

## Septata intestinalis N. G., N. Sp., an Intestinal Microsporidian Associated with Chronic Diarrhea and Dissemination in AIDS Patients

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**ABSTRACT.** Intestinal microsporidiosis in patients diagnosed with acquired immunodeficiency syndrome (AIDS) and having chronic diarrhea was first reported in 1985 and the associated microsporidian was named *Enterocytozoon bieneusi*. The intracellular developmental cycle of *E. bieneusi* in enterocytes has been demonstrated and many cases have been reported worldwide. This report presents the life cycle of a second intestinal microsporidian, associated with the same symptoms, in five AIDS patients. This new microsporidian also infects enterocytes but its pathology and morphology differ from that of *E. bieneusi*. It involves lamina propria macrophages, fibroblasts, and endothelial cells and can disseminate to infect other parts of the body, e.g. the kidney and gall bladder. The parasite cycle includes development of rounded uninucleate and elongated bi- or tetranucleate cells without the formation of plasmodial stages. Sporogony is similar to the more typical development of microsporidia with sporoblast morphogenesis occurring after the last cell division. The development of cells within chambers of a septate, honeycomb-like, parasite-secreted fibrillar network and surrounded by a parasitophorous vacuole, however, is unique to this microsporidian, justifying the establishment of a new genus and species, *Septata intestinalis* n. g., n. sp.

**Supplementary key words.** Human infection, life cycle, Microspora, Microsporida, morphology, ultrastructure.

MICROSPORIDIA are obligate intracellular protozoan parasites in the phylum Microspora. They have been reported to infect every major animal group, primarily arthropods and fish [17]. There are tens of genera and hundreds of species. In mammals, *Encephalitozoon cuniculi* has been the principal infecting organism [3, 6]. In humans, four microsporidian genera have been reported, i.e. *Nosema*, *Encephalitozoon*, *Pleistophora*, and *Enterocytozoon* [1-3, 6, 8, 10, 19]. The first three genera were originally reported from animals and later found in humans. *Enterocytozoon*, however, was originally described from a human infection [8] and has only recently been demonstrated in animals [7].

The genus *Enterocytozoon* was created in 1985 for the organism identified infecting enterocytes in small intestine biopsies from AIDS patients with chronic diarrhea and wasting [8, 11]. There have been over 100 similar cases of this parasite, *Enterocytozoon bieneusi*, subsequently reported [1, 3, 9, 13, 16]. The ultrastructural morphology of all transmission electron microscope (TEM)-studied cases apparently have been identical. The patients reported here had similar chronic diarrhea and wasting. Consequently, they were similarly evaluated [15]. Both at the light microscope and TEM levels, the microsporidia in these patients' biopsies were distinct from *E. bieneusi* [5]. The spores of this parasite appeared to be suspended in a honeycomb with one spore in each chamber, instead of being in direct contact with the host cell cytoplasm. This is not typical of any previously described microsporidian genus. Additionally, infected mucosal macrophages were observed, a feature not seen with *E. bieneusi* infection. It was these differences which led to the present study.

### MATERIALS AND METHODS

Small intestine biopsies fixed in 2.5% glutaraldehyde (pH 7.4, phosphate or cacodylate buffer) at the time of endoscopy were available from four of the five patients diagnosed with intestinal microsporidiosis due to this second intestinal species [14-16]. The diagnosis for the fifth patient was made on paraffin sections and confirmed on tissue retrieved from the paraffin block and processed for TEM [14]. Portions of each biopsy were divided into 1-mm pieces, washed with cacodyl-

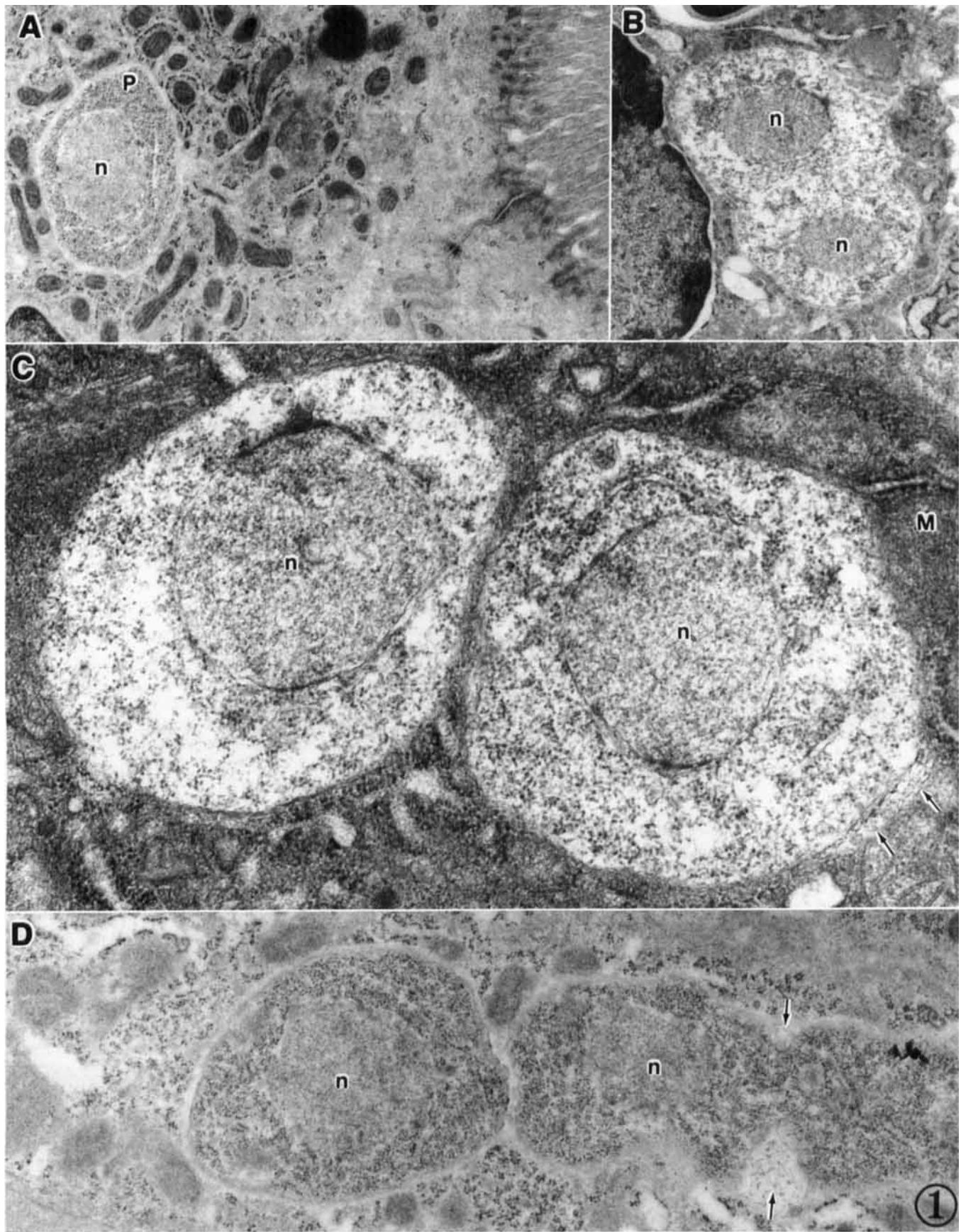
ate buffer, post-fixed in 1% OsO<sub>4</sub>, dehydrated in graded ethanol and propylene oxide, and embedded in Spurr's plastic [16]. Semi-thin plastic sections (1-2 µm) were cut with glass knives from the blocks (6-8) of embedded tissue, then stained with the combined methylene blue-azure II, basic fuchsin stain. Thin sections (60 nm) from at least two paraffin blocks were cut with diamond knives and stained with uranyl acetate and lead citrate for TEM (Zeiss EM 10A).

