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Ultrastructure and Development of *Pleistophora ronneafiei* n. sp., a Microsporidium (Protista) in the Skeletal Muscle of an Immune-Compromised Individual

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ABSTRACT. This report provides a detailed ultrastructural study of the life cycle, including proliferative and sporogonic developmental stages, of the first *Pleistophora* species (microsporidium) obtained from an immune-incompetent patient. In 1985, the organism obtained from a muscle biopsy was initially identified as belonging to the genus *Pleistophora*, based on spore morphology and its location in a sporophorous vesicle. Since that initial report, at least two new microsporidial genera, *Trachipleistophora* and *Brachiola*, have been reported to infect the muscle tissue of immunologically compromised patients. Because *Trachipleistophora* development is similar to *Pleistophora*, and as *Pleistophora* was only known to occur in cold-blooded hosts, the question of the proper classification of this microsporidium arose. The information acquired in this study makes it possible to compare *Pleistophora* sp. (Ledford et al. 1985) to the known human infections and properly determine its correct taxonomic position. Our ultrastructural data have revealed the formation of multinucleate sporogonial plasmodia, a developmental characteristic of the genus *Pleistophora* and not *Trachipleistophora*. A comparison with other species of the genus supports the establishment of a new species. This parasite is given the name *Pleistophora ronneafiei* n. sp.

Key Words. Human myositis, microsporidia, Microspora, sporogonial plasmodium.

THE Microsporidia are a phylum of obligate intracellular protistan parasites. They are ubiquitous in nature, with the majority of described species from insect and fish hosts. In mammals, the field has been dominated by the microsporidium, *Encephalitozoon cuniculi*. In man, several genera have been identified, primarily from immunologically incompetent individuals. The most common organisms include *Enterocytozoon bieneusi*, *Encephalitozoon (Septata) intestinalis*, and *Encephalitozoon hellem*. Infections with species of the genera *Vittaforma*, *Brachiola*, *Pleistophora*, and *Trachipleistophora* are less common. Several of these genera were established to describe new organisms in humans. However, *Pleistophora* sp. infections, described from human skeletal muscle (Chupp et al. 1993; Grau et al. 1996; Ledford et al. 1985), are notable because the genus *Pleistophora* is known as a parasite of fish muscle, with a few reports from amphibians and reptiles. As all other reports are from poikilothermic animals and microsporidia are intracellular parasites, intimately associated with their hosts, this presents some interesting questions. The most obvious being: are humans infected with species of the genus *Pleistophora*? In 1996, the genus *Trachipleistophora* was established for a microsporidium from the skeletal muscle of an AIDS patient (Hollister et al. 1996) and it has subsequently been reported several times. This brought the previous reports of *Pleistophora* into question since *Trachipleistophora* is a closely related genus possessing a different sporogonic developmental sequence. The present report describes the development and morphology of the microsporidium, *Pleistophora* sp., from human skeletal muscle, first reported by Ledford et al. (1985). Evidence is presented that validates the placement of

this organism in the genus *Pleistophora* and it is given the species name, *ronneafiei*.

MATERIALS AND METHODS

Muscle biopsies were taken in 1984 from a severely immunodeficient male with generalized muscle weakness and contractures (Ledford et al. 1985; Macher et al. 1988). Biopsies from the left quadriceps and left deltoid muscles were fixed in 10% neutral buffered formalin and processed for light microscopy. Subsequently, some of this tissue was reprocessed for electron microscopy (EM) by removal of paraffin, rehydration and post-fixation in 1% OsO₄. These tissues were embedded in Epon, thin-sectioned, stained with uranyl acetate and lead citrate, and examined at the Rutgers-Newark Philips EM Facility.

