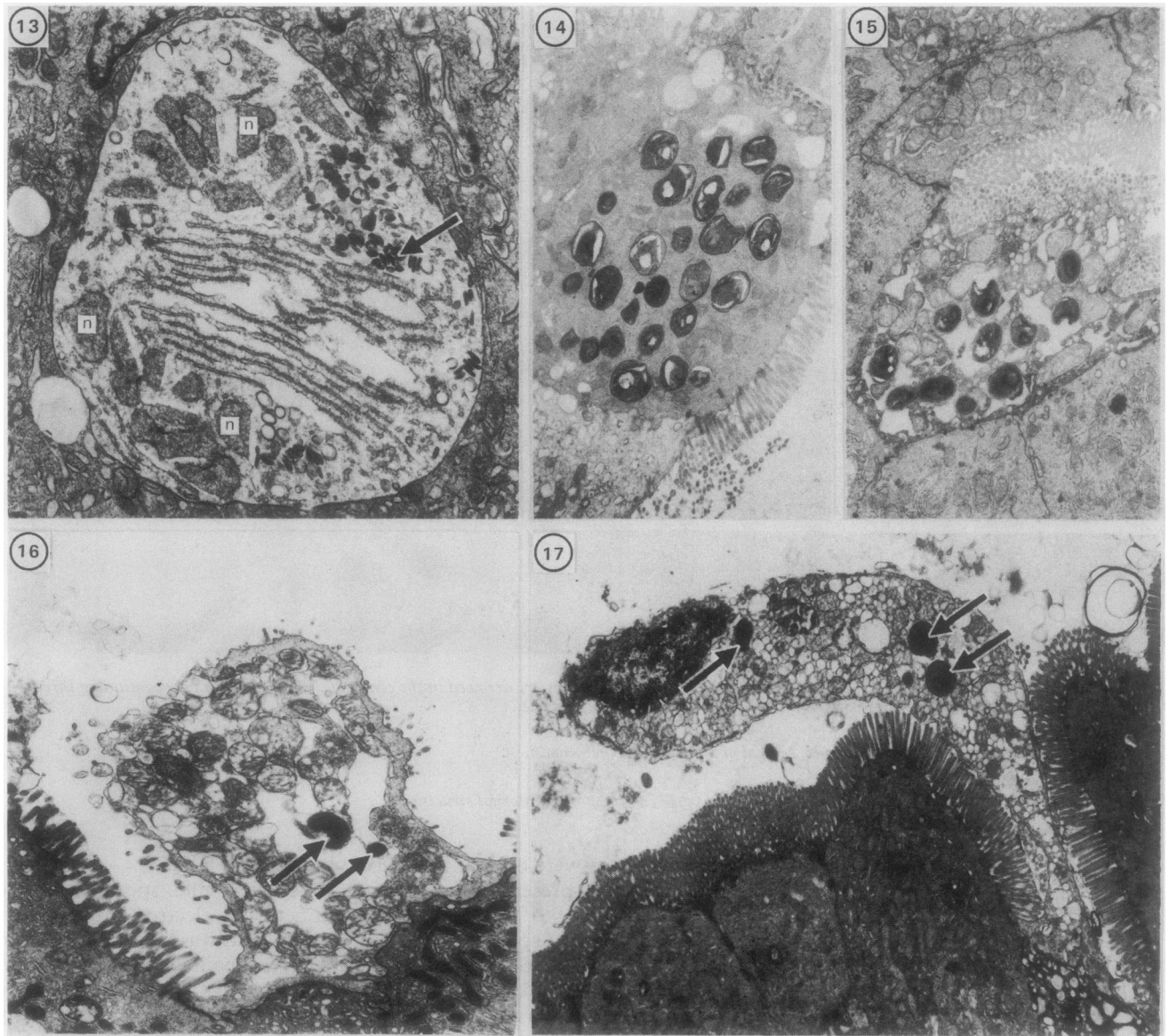


Figure 13 A meront containing at least 16 nuclei (three marked "n") and extensive central endoplasmic reticulum. The arrow marks a group of, as yet, unallocated filaments awaiting their incorporation individually into the developing spores.

Figure 14 Profiles of some 30 spores no longer surrounded by any sporont plasmalemma. Once these latter have disintegrated it is impossible to tell, as here, whether one or more sporont gave rise to these spores. This host cell still appears viable with an intact brush border.

Figure 15 Parasite spores lying within a vacuolated and dying enterocyte before the process of its extrusion.

Figure 16 Profiles of two spores (arrowed) within a dead enterocyte which is being extruded into the gut lumen, but whose plasmalemma is still adherent to those of the adjacent cells. Note disappearance of any proper brush border.

Figure 17 The final stage in the extrusion process of an infected enterocyte (two spores arrowed). Note the healthy condition of the adjacent gut cells with their normal microvilli.

The incidence in the present study, where five of 15 patients in whom no other pathogen had been found as a cause of diarrhoea had evidence of microsporidiosis, is very similar to a large New York experience reported recently.<sup>1</sup>

Our study suggests that light microscopy is a satisfactory technique for showing the presence of microsporidia. The organism was seen in all patients in whom it could also be identified by electron microscopical examination. This contrasts with the study from New York<sup>1</sup> where only five of 20 cases were initially suspected by light microscopy, and it was suggested that this might have been because of

low numbers of infecting particles or poor tissue preparation. The lower definition and magnification of light microscopy compared with that of electron microscopy, however, should be compensated for by the ability to scan larger areas of tissue, and poor tissue preservation should only be a problem with necrotic tissue or necropsy material. The use of 1  $\mu$ m glycolmethacrylate resin sections stained with Giemsa and examined at light microscopy gave the clearest identification of the various stages of microsporidiosis, but thicker cut paraffin wax sections stained with haematoxylin and eosin clearly showed microsporidial infec-

tion, particularly the spore stage which was basophilic and highly refractile.

It is still only possible to classify the individual species of microsporidia using electron microscopy although the characteristic size, site, and appearance at light microscopy should suggest the diagnosis of *E. bienewisi*. Other intracellular parasites of enterocytes including *Cryptosporidium*, *Isospora*, and *Sarcocystis* species are easily distinguished from microsporidial infection at the light microscopic level.

The light and electron microscopic appearances of the life cycle of this parasite studied in this paper confirm many of the previously reported features. Electron microscopy also clearly showed division of the sporont by endodyogony, which has not been reported before. Both light and electron microscopy showed extrusion of necrotic cells with large numbers of spores contained within them, which is a method of shedding and transmitting this parasite that has not been previously recorded.

Proof that microsporidiosis is responsible for diarrhoea in immunodeficient patients is unlikely to be obtained until a suitable animal host is identified or there is an effective treatment capable of eradicating the protozoan. Microsporidia were only found in our patients with diarrhoea, however, which might be caused by the observed degeneration of infected epithelial cells.

Much remains to be learnt about the clinical characteristics and prognosis of patients with

microsporidiosis. This should become much easier if the organism can readily be identified in light microscopic samples. Duodenal pinch biopsy specimens are particularly easy to perform, and clinically it seems that this procedure is as likely to yield a positive histological diagnosis as larger "Crosby" capsule samples.

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