### RESULTS

**Life cycle and host/parasite interface.** *Proliferative development.* The earliest proliferative stages observed appear to be in direct contact with the enterocyte cell cytoplasm, at times abutting host cell mitochondria or nuclei. These parasites are relatively simple. Each contains a single large round nucleus, short lengths of rough endoplasmic reticulum, and free ribosomes (Fig. 1A, C). During karyokinesis the cells elongate. When nuclear division is complete the products separate so that binucleate cells are observed. Nuclei are not elongated or abutted (i.e. not diplokaryotic) (Fig. 1B). Cytokinesis occurs as binary fission following karyokinesis, resulting in elongated chains of cells (Fig. 1C, D). Sometimes the karyokinetic process is repeated before cytokinesis occurs, resulting in a long ribbon-like cell with four nuclei and cytoplasmic indentations (Fig. 2A). As elongation and cell division continues, multiple organisms are produced, usually as clusters (Fig. 2B), but occasionally as long chains of cells (Fig. 1D). Study of innumerable micrographs of these early developmental stages revealed a very fine meshwork of secreted osmophilic material at the parasite surface (Fig. 1C, D). Scattered electron-dense secretory droplets are first to appear (Fig. 2B, C). These fuse, forming a network of fine fiber-like material in which the developing parasites become embedded. As parasite development proceeds, this fibrillar network becomes more prominent (Fig. 3A, B). Since each organism is apparently surrounded by this material, it seems likely that it is continuously secreted.

**Sporogonic development.** The commencement of sporogony is morphologically characterized by two changes in parasite appearance. The plasmalemma thickens in a progression from small scalloped dense areas (Fig. 3A, B) to a uniformly dense membrane (Fig. 4A, D). Meanwhile, the parasite plasmalemma shrinks away from the fibrillar network, making its presence

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more obvious and chamber-like. At about this time, the presence of a phagosome-like membrane envelope, i.e. the parasitophorous vacuole, becomes evident around the parasite cluster (Fig. 3B).

**Sporont cell division.** Division of the parasite cells continues after the plasmalemmal thickening has been observed (Fig. 4B-D). The longest sporont cells observed contained four nuclei, so this organism is at least tetrasporous (Fig. 4C). However, it is difficult to determine the exact number of cell divisions in sporogony prior to sporoblast formation because binucleated dividing cells with dividing nuclei were observed, indicating further division (Fig. 4D). These binucleated dividing cells were seen more often than the tetranucleate cells (Fig. 4B).

**Sporoblasts.** Single nucleated cells begin development of spore organelles after the last cell division (Fig. 5). The commencement of polar tubule formation is visualized by the presence of a vesicular branching Golgi-like complex, first seen in association with an electron-dense mass (Fig. 5A, B), then associated with electron-dense rings with dense cores, i.e. the polar tubule primordia (Fig. 5C, D). One to seven polar tubule coils have been observed within developing sporoblasts and spores (Fig. 5E-I). The developing polar tubule, also referred to as a polar filament, contains an electron-dense core (Fig. 5C, F) which is present through spore maturation (Fig. 5I, J).

Meanwhile, the sporoblast cell cytoplasm becomes increasingly electron-dense. The polar tubule establishes the anterior/posterior axis of the developing spore; the coiled region is in the posterior, while the attachment complex is in the anterior pole (Fig. 5E-I). The first signs of the posterior vacuole are seen as irregular clusters of smaller vacuoles (Fig. 5H, I). Externally, the fibrillar matrix separates individual sporoblast cells (Fig. 5M).

As sporoblast maturation proceeds, the cells condense, that is, the cytoplasm becomes more dense and a clear zone appears between the outer surface of the thickened sporoblast plasmalemma and the darkening fibrillar matrix. The honeycomb appearance of sporoblasts and spores, isolated in

individual chambers within the fibrillar matrix (Fig. 5M, 6A, B), is thus produced.

Tubular appendages appear within the clusters of organisms during sporogony. These appendages are long and measure up to 1.2  $\mu\text{m}$  in length in a single plane of section. They usually appear to continue beyond the section and are approximately 50 nm in diameter. These tubular appendages appear to occur singularly, apparently originating from the sporont surface (Fig. 5K) and terminating in an enlarged bulb-like structure (Fig. 5L) measuring 120 x 200 nm. Occasionally, a sphere-like bulge appeared along the tube; these were somewhat smaller than the terminal bulbs and measured approximately 120 nm in diameter.