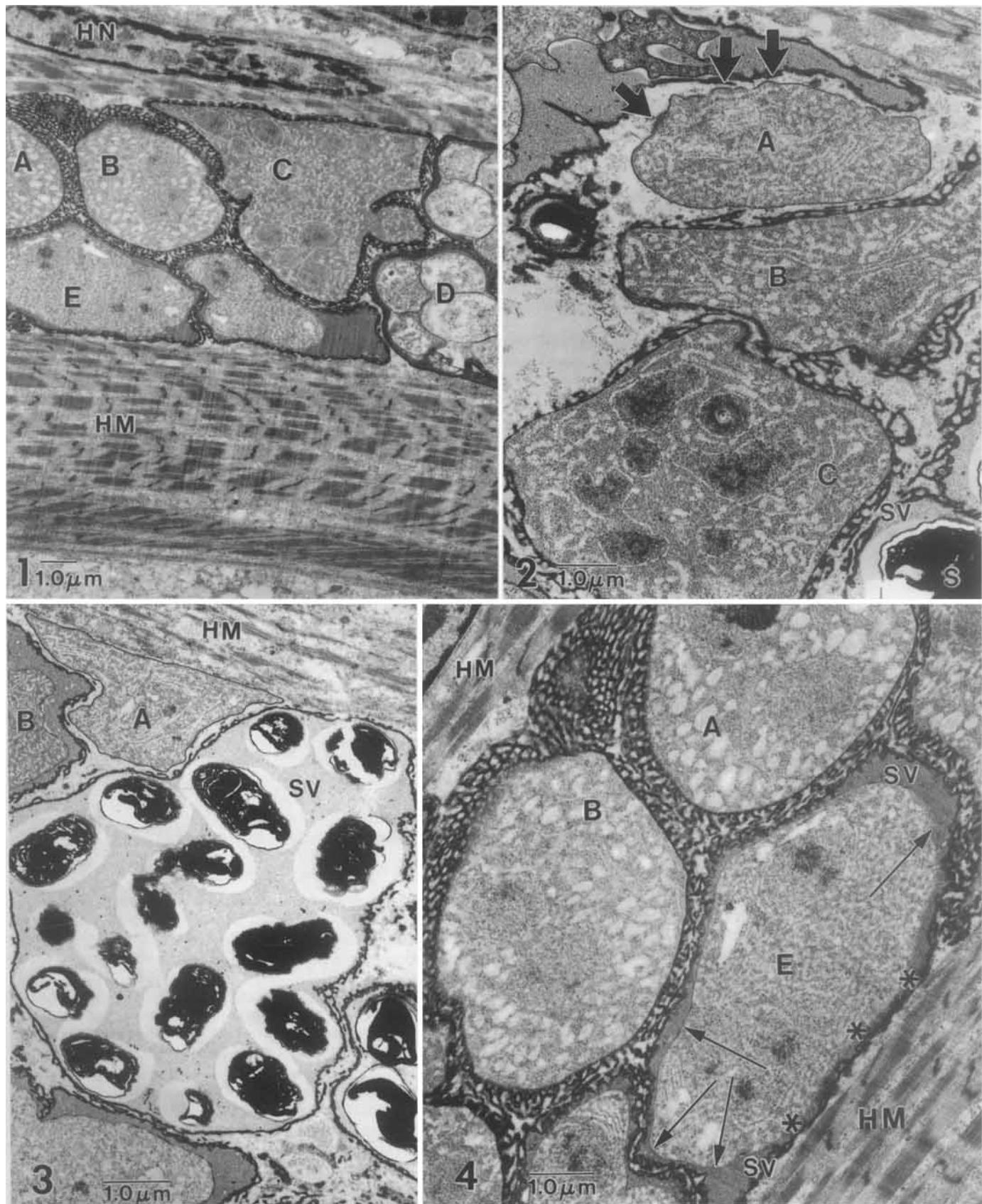
RESULTS

The parasites were found in the cytoplasm of striated muscle cells. They were synchronized within each sporophorous vesicle, but within one muscle cell there were several proliferative forms and sporophorous vesicles at different stages of development (Fig. 1).

Proliferative phase. The earliest stages observed were small “thick”-membraned cells whose plasmalemmal surface is relatively smooth, lacking most of the secretion projections (Fig. 2, 3). The beginning of the formation of the projections is present in small areas of the surface of these cells (Fig. 2, cell A). As development proceeds, the secretion material forms extensive branched complexes protruding from the cell surface. Once they were formed the branched complexes were maintained, even on dividing cells, which will be referred to as the typical proliferative cells.

The earliest typical proliferative cells observed (Fig. 1, 2, 4–7) ranged from small dividing cells with 1–4 nuclei to large multinucleate plasmodia containing approximately 10 nuclei

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visible per plane of section, all with secreted dense material surrounding the plasmalemmal periphery. The secreted dense material is convoluted and branched. In some sections it is quite elaborate and provides an almost honey-combed appearance (Fig. 4). The cytoplasm is vesicular, contains fine granular material (probably ribosomes), endoplasmic reticulum, and multiple, single nuclei. In this phase the cells divide (Fig. 5, 6), producing additional multinucleate proliferative plasmodia (Fig. 7). Since the secretions invaginate with the dividing plasmalemma, the daughter plasmodia remain enclosed by the thick wall (Fig. 7). At this phase of development, the dense surface secretions are most elaborate (Fig. 4), extending for some distance beyond the parasite surface, branching (Fig. 8), and sometimes producing an extensive tubular appearance (Fig. 9).

Sporogony. The next morphological change observable was the formation of a space between the secreted dense surface material (now the sporophorous vesicle wall) and the plasmalemma of the plasmodium (Fig. 4, 10, 11). A matrix of homogeneous granular material fills the space between the membrane and the thick vesicle wall as the membrane retreats (Fig. 4, 10). As it pulls further away from the wall, the amorphous material becomes more obvious (Fig. 11, 12). The plasmalemma becomes denser in small patches (Fig. 11) until the entire "plasmalemma" is thickened (Fig. 13, 14). This membrane thickening usually occurs very shortly after retraction from the sporophorous vesicle wall. However, dividing plasmodia with scant membrane thickening were occasionally observed (Fig.

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Fig. 1-4. Developmental stages (A,B,C,D,E) of *Pleistophora* in human skeletal muscle. 1. Low-power micrograph demonstrating the location of the infection, abutting bundles of actin and myosin filaments arranged as normal, functional contractile units of host muscle (HM) and containing a host cell nucleus (HN). The early stages of parasitic development are each surrounded by parasite-secreted dense material. The vesicle wall is most elaborate in proliferative development as illustrated between proliferative cells A and B (enlarged in Fig. 4). The proliferative cells A and B are probably sister cells as in early proliferative development the cells divide with the secretions. Later, the proliferative cells undergo several karyokinetic divisions without cytokinesis, resulting in the formation of large plasmodial cells (C). In sporogony, multiple parasite cells (cluster D) may be found within each sporophorous vesicle, which becomes more homogeneously dense in sporogony. 2. Cell A is the earliest stage observed. It has a thickened membrane but is only just starting to develop the elaborate secretions (arrows) present on proliferative cells B and C. The edge of a sporophorous vesicle (SV) containing spores (S) is in close proximity. 3. A sporophorous vesicle (SV) containing many (17) spores and late sporoblasts. Note the loss of branching surface secretions. An early stage parasite (A) abutting it, host muscle (HM), and an early sporophorous vesicle (B). 4. Enlargement of part of Fig. 1. Note the elaborate honeycomb-like appearance of the branched surface secretions on proliferative cells A and B abutting the host muscle cell cytoplasm (HM). Cell E is an early sporont. Note the decrease in branched surface secretions (*) after the cell plasmalemma (arrows) begins to pull away from them. The secretions become the wall of the sporophorous vesicle (SV).