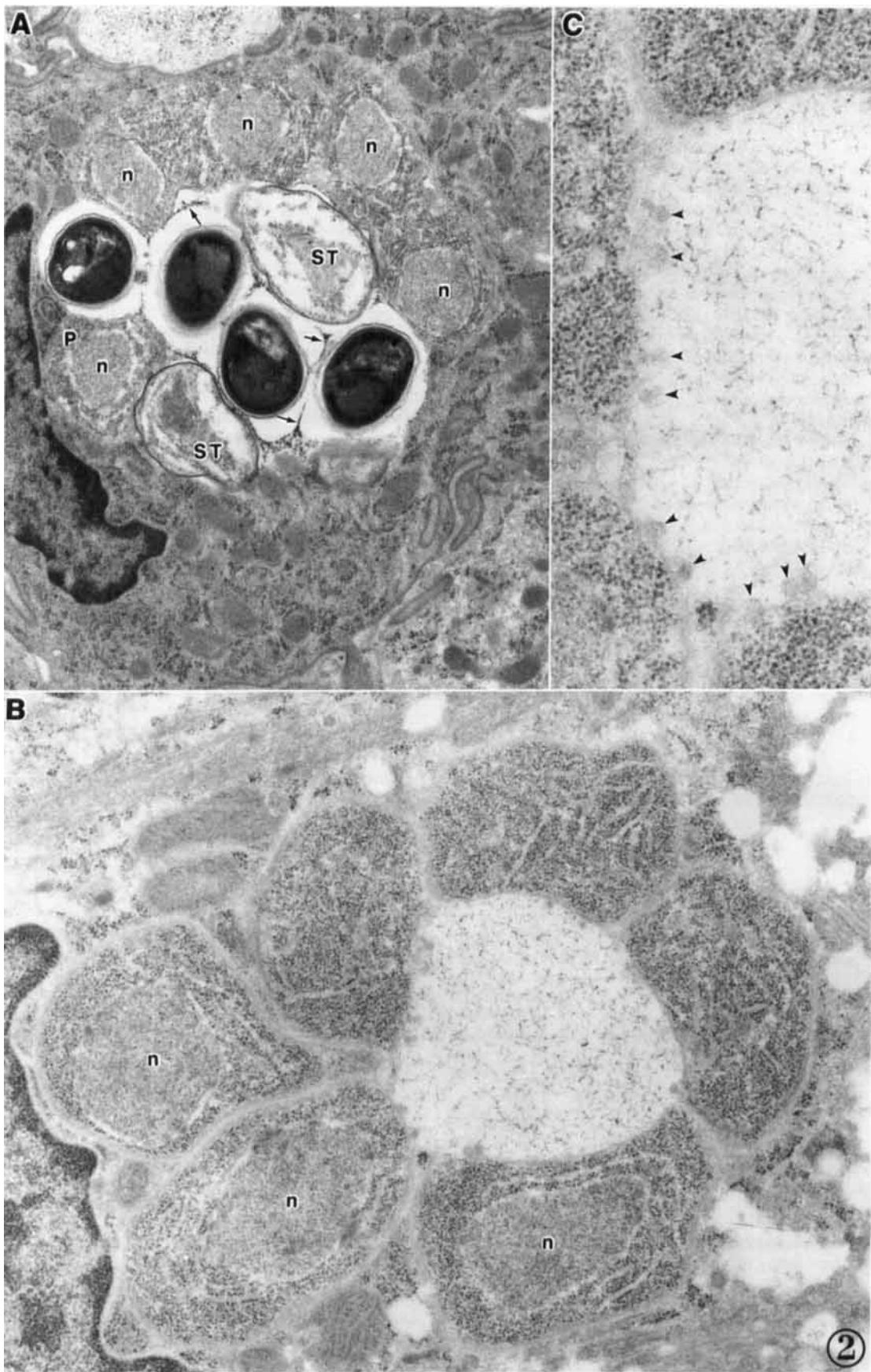
**Spores.** The mature spores measure 2.0  $\mu\text{m}$  x 1.2  $\mu\text{m}$ , contain 4-7, but usually 5, polar tubule coils, and a single nucleus. The anterior polaroplast develops many smooth membrane laminations surrounded by densely packed laminations of rough endoplasmic reticulum and ribosomes that fill the anterior third of the spore (Fig. 6A, B). Within the sporoplasm, ribosomes pack in a regular array, forming several layers around the polar tubule shaft. The dense core of the tubule is still maintained in the anterior, manubroid region (Fig. 6A, B). The electron-lucent endospore coat is the last feature of a mature spore to develop. The spores remain embedded in the fibrillar network of the septate vacuole. Development within parasite clusters appears asynchronous. Cells ranging from early proliferative stages to sporonts and spores are found within a single parasitophorous vacuole (Fig. 6A). The spores are usually more centrally located while the earlier stages tend to be at the periphery of the cluster (Fig. 2A, 4C, 5M).

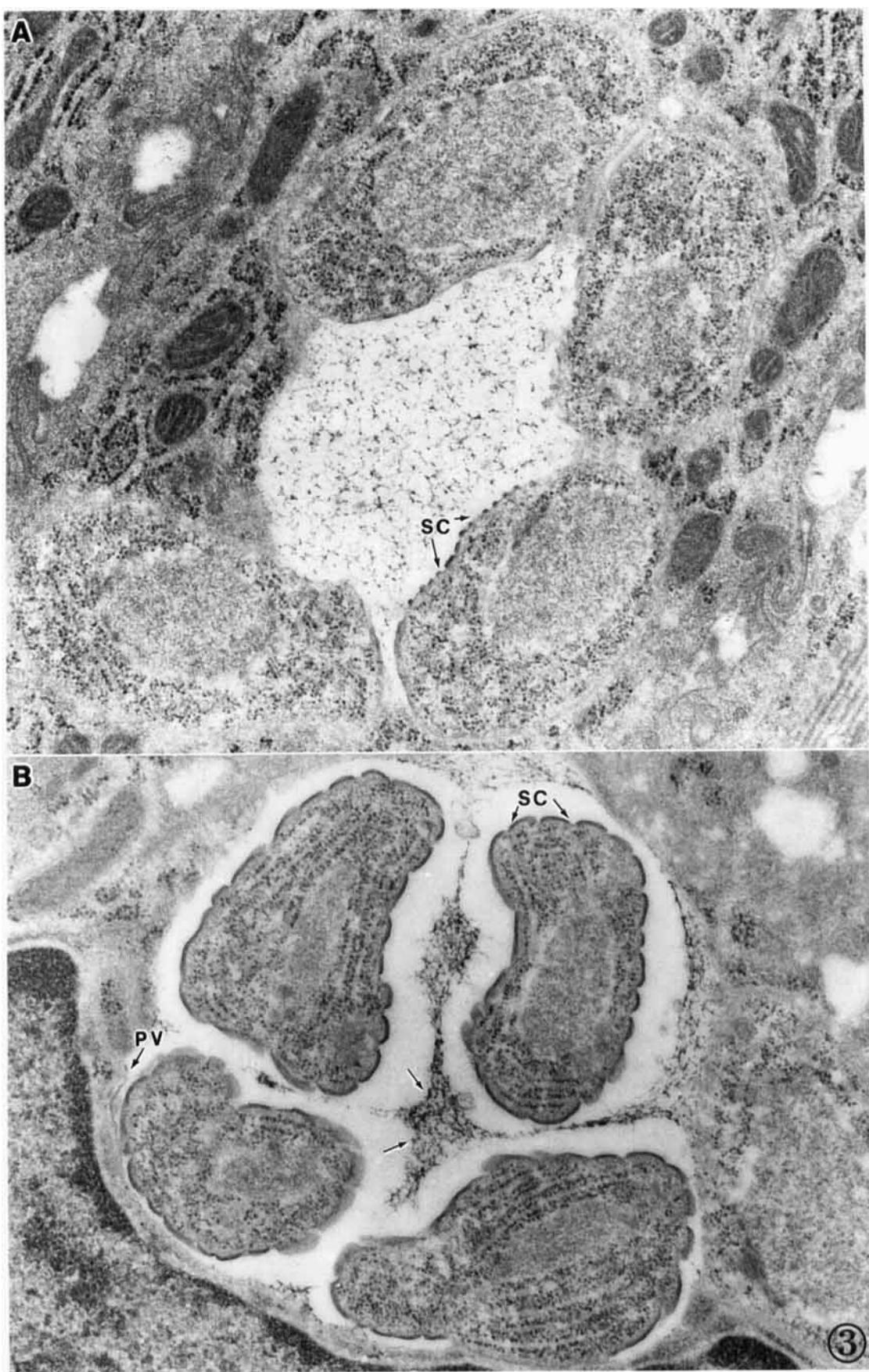
**Location of infection.** The species presented here was originally observed in villus enterocytes and lamina propria macrophages. However, they have occasionally been observed developing in fibroblasts and endothelial cells of the lamina propria (Fig. 7A, B). Additionally, they have been found in the epithelium and macrophages in the gall bladder of one of these patients and free or within tubular and transitional cells in the urine of two patients [14].

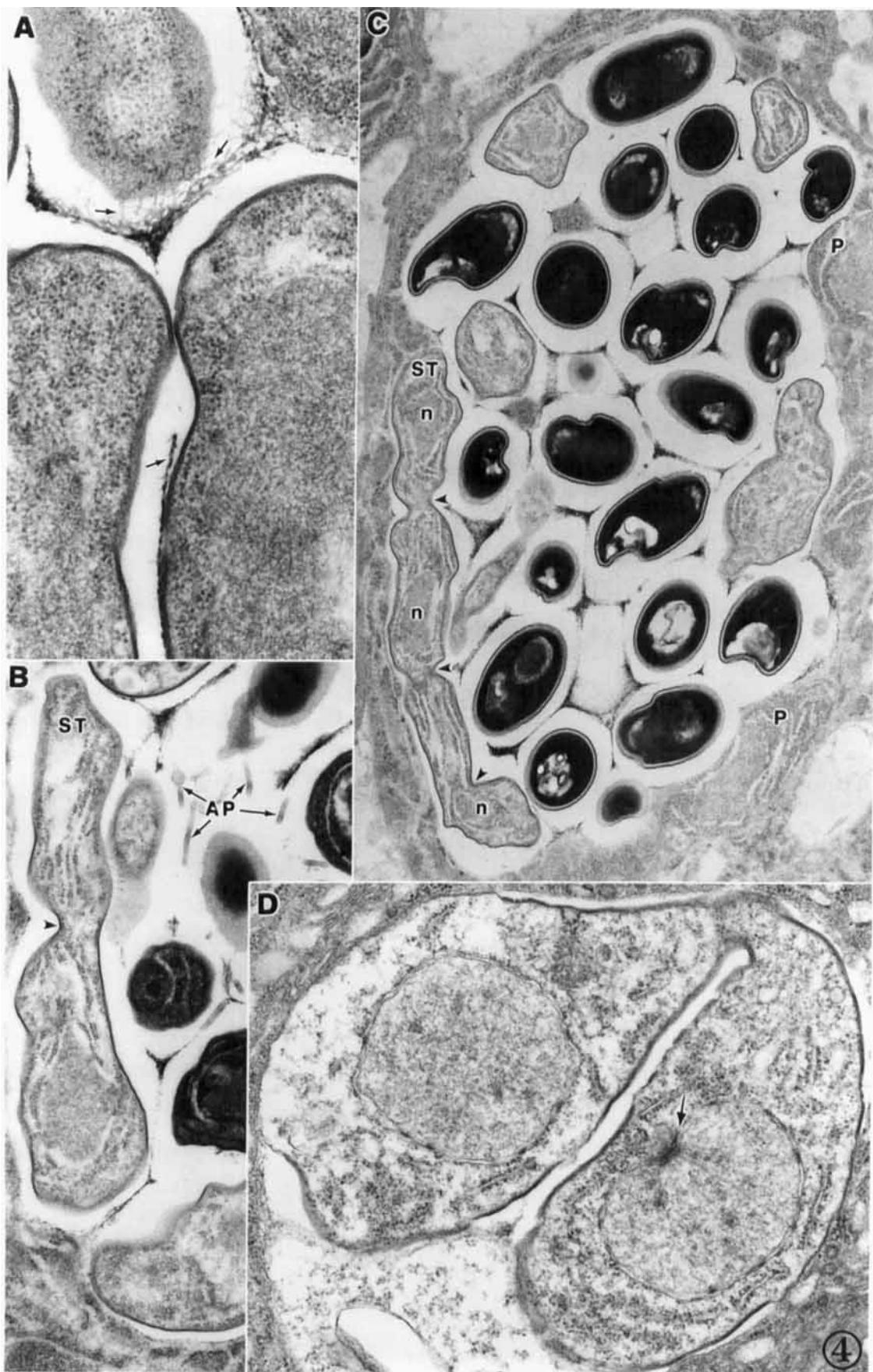
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**Fig. 1.** Early proliferative development of microsporidian in intestinal enterocytes. A. A single round uninucleate (n) early proliferative cell (P) near the apical pole of an enterocyte (microvilli of luminal surface at right). X18,800. B. Elongated binucleate (n), but not diplokaryotic, proliferative cell, closely abutting enterocyte nucleus (left). X12,400. C. Two uninucleate proliferative cells closely abutted (probably recently completed binary fission). They appear to be in direct contact with the host cell cytoplasm, abutting mitochondria (M), except for the lower right corner where some light-staining fine fibril-like material is present (arrows). X43,000. D. Elongated chain of proliferative cells (n, nuclei). The first cell (left) is round, the second is elongated and pinched (arrows) in the process of cytokinesis. Note the "clear spaces" at the cytoplasmic indentations and the fine fibrils of material (arrows). X25,000.