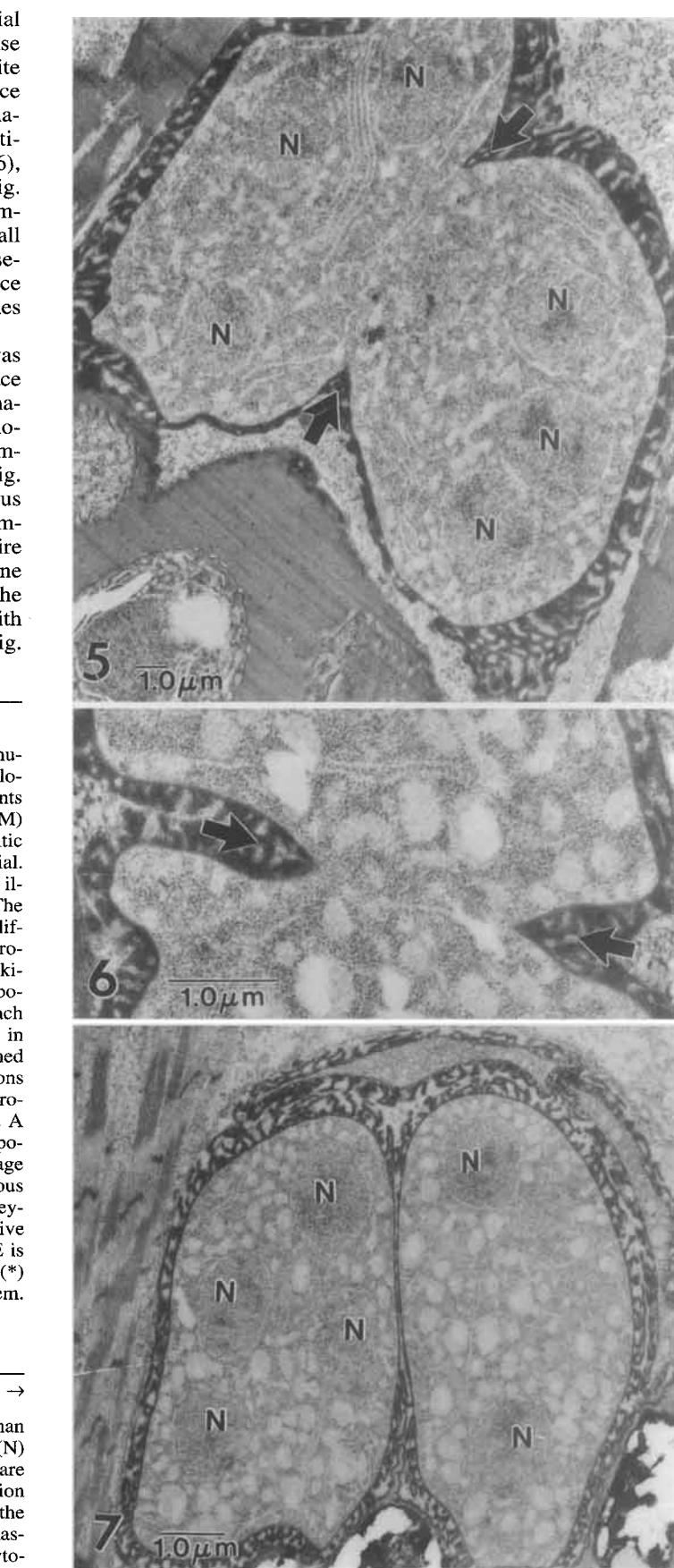
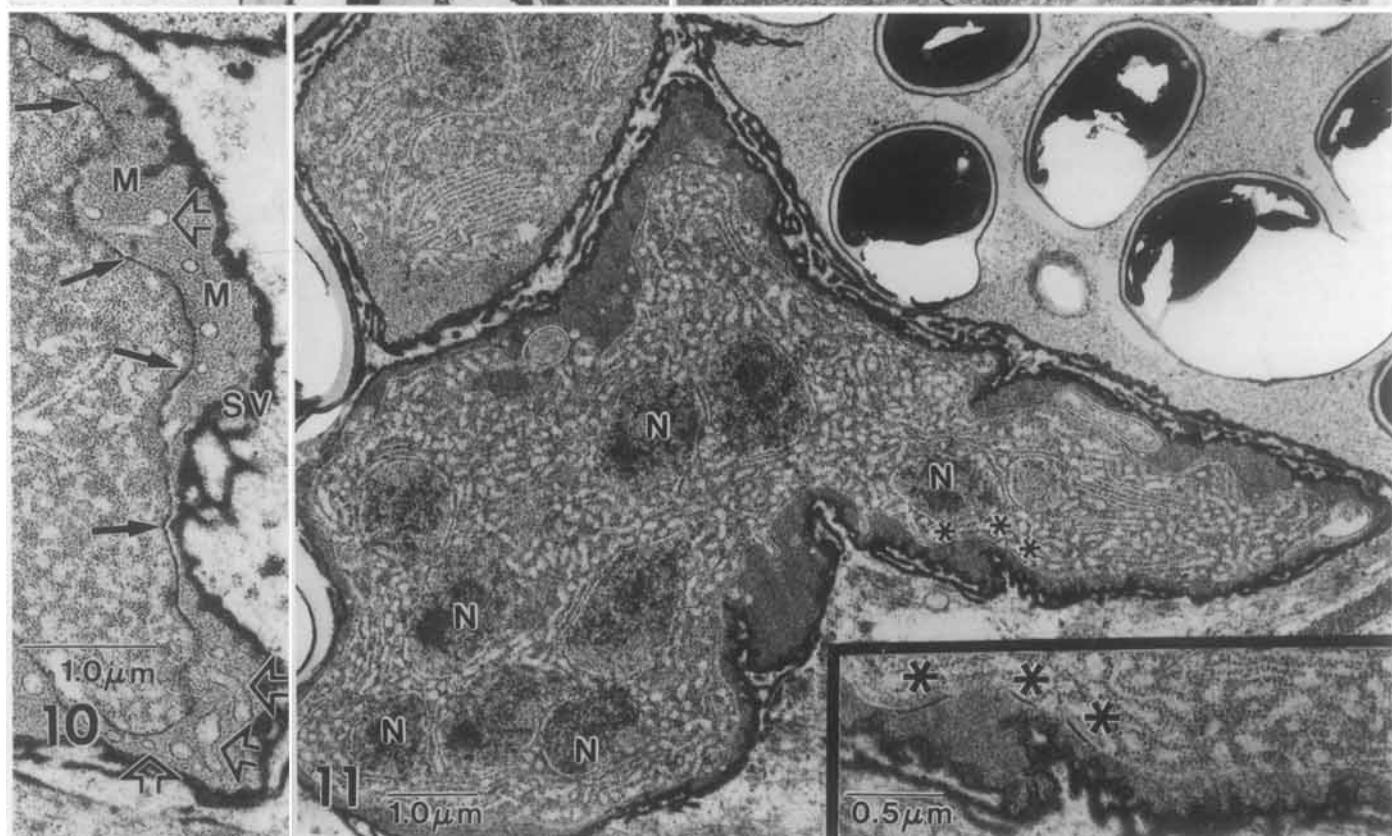
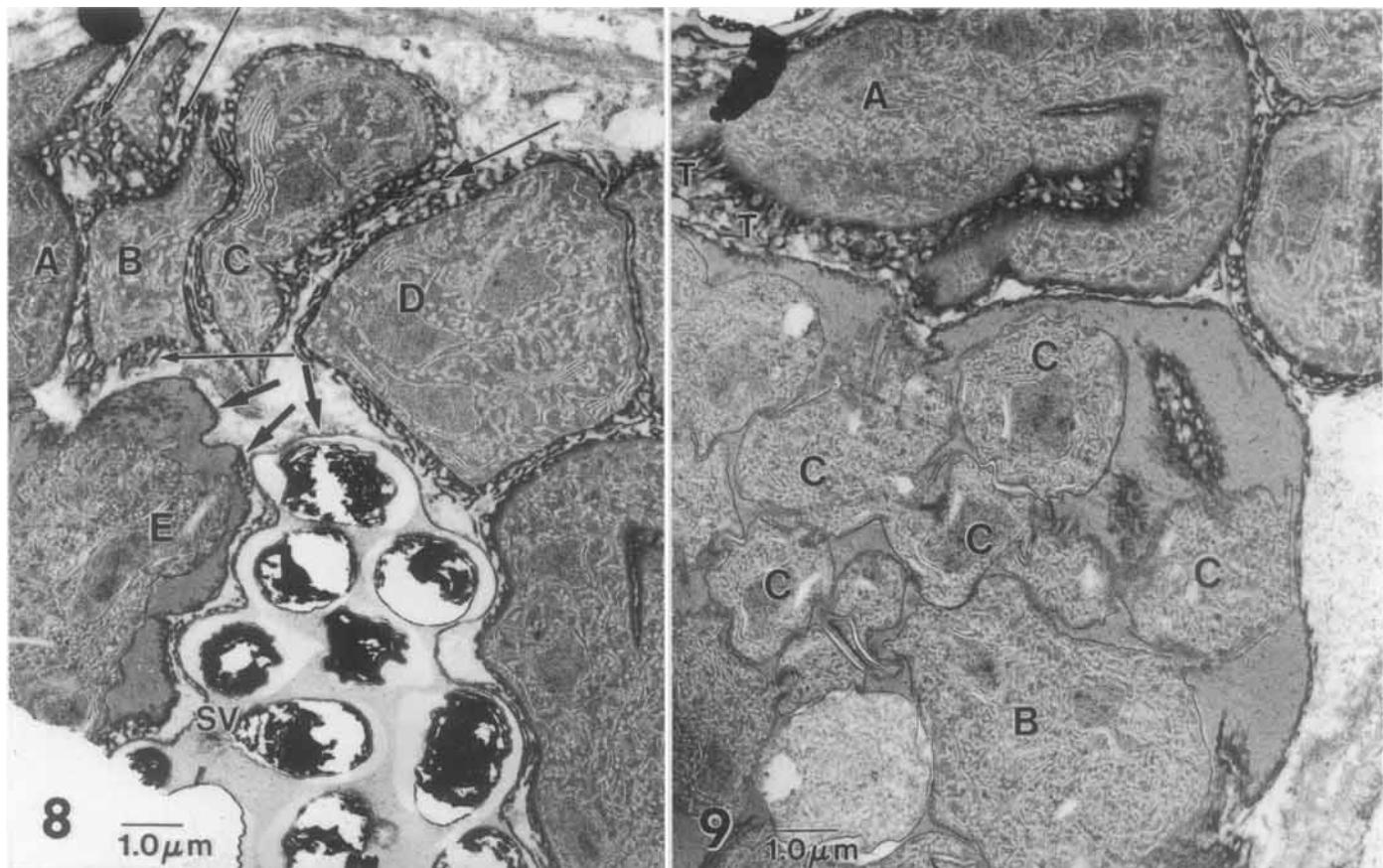