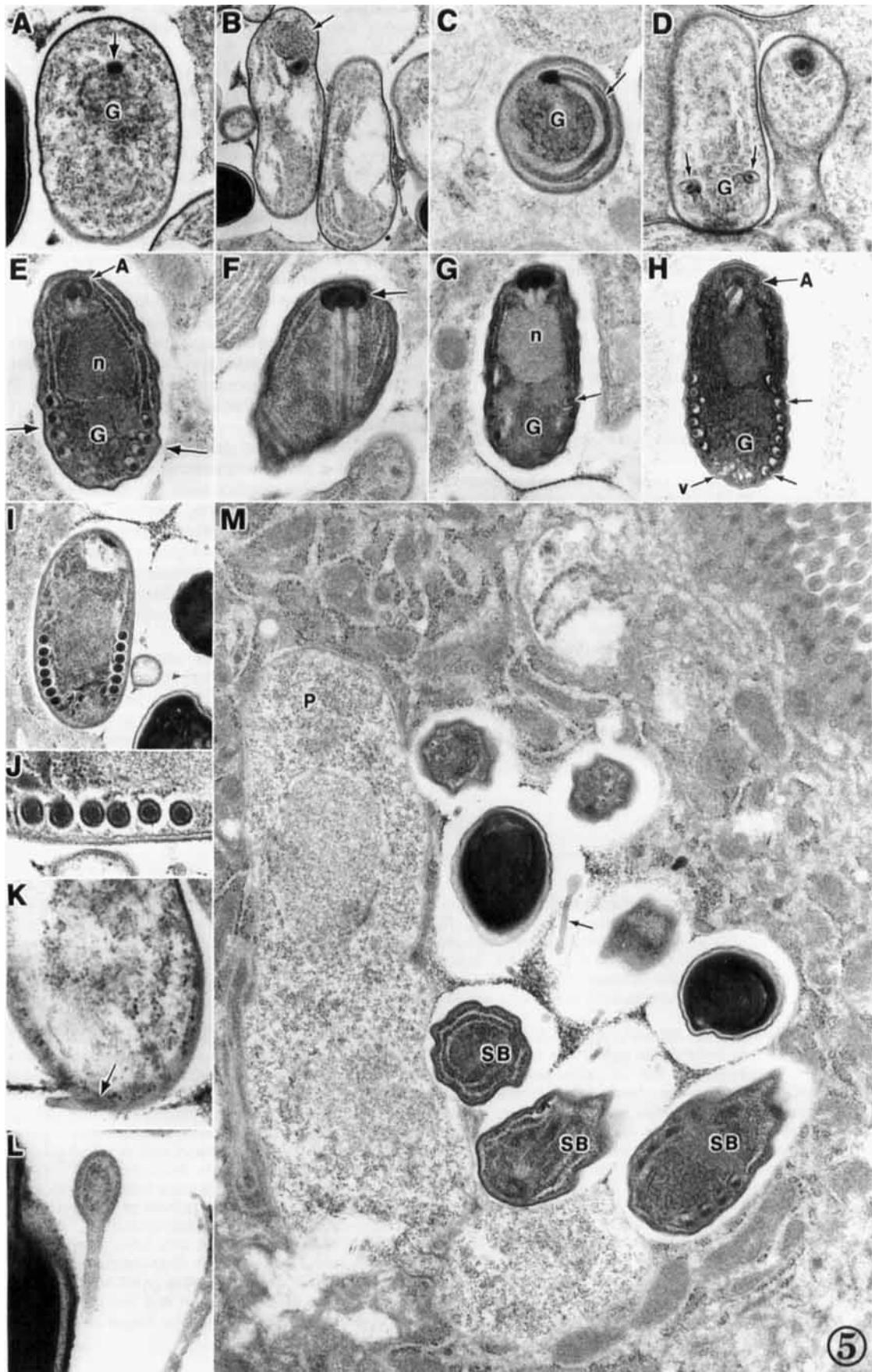
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**Fig. 2.** Multicellular proliferative clusters in enterocytes. A. Asynchronous cluster of parasite cells including a tetranucleate (n, nuclei), elongated ribbon-like proliferative cell, and a uninucleate proliferative cell (P), two sporonts (ST), and four electron-dense spores. Note the fibrous matrix (arrows). X20,300. B. Six proliferative cells, closely abutted as if they just divided from each other, and three with nuclei (n) in plane of section, are arranged in a circular cluster next to the host cell nucleus (lower left). Note the presence of the fibrillar network in the center of the cluster of the parasites. X32,300. C. Enlargement of area in B, showing secretory droplets (arrowheads) all along the parasites' plasmalemmal surface, facing the area filled with the branching fibrillar matrix. X61,000.

**Fig. 3.** Commencement of sporogony and the formation of sporonts. A. The first sign of sporogony is the deposition of secretory material (SC) on the plasmalemmal surface of uninucleate cells, now called sporonts. X43,000. B. As deposition of this material continues, the sporont cell surface takes on a scalloped appearance and pulls away from the fibrillar network (arrows). It is at this stage that the presence of a parasitophorous vacuole membrane becomes evident (PV). X43,000.









## DISCUSSION

The organism described in this paper has morphological and developmental features similar to several established microsporidian genera. However, the combination of features is unique to this organism, clearly setting it apart from the previously established genera and justifying the establishment of a new genus. This microsporidian infection represents a new infection in humans, different from the four genera of microsporidia previously reported in humans.

**Comparison with the four other microsporidian genera found in man.** Both *Nosema* and *Encephalitozoon* proliferate by cellular elongation and cytoplasmic invagination between nuclei. However, *Nosema* has paired abutted nuclei (diplokarya) throughout development and all stages are in direct contact with the host cell cytoplasm. *Encephalitozoon* has single rounded nuclei and develops within a parasitophorous vacuole but lacks both a secreted fibrillar network surrounding the parasite cells and tubular appendages in sporogony. Additionally, *Encephalitozoon* is diporous and this new species is tetrasporous [3].

*Pleistophora* and *Enterocytozoon* both have single nuclei but they form large, rounded multinucleate plasmodia. In *Pleistophora*, the nuclei are round and the parasite secretes material, but it forms a thick electron-dense sporophorous vesicle around the cluster of developing parasite cells [6, 10]. In *Enterocytozoon*, the parasites are in direct contact with the host cell cytoplasm. Their nuclei are greatly elongated during some stages of development and rounded in others, and these parasites contain specialized organelles that provide for a unique sporogony cycle, including sporoblast morphogenesis prior to plasmodial division, resulting in multiple polar tubules within a single cell. Finally, their mature spores are located in direct contact with the enterocyte cell cytoplasm [4, 5]. The microsporidian in this report is not compatible with any of these genera or with any other microsporidian genus found in animals.

**Comparison with the other related genera.** Additional genera with related features include *Unikaryon*, *Glugea*, and *Tetramicra*. *Unikaryon* does not form diplokarya or plasmodial stages during any phase of its development and it reproduces by continuous binary fission of cytoplasm after nuclear division. However, *Unikaryon* develops in direct contact with the host cell cytoplasm and does not secrete a fibrillar network as does the new microsporidian. The tubular appendages, demonstrated in the sporogony of our parasite, have only been reported in one other genus, *Glugea*, which forms three types of tubular appendages during sporogony [18]. The tubular appendages of this new species conform to the "type I" appendages of *Glugea* and will be referred to as such. None of these genera are tetrasporous. The genus *Tetramicra* forms four spores but from a rosette-like dividing stage [6].

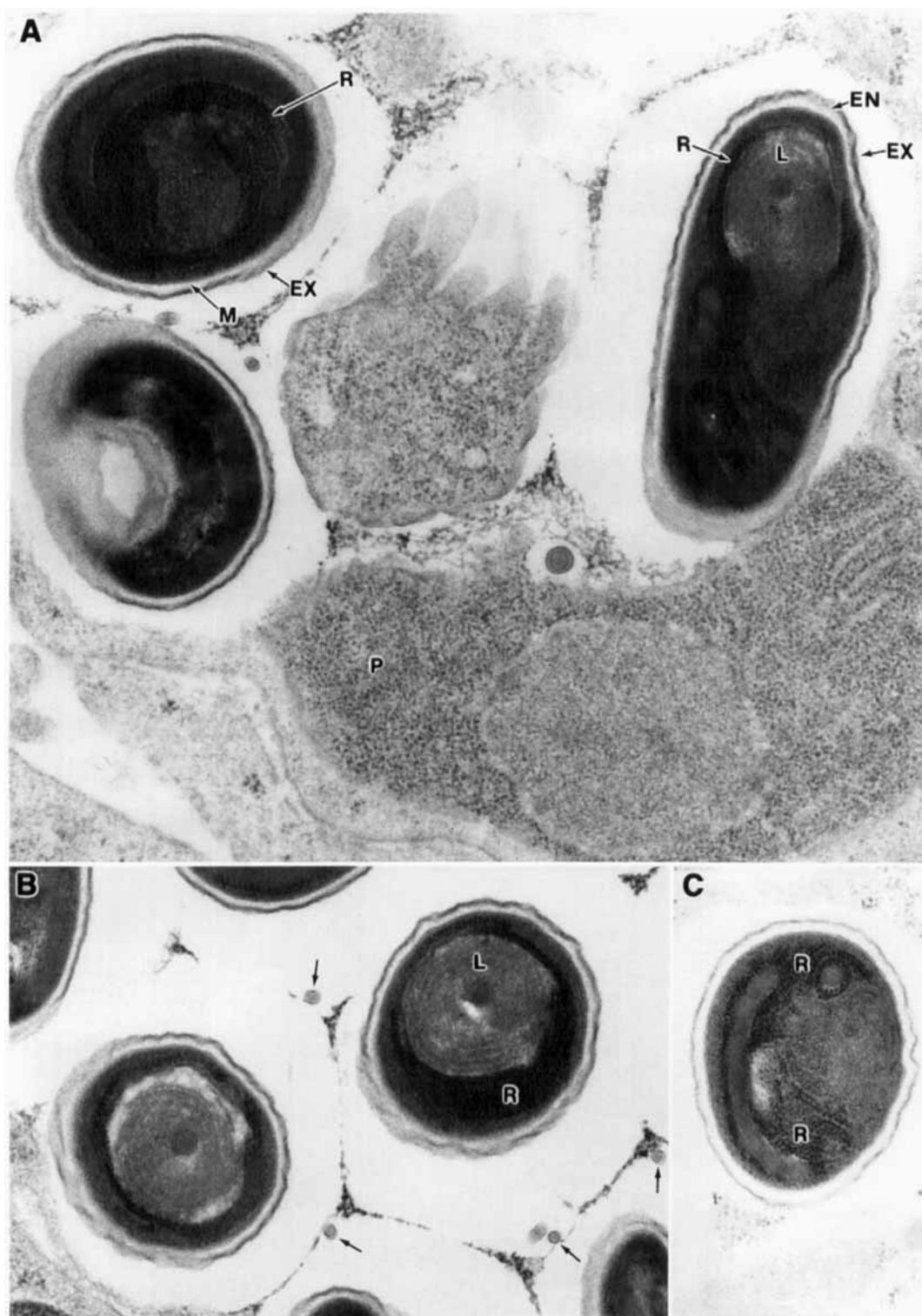
Several genera, such as *Pleistophora* and *Vairimorpha* are known for secreting material, but they are pansporoblastic and plasmodial in their development and our parasite is not [6, 12].

This new parasite possesses unique features. The material it secretes forms a fibrillar network external to the plasmalemmal surface of the developing stages. This material is present from the earliest stages of development through spore formation and forms a matrix separating the parasites from each other and from direct contact with the host cell cytoplasm. It is secreted from proliferative, sporont, and sporoblast cells as demonstrated by its continued presence between individual cells regardless of the developmental phase. There are several microsporidia that secrete material, but they are plasmodial and form sporophorous vesicles. Also, this new parasite is tetrasporous yet it divides by binary fission or multiple fission of elongated cells.

This new parasite shares some characteristics with several other microsporidian genera, but represents a unique combination of characteristics, which is justification for a new genus and species designation. Because it was first identified in the intestine, and because the fibrillar network, a unique feature of

Fig. 4. Sporogony. A. The deposition of material continues, resulting in a uniformly thick plasmalemma surrounding the sporont cells. These sporonts continue to secrete the fibrillar matrix (arrows). X52,000. B. The sporonts (ST) are often seen as elongated binucleate cells in the process of cytokinesis (arrowhead). Pieces of tubular appendages (AP) are scattered between the parasite cells. X25,000. C. Occasionally, dividing (arrowheads) tetranucleate sporont cells (ST) are seen (n, nuclei). This cluster of parasite cells also contains many mature electron-dense spores, proliferative cells (P), and a dense fibrillar network separating the individual parasite cells. X16,300. D. Dividing binucleate sporont with a spindle plaque (arrow) forming on one nucleus, indicating that it will divide again. X32,300.