Fig. 5-7. Dividing proliferative forms of *Pleistophora* in human skeletal muscle. 5. Proliferative plasmodium, containing six nuclei (N) in plane of section, is undergoing cytokinesis. The dense secretions are present on the invaginating surface (arrows). 6. Higher magnification of an invagination area. Note the presence of secretion material on the cleavage furrow membrane (arrows). 7. Two abutted proliferative plasmodia containing multiple nuclei (N) shortly after completion of cytokinesis.

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12). The envelope forming the surrounding vesicle remains intact, losing the elaborate branching, appearing thicker and denser, forming the persistent sporophorous vesicle (Fig. 15).

In a single plane of section through the sporogonial plasmodium, as many as 9 to 10 nuclei have been observed (Fig. 11). Cell division occurs coincident to membrane thickening, in several stages of irregular fragmentation (Fig. 12–15) resulting in many uninucleate thick-membraned cells inside the sporophorous vesicle (Fig. 16). These uninucleate cells will metamorphose into spores without any further nuclear or cytoplasmic division; therefore they are sporoblasts (Fig. 16, 17). Each of the sporoblasts contained a developing polar tube complex, Golgi, rough endoplasmic reticulum, and a single nucleus. Metamorphosis is completed by the formation of the spore wall (Fig. 18, 19). The mature spores are typical of the microsporidia, with a thick spore coat, limited on the inside by a thin membrane. The most diagnostic organelle inside the spores is the polar filament. In longitudinal section, the number of polar filament coils was typically nine, but as many as 11 were observed (Fig. 18). Due to the angle cut and availability of material, it was not possible to determine if the polar filament was isofilar or anisofilar. The uncoiled portion of the polar filament and its attachment complex is in the anterior portion of the spore. The sporoplasm contains a single nucleus and is rich in ribosomes (Fig. 19).

The matrix surrounding the protozoan cells changes in appearance as the parasite development proceeds through sporogony. In the early phase of sporogony (Fig. 11, when the thickened plasmalemma is first formed), the matrix looks relatively medium-dense and homogeneously granular with tubular channels visible in some micrographs (Fig. 10). After the last cell division, sporoblast metamorphosis progresses (Fig. 16) and the matrix breaks down considerably, providing a much less dense medium (Fig. 17, 18). When the sporophorous vesicle is filled with spores, they are dispersed in a less dense matrix (Fig. 19). The surface of the vesicle containing sporoblasts is a somewhat homogeneous, dense thick envelope (Fig. 16) as compared to the dense irregular branching secretions of the proliferative plasmodial surface (Fig. 4).