Fig. 5. Sporoblast development. A. After the last cell division, sporoblast metamorphosis begins. These cells are oval and a little larger than the mature spores. The first new organelle to appear is a vesicular Golgi-like mass (G) associated with a small dense mass (arrow). X25,000. B. When these cells are cut longitudinally, this Golgi-like complex (arrow) appears at one end of the cell. X16,300. C. Cross section of early sporoblast through the Golgi-like complex. Note longitudinal section through the developing polar tubule (arrow). X28,200. D. Longitudinal section of early sporoblast through Golgi-like complex with two cross sections of the forming polar tubule (arrows). X18,000. E. Longitudinal section of later sporoblast. The cytoplasm is denser, there are four coils of the developing polar tubule cut in cross section (arrows), a centrally located nucleus (n) and, at the opposite end, the anterior attachment complex (A). X25,000. F. Anterior end of developing sporoblast. The developing anterior attachment complex (arrow) of the polar tubule with its shaft containing an electron-dense core. X27,000. G. Longitudinal section of sporoblast with a large single nucleus (n) directly below the anterior attachment complex of the polar tubule and above the polar tubule coils (arrow) surrounding the vesicular Golgi (G). X20,300. H. Sporoblast with seven coils of polar tubule in the posterior end of the cell, beginning of posterior vacuole (V), and anterior attachment complex (A) are visible. X23,500. I. This is a late sporoblast. The morphology of the polar tubule cross section has changed to alternating dense and lucid concentric rings around a dense core (enlargement in J); the Golgi-like complex is no longer visible. X18,800. J. Enlargement of polar tubule from I demonstrating the concentric rings around a dense core. X52,000. K. Attachment of tubular appendage (arrow) at one end of an early sporoblast cell. X52,000. L. Bulb-like end of type I tubular appendage. X78,300. M. Low magnification of a parasite cluster illustrating both the asynchronous nature of development and the presence of type I tubular appendages (arrow). In such clusters, the proliferative cells (P) are generally at the periphery and the later stages are more centrally located. Sporoblasts (SB) and electron-dense spores are also visible. X25,000.



**Fig. 6.** Spores. **A.** Longitudinal and cross sections of spores. The spore coat consists of an outer electron-dense layer or exospore (EX), an inner thick electron-lucent layer (EN), and a membrane surrounding the inner spore contents (M). In the anterior end of the longitudinally cut spore (right), the lamellar polaroplast (L) is visible surrounding a cross section of the polar tubule. Surrounding the polaroplast and the polar tubule are several layers of densely packed ribosomes (R). Note the proliferative cell (P) in close proximity to the fibrillar matrix and the clear spaces between it and the spores. X52,000. **B.** Cross section of the anterior of two spores. The densely packed ribosomes (R) completely surround the polaroplast (L). Four cross sections of appendage tubules (arrows) are visible scattered between the spores. The fibrillar network isolates each spore, giving a septate appearance to the cluster, characteristic of only this microsporidian species. X52,000. **C.** This spore shows cross and longitudinal sections of the polar tubule, surrounded by densely packed ribosomes (R). X52,000.

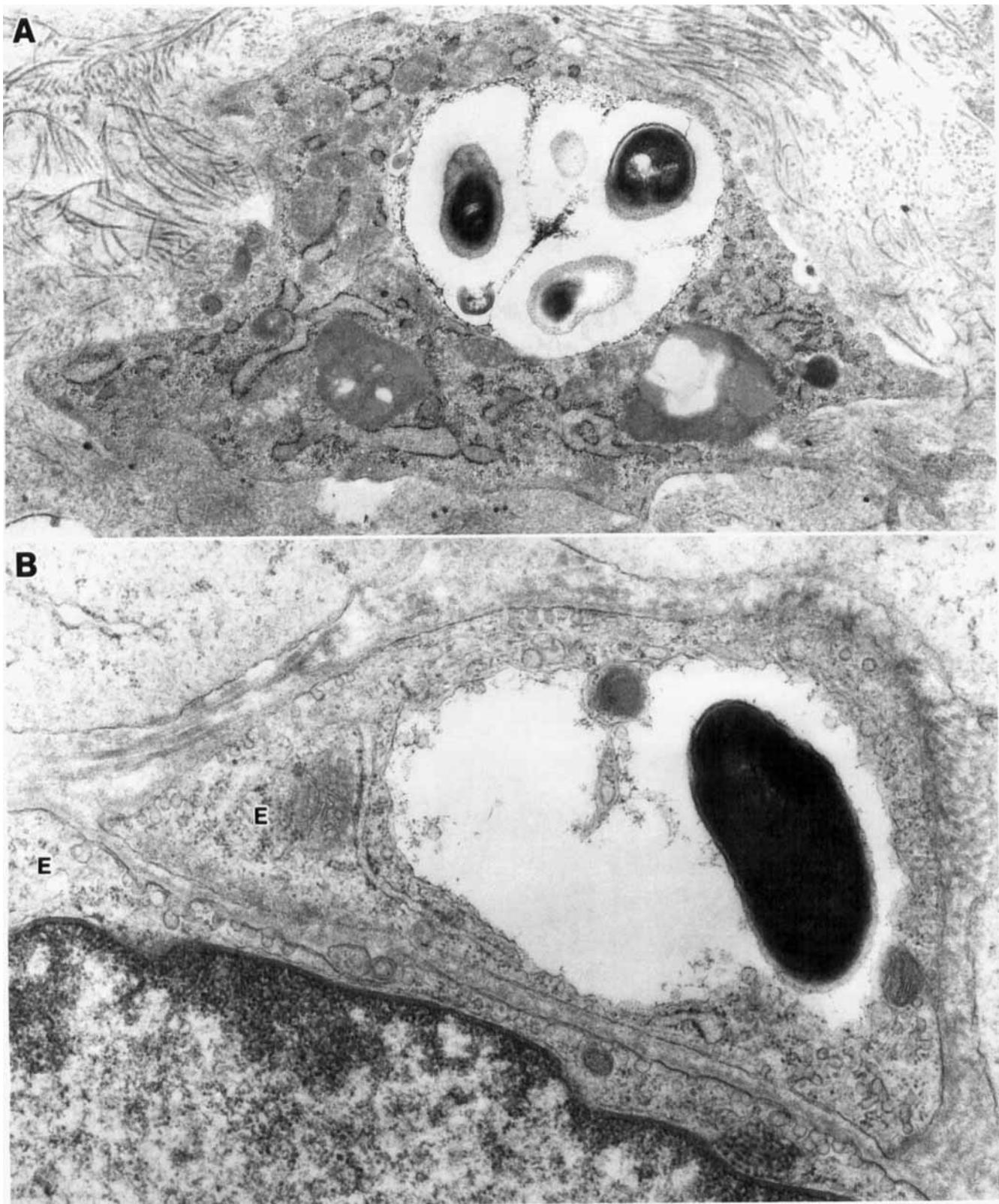


Fig. 7. Infection in other types of host cells besides enterocytes and macrophages. A. Fibroblast surrounded by secreted collagen fibrils and containing a cluster of parasite cells, each surrounded by the parasite-secreted fibrillar matrix. X20,300. B. Endothelial cell (E) with abundant pinocytic vesicles and external laminas. Cytoplasmic parasitophorous vacuole contains electron-dense spore and fibrillar matrix material. X32,300.

this organism, forms septations between the spores, we name it *Septata intestinalis* n. g., n. sp.