Pathology. The sporophorous vesicles are found in the cytoplasm of the striated muscle cells. As multiplication and development of the parasite proceeds, it replaces a large portion of the functional elements of the host cell. Immediately outside the sporophorous vesicles, the muscle cell myofibrils are present (Fig. 1, 4, 16). During early parasite development, the vesicular surface of the parasite is in intimate contact with the host cell contractile elements (Fig. 1, 4).

DISCUSSION

There are several documented cases of human muscle microsporidiosis. Two different types of parasite development have been recorded. *Brachiola* species are diplokaryotic and develop

in direct contact with the host cell cytoplasm (Cali et al. 1998) while *Pleistophora* and *Trachipleistophora* have isolated nuclei and develop in sporophorous vesicles (Cali and Takvorian 1999). Since *Brachiola* is morphologically and developmentally different from the other two genera, it will not be discussed further.

Pleistophora species. *Pleistophora typicalis* is the type species for the genus *Pleistophora* (Gurley 1893). It was originally found in skeletal muscle in the fish, *Myoxocephalus scorpius*. Since that time, many species of *Pleistophora* have been described. Canning and Nicholas (1980) emended the characteristics of *Pleistophora* on the basis of ultrastructural findings made on the type species. The proliferative phase includes plasmodia that divide producing smaller plasmodia (plasmotomy). The plasmalemma of these cells is coated with a dense layer of secretions that divides along with the cells. The plasmalemma retracts from the dense coat and thickens at the onset of sporogony. Sporogony is polysporous, occurring by stepwise division of the sporogonial plasmodium through multinucleate segments into uninucleate sporoblasts, all within the dense coat now known as the sporophorous vesicle. Although two spore types were included in the genus redescription, Canning and Lom (1986) subsequently stated that the apparent absence of macrospores in the remaining *Pleistophora* species does not constitute grounds for transfer to a new genus.

The above findings resulted in the establishment of several new genera when species of *Pleistophora* were ultrastructurally reexamined. Cali and El Garhy (1991) reviewed and summarized the features of these new genera. Ultimately, the microsporidian species in invertebrates, which have been ultrastructurally studied, have been transferred to genera other than *Pleistophora*, thus restricting species of this genus to vertebrate hosts.

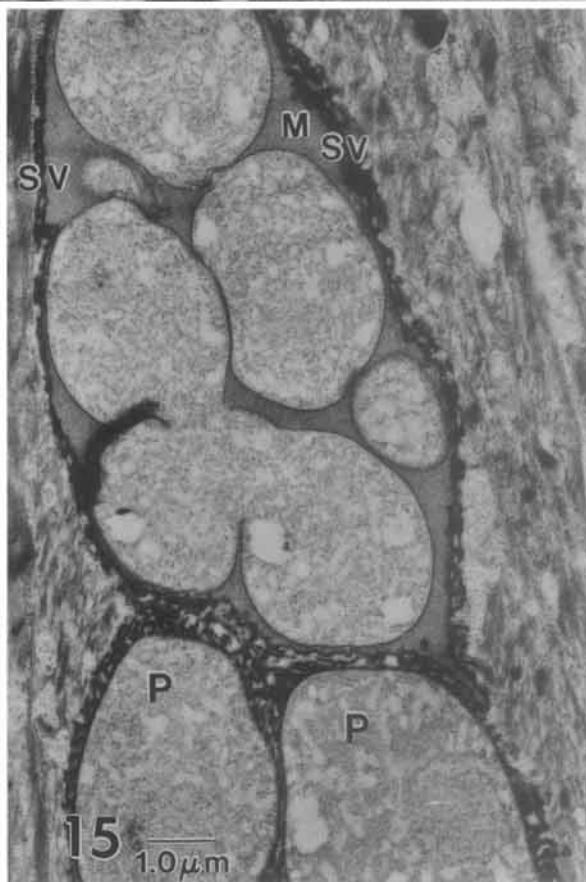
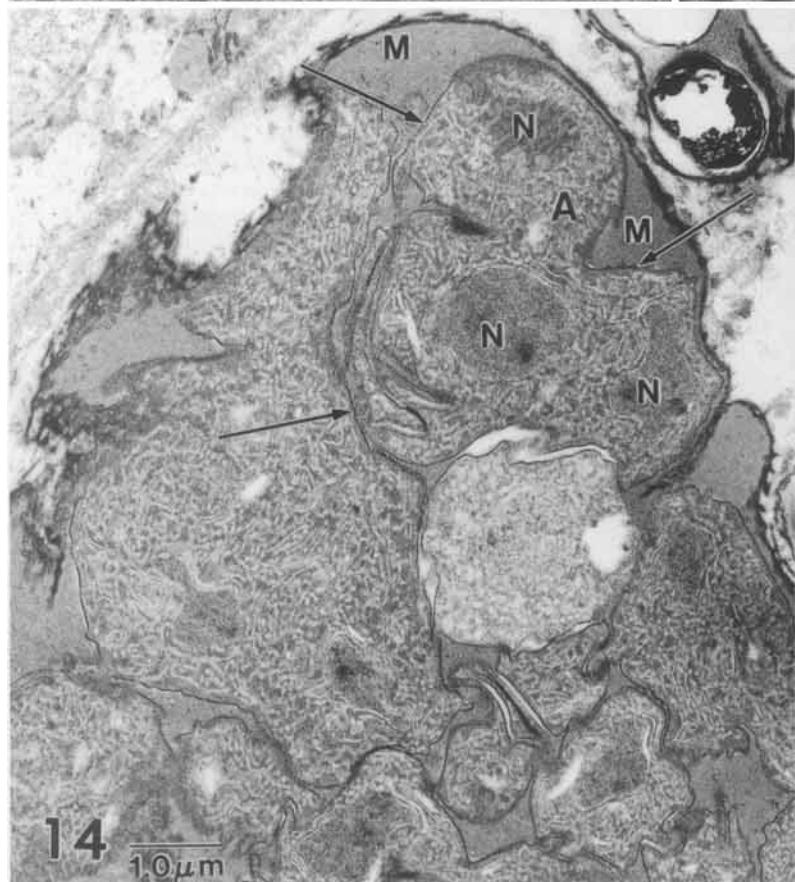
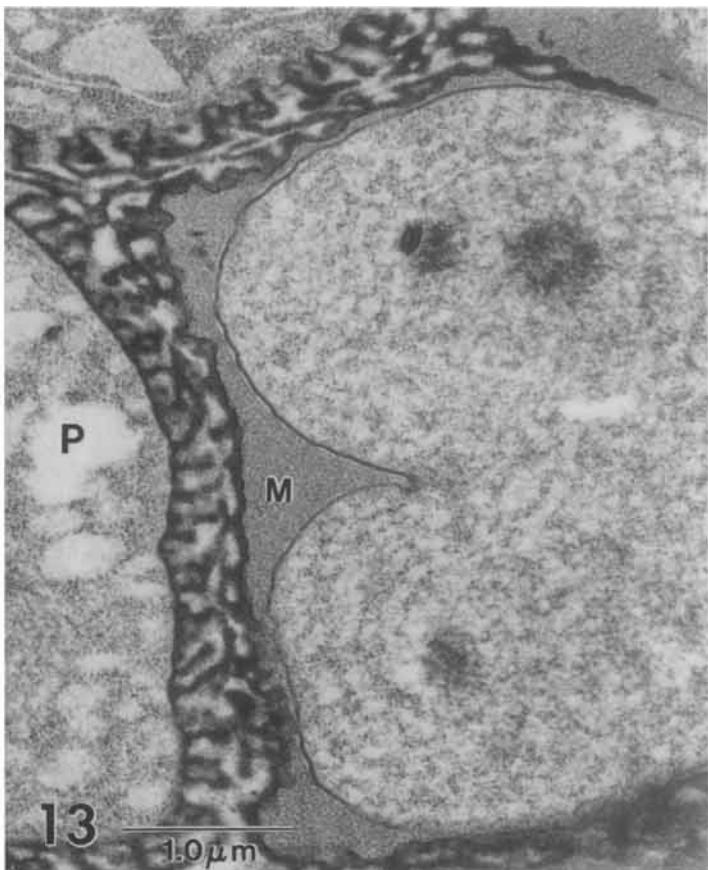
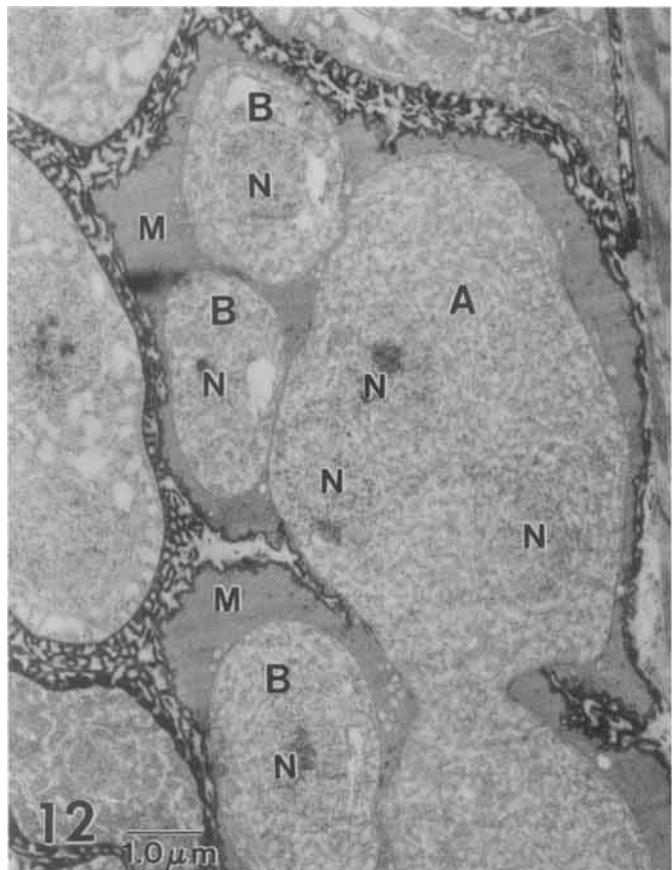
Human infections. In 1985, the human-infecting organism, that is the subject of the current report, was placed in the genus *Pleistophora* based on the presence of spores in sporophorous vesicles, unpaired nuclei in observed stages of development, and its location within skeletal muscle (Cali and Owen 1988; Ledford et al. 1985). Matcher et al. (1988) described the clinical manifestations of this case; the disease is characterized by "extensive and marked myositis". Using several different stains, they presented histopathological data that revealed parasite spores in sporophorous vesicles.

Subsequently, two reports of *Pleistophora* sp. induced myositis in humans (Chupp et al. 1993; Grau et al. 1996) described sporophorous vesicles containing spores with 9 to 12 polar filament coils. This number overlaps with the 11 coils reported by Ledford et al. (1985).

Trachipleistophora. In 1996, a new microsporidium was identified in human skeletal muscle that was similar to *Pleistophora* but did not form multinucleate plasmodia in the sporogonic phase of development. Consequently, a new genus,

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Fig. 8–11. Secretions and tubules present during late proliferative development and changes during early sporogony of *Pleistophora* in human skeletal muscle. **8.** The very elaborate and branching surface secretions extend for some distance from the parasite cell surface during late proliferative development on cells A, B, C, and D (long arrows). Note the difference between the surface secretion of these cells and the surface of sporophorous vesicles (short arrows) in early sporogony (E) and at the end of sporogony when the vesicle (SV) contains spores. **9.** Proliferative cell (A) with tubular extensions (T) from cell surface. Sporophorous vesicle containing plasmodium (B) that is undergoing several stepwise cell divisions, producing multiple smaller cells (C). **10.** Early sporont. The plasmalemma has just started to separate (arrows) from the sporophorous vesicle wall, which has already become less vesicular and more densely coated with the dense secretions. Note the presence of tubules (open arrowheads) in the matrix (M) between the plasmalemma and the sporophorous vesicle wall (SV). **11.** Early sporont. The plasmalemma has pulled away from the vesicle wall and has begun to invaginate starting the plasmodial division process. Note the presence of many nuclei (N) in the plasmodium and the beginning of plasmalemmal thickening in small patches (*) along its surface. Insert is enlargement of membrane area with thickening (*).