**Microsporidial family designation.** Because of the features enumerated above, *S. intestinalis* does not fit any established family. However, it probably is most closely aligned with the family Encephalitozoonidae, which contains only the genus *Encephalitozoon*. This assignment requires a modification of the family definition to include only the more general features such as the presence of only single rounded nuclei, no diplokarya; cytokinesis by elongation and fission; the presence of a parasitophorous vacuole membrane during all or at least in the latter part of the developmental cycle; sporogony indicated by thickening of the parasite plasmalemma; sporoblasts produced by elongation and fission of sporonts and sporoblast metamorphosis after the last cell division; spores uninucleate. The separating features will be considered genus specific characters.

**The genus *Septata*.** Genus characteristics include those of the family together with the presence of a host-formed membrane that surrounds the parasite clusters at least during sporogony; continuous secretion of material, forming a network of electron-dense fibrillar material from early proliferative development through to sporoblast metamorphosis, resulting in the appearance of parasite cells in isolated chambers; long tubules, also known as type I tubular appendages, formed in the sporogony phase; tetrasporous.

Description
<i>Septata intestinalis</i>

**Life cycle.** The proliferative phase includes single-nucleated rounded, or binucleated or tetranucleated elongated cells which multiply by repeated nuclear and cytoplasmic division. They are embedded in a very fine fibrillar network that is not easily detected during the proliferative phase of parasite development. This material is secreted by the parasite cells as medium dense amorphous droplets and is most obvious in tangential sections cut just outside the parasite plasmalemma. Proceeding away from the parasite surface, this material takes on a fibrous appearance and as parasite development progresses it becomes more electron-dense and abundant (Fig. 2-4, 6). The material condenses into a network that appears to be composed of cross connecting fibers. Because it appears as fine filamentous material, it is referred to as a fibrillar network. It is extracellular to the parasite and is in direct contact with the host. This network causes the proliferative parasite cells to appear tightly packed together (Fig. 2) as opposed to the dispersed appearance of microsporidia that are in direct contact with host cell cytoplasm, such as members of the genera *Nosema* and *Enterocytozoon*.

The sporogonic phase of development is characterized by the appearance of electron-dense secretions of material on the surface of the plasmalemma. This is a progressive process, initially giving the cell surface a scalloped appearance and eventually appearing as a homogeneously dense, thick coating that is characteristic of many microsporidia and referred to as a "thick membrane." This becomes the exospore coat of the spore stage (Fig. 3-6). The secretory process that forms the thickened membrane is variable: on some cells the secretory deposits are uniform in size and on other cells they are quite irregular (Fig. 3, 4D) with both large and small patches of secretory material.

Cells with thickened plasmalemmal surfaces undergo nuclear and cytoplasmic division (Fig. 4B-D), but it is difficult to determine the actual number of cell divisions. The divisions

observed have occurred by multiple or binary fission of tetranucleate or binucleate cells respectively; thus, this parasite is tetrasporous. However, because primarily binucleate dividing sporonts were observed, the timing of the cytokinetic cycles could be variable. That is, sometimes the cytokinesis is slow enough to allow for a tetranucleate cell (Fig. 4C); at other times cytokinesis follows the first nuclear division and occurs again after the second nuclear division, a biphasic process (Fig. 4D). Additionally, the tetranucleate cells may seem less common than the binucleate cells because of the improbability of cutting a single section through the entire length of such a long cell.

Along with the commencement of sporogony is the appearance of a parasitophorous vacuole-like envelope surrounding the parasite cluster (Fig. 3B). This is apparently of host origin and is similar to the parasitophorous vacuole of *Encephalitozoon* [3].

Tubular structures, similar to the type I tubular appendages described in the sporogony phase of *Glugea stephani* [18], are also present during sporogony in this life cycle. The actual length of these appendages is difficult to determine because of the planes of sections, but measurements as long as 1.2  $\mu\text{m}$  have been recorded. The appendages appear to be attached to the sporont (Fig. 5K), but they can pass through the fibrillar network and into adjacent chambers. They possess a single bulb-like structure at the distal end (Fig. 5L).

**Sporoblasts and spores.** Following the last cell division, spore-forming organelles appear. One of the earlier changes is the appearance of a vesicular Golgi-like complex followed by the formation of the developing polar tubule, seen as round dense rings with a dense core in cross section (Fig. 5D, E, H, I). These cells contain a single large nucleus. The spore metamorphosis is that of a typical microsporidian, producing a uninucleate spore with a thick electron-lucent endospore coat and a thin electron-dense exospore coat, and, on average, five coils of the polar tubule. This tubule is also called a polar filament at this stage, when it is still within the sporoblast or spore and appears to have a solid core (Fig. 5C, F, J & 6B). The tubule is uniform in diameter, isofilar, and forms a single row of coils inside the spore coat. Surrounding the polar tubule shaft and polaroplast are ribosomes, which are tightly packed in a regular array of several rows. They form what appears to be a "sheath" of ribosomes (Fig. 6A-C).

The mature spores are each surrounded by an electron-lucent space within the fibrillar network, which now appears more dense than in earlier development (Fig. 6). The spores appear to be suspended in a honeycomb with one spore in each chamber. These parasites are asynchronous, i.e. multiple stages occur within a cluster-spores as well as early and late developing stages. Usually the earliest stages are peripheral and the later stages are more centrally located within the clusters (Fig. 5M, 6A). Spores with extruded polar tubules were not observed in any of the infected host cells from intestine biopsies.

Since developing parasite stages were observed in both the intestinal enterocytes of the mucosa and in the fibroblastic, endothelial, and macrophage cells of the lamina propria, it is probable that this infection can be carried to other locations of the body via these cells.

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