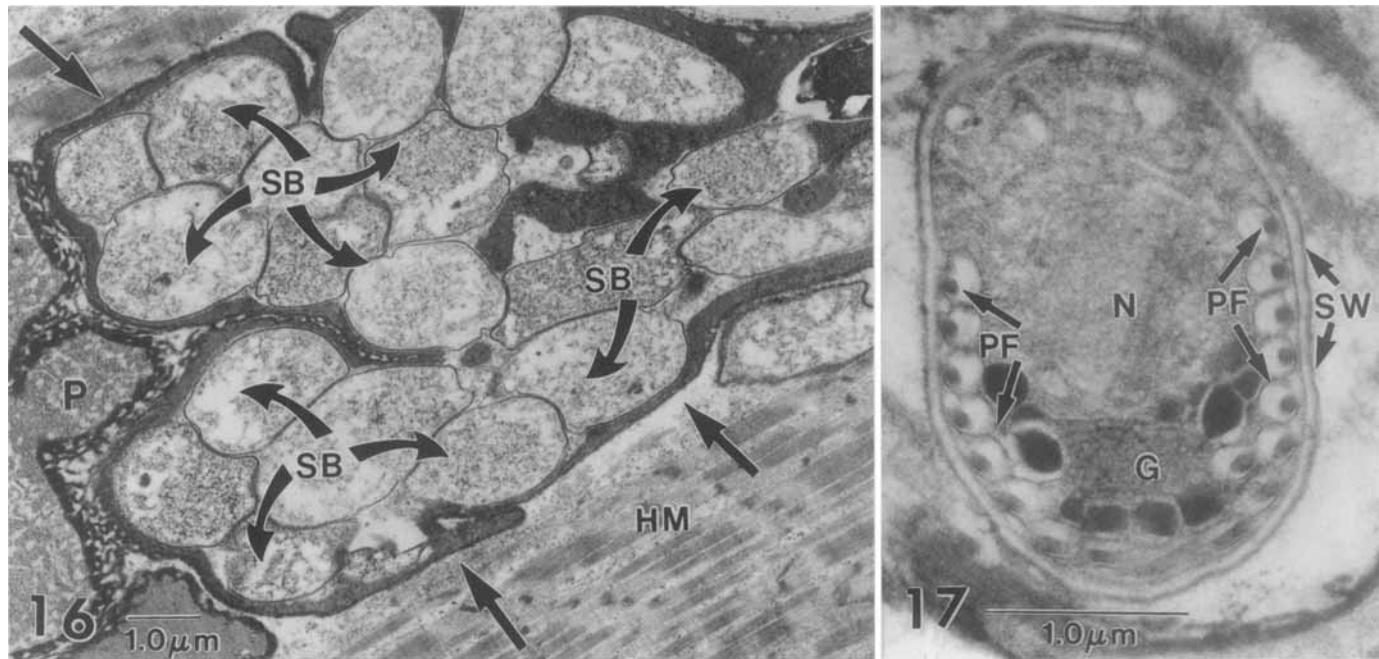


Fig. 16–17. Sporoblasts of *Pleistophora* in human skeletal muscle. After sporogonial plasmotomy is completed, the sporophorous vesicles are filled with many uninucleated cells, the sporoblasts. **16.** Sporophorous vesicle containing early sporoblasts (SB) abutted to a proliferative cell (P) and the lack of projections on the vesicle walls (straight arrows) where it abuts the host cytoplasm (HM). **17.** Late sporoblast or early spore contains a single nucleus (N), the developing polar filament (PF), the Golgi (G), and the beginning of spore wall (SW) thickening.

Trachipleistophora, was erected for this human infection (Hollister et al. 1996). The taxonomically significant features of the genus *Trachipleistophora* from Hollister et al. (1996) include uninucleate sporonts that retract within the surface coat, which becomes the envelope of the sporophorous vesicle. Within the vesicle, a series of binary fissions of binucleate stages, produce uninucleate sporoblasts and spores. Multinucleate sporogonial plasmodia are not formed. The features of the sporogonic phase of the genus *Trachipleistophora*, represent the taxonomically significant differences between it and the genus *Pleistophora*.

At this time it is not possible to determine the correct taxonomic position of the organisms described by Chupp et al. (1993) and Grau et al. (1996). In light of the documentation for both *Pleistophora* and *Trachipleistophora* occurrence in humans and the similarity of the spores, it is important for researchers reporting infections to include the early stages of sporogony in order to differentiate between the two.

***Pleistophora* sp.** The present observations of the detailed development of the organism identified as *Pleistophora* sp. (Ledford et al. 1985) have made it possible to decide on its placement. It is consistent with those of the genus *Pleistophora* not *Trachipleistophora*, which does not form sporogonial plasmodia.

Species comparisons. Those organisms remaining in the ge-

nus *Pleistophora* are found in poikilothermic vertebrates, primarily fish and in their muscle tissue. The life cycle of the genus *Pleistophora* has a distinctive pattern but most of the species are poorly described; consequently, comparisons are made to other species based on their host group, spore size, and number of polar filament coils. No other vertebrate-infecting *Pleistophora* species (Canning and Lom 1986; Shaw and Kent 1999) have spores matching the dimensions of the spores in the present study or infect homothermic hosts.

TAXONOMIC SUMMARY

Pleistophora ronneafiei n.sp.
(Fig. 1–19)

Description. *Life cycle stages.* All developmental stages contain unpaired nuclei. The proliferative forms possess the typical elaborate *Pleistophora* secretions on their cell surface. These cells range from small uninucleate forms to multinucleate plasmodia that are often observed in the process of division. As cell division occurs in the proliferative phase, multinucleate daughter cells are produced. The thickened surface material invaginates along with the plasmalemma as cytokinesis proceeds.

Sporogony is indicated by the retraction of the plasmodial

Fig. 12–15. Sporogonial cell division of *Pleistophora* in human skeletal muscle. Each plasmodium, in sporophorous vesicles, divides serially, producing smaller multinucleate daughter cells and ultimately single nucleated cells. The timing of membrane thickening is variable. **12.** Multinucleate plasmodial division (A) and single nucleated cells (B) in a homogeneous matrix (M) with little membrane thickening. **13.** Higher magnification of plasmalemma retracting from the sporophorous vesicle wall. Plasmodial cytokinesis has commenced and the plasmalemma is already completely thickened. Note the granular appearance of the matrix (M) and loss of branching of the sporophorous wall as compared to the abutting proliferative cell (P). **14.** A sporogonial plasmodium that has undergone a few cell divisions and has a completely thickened plasmalemma. The cell (A) on the right is dividing into three cells, each containing one nucleus (N) and all with thickened membranes (arrows). **15.** A sporophorous vesicle (SV) containing a cell dividing into at least three cells. There are two proliferative cells (P) abutting the sporophorous vesicle.

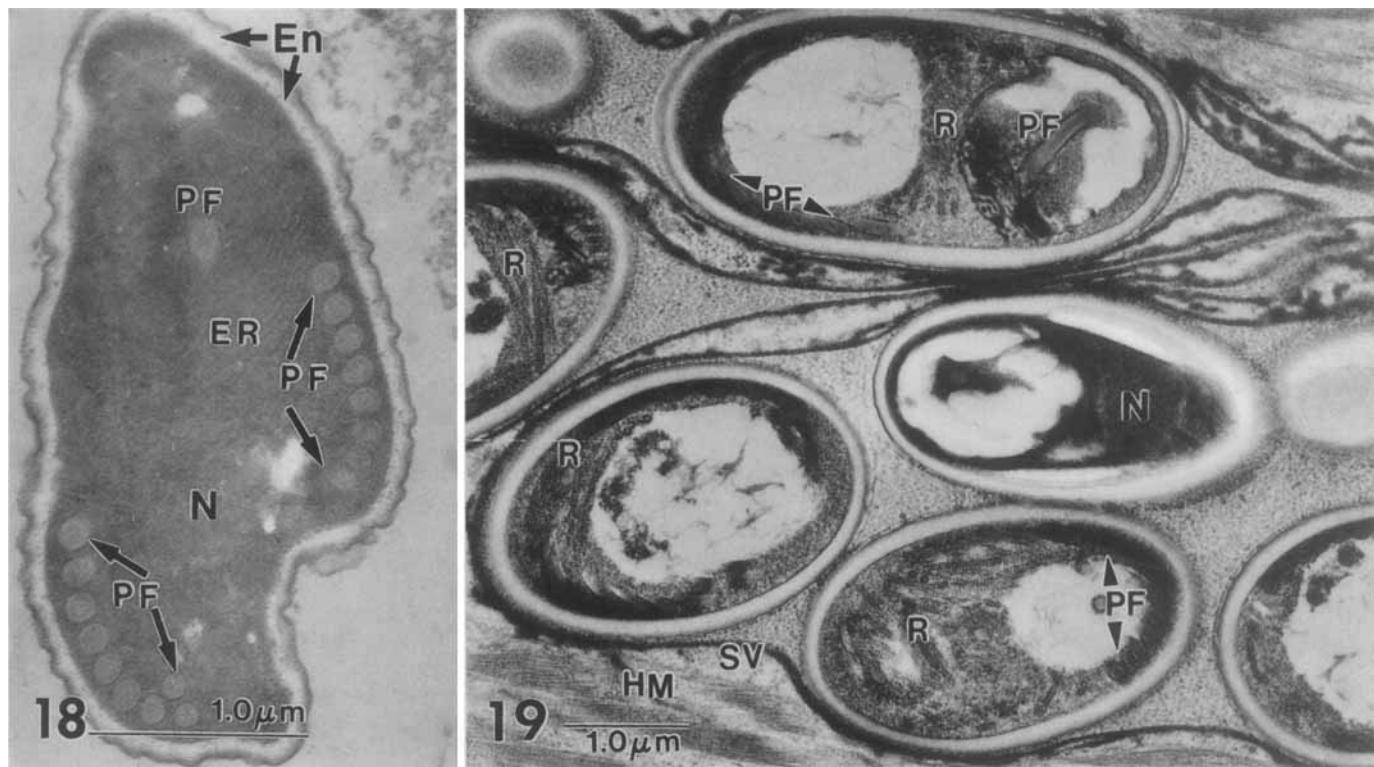


Fig. 18-19. Spores of *Pleistophora* in human skeletal muscle. **18.** A mature spore within a sporophorous vesicle. Note the row of nine polar filament cross-sections (PF) and the thickened endospore (EN) layer of the spore wall. **19.** Mature spores within intact sporophorous vesicles (SV) in skeletal muscle tissue (HM). Note the presence of a single nucleus (N), ribosomes (R), and the polar filament (PF) in the spores.

plasmalemma from the surface secretions which no longer divide with the cell. The retraction of the plasmalemma is accompanied by the deposition of granular-appearing material between it and the surrounding sporophorous vesicle. The cell is a plasmodium containing many nuclei, as many as nine in a single plane of section. Plasmalemmal thickening, analogous to that usually observed in microsporidia at the onset of sporogony, occurs during plasmodial division into multiple smaller cells. These cells divide repeatedly by "stepwise division" (plasmotomy, the process of successive divisions with the daughter cells containing multiple nuclei, which therefore will divide again, into multiple uninucleate cells), resulting in the formation of many single nucleated cells. The last cell division produces the sporoblasts, which metamorphose into spores. The uninucleate spores were measured from fixed and stained tissue. They range from $3.3-4 \times 2-2.8 \mu\text{m}$ and contain 9 to 11 polar tube cross-sections. The persistent sporophorous vesicles contain many spores (over a dozen in a single plane of section).

Type host. Humans, *Homo sapiens*

Other hosts. Unknown

Type locality. Florida, USA

Other localities. Unknown

Prevalence. Unknown

Site of infection. Skeletal muscle cells

Material deposited. Histological sections grid deposited in the International Protozoan Type Slide Collection, Smithsonian Institution, Washington, D.C. USNM no 1009174.

Etymology. This species is named in honor of Ronald Neafie of the Armed Forces Institute of Pathology, Infectious Disease Branch, Washington, D.C., who originally observed the parasite.

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