[Jap. J. Parasit., Vol. 18, No. 1, 8-20 1969]

Studies on the ultrastructure of *Nosema cuniculi*, a microsporidian parasite of rodents

SHINKICHI AKAO

Department of Parasitology, School of Medicine, Keio University
Shinjuku-ku, Tokyo, Japan

(Received for publication; November 6, 1968)

Nosma cuniculi was first found by Wright & Craighead (1922) in 1922 when they were trying to grow poliomyelitis virus in rabbits. They found small elongated bodies measuring less than 4.0 μ in length and 1.5 μ in breadth, associated with lesions in brain, spinal cord and kidney. The same parasite was found subsequently from rabbits and/or mice by Doerr & Zdansky (1923), Levaditi *et al.* (1923), Cameron & Maitland (1924), Da Fano (1924), Cowdry & Nicholson (1924), and Goodpasture (1924), Among others, Levaditi *et al.* (1924) studied most extensively on this parasite and named it *Encephalitozoon cuniculi*. They suggested the allocation of the parasite to the Order *Microsporicia*, although the basis of this allocation and proposal of new generic name was not always convicing. Perrin (1943a) examined a large number of laboratory animals and found the parasite in white mice, albino rats, guinea pigs, though the incidence of infection was less than 1.0%. He could not find the parasite in rabbits. Morris *et al.* (1956) found "*Encephalitozoon*-like" bodies in mice and considered them as a new agent causing a heavy ascites. Although he called it MAA (mouse ascites agent). this might have been the same parasite hitherto called *Encephalitozoon*. Innes *et al.* (1962) called attention to the fact that laboratory mice are very often infected spontaneously with this parasite.

Nelson (1962) could burst the macrophage cells infected with the organism by giving pressure upon the cover glass of fresh smear preparations and found the freed organisms which released a wavy filament. Due to this finding, Nelson suggested that this organism might be a member of the Order *Microsporidia*, as the presence of the polar filament is the most prominent feature of the microsporidian spores. Lainson *et al.* (1964) reported the morphological details and life cycle of a rat strain of the paraste and using a hydrogen peroxide solution, recognized the polar filament shooting out from the organism. They also successfully demonstrated the presence of coiled filament in spores by electron microscope observation, although their pictures did not reveal the minute details of the structures. Thus the microsporidian nature of this parasite was definitely established. Lainson *et al.* proposed the name *Nosema cuniculi* based upon the morphological details of the parasite. Recently, Petri (1966) found *Nesema cuniculi* in Yoshida sarcoma cells in rats. The organisms had been transferred serially in rats along with the sarcoma cells. He together with Schiodt (1966) further studied the ultrastructure of this organism and demonstrated the polar filament and other organelles which are commonly found in microsporidian spores of other species.

The purpose of the present study is to elucidate the ultrastructure of proliferative form, spore and intermediate stages of these two forms of *N. cuniculi* by electron microscope observations.

* The research reported in this document has been made possible through the support and sponsorship of U. S. Department of Army, through its Far East Research Office.

Abstract

FLUORESCENT ANTIBODY TECHNIQUE IN THE FILARIAL INFECTION IN THE COTTON RAT, *SIGMODON HISPIDUS*, WITH THE FROZEN SECTION OF VARIOUS STAGES OF *LITOMOSOIDES CARINII*

AKIRA ISHII, HAJIME MATSUDA, MASAO KAMIYA AND JUNZO KOBAYASHI
(Department of Parasitology, the Institute of Medical Science, the Universtiy of Tokyo, Tokyo, Japan)

The cotton rat, *Sigmodon hispidus*, was experimentally infected with the cotton rat filaria, *Litomosoides carinii*, by utilizing the tropical rat mite, *Ornithonyssus bacoti*, as the experimental intermediate host. Around eleven weeks after the infection, the cotton rats were bled to take sera. Adult worms of cotton rat filaria were collected from pleural cavity and maintained in Simm's solution with 30% of horse serum for a day to collect microfiariae. Infective larvae of cotton rat filaria were collected by disection of the infected mites. Adult worms and microfilariae were mounted in egg-white and gum-arabic medium, and were frozen rapidly in dry ice and acetone. Frozen sections of 5 micron in thickness were prepared by a cold microtome, and were utilized as the antigen after treated with 95% ethanol for eight minutes. Anti-cotton rat globulin rabbit antibody was conjugated with fluorescein isothiocyanate (FITC) and was used at four fold dilution. Indirect fluorescent antibody staining was conducted on cover slips with the frozen sections. Staining of the infective larvae were carried out with the whole body.

Sera of infected cotton rats reacted to the sections of adult worms up to 3,125 fold dilution, and these were found to be the most reactive antigen at this stage of infection when the adult worms matured and began to shed microfilariae. The intensity of the specific fluorescence was seen to be the strongest at the site of musclature inside of cuticle of adult worms. Next to the musclature were the lateral lines and the internal cavity of digestive canal. Cuticle and ovary did not take specific fluorescence, but in some cases specific fluorescence was observed around cuticle outside of the lateral line. Sections of microfilariae also reacted to the sera, but the titers were lower than with adult worms. No positive reaction was seen with the whole body of the infective larvae. At this stage when the infection is well established, adult worm had the most reactive antigen, and the most reactive antibody binding site was the muscle layer.

Materials and Methods

To get a strain of *N. cuniculi* to be used in this study, Giemsa stained smears of peritoneal fluid of mice were examined. Although mice are believed to be infected very often, the parasites were rarely demonstrated by the examinations, probably due to their extreme paucity in number, even if the mice were infected. Koike *et al.* (1960) tried serial blind passages of emulsions of brain, liver and spleen of these apparently negative mice. Among 20 series of the passages, 10 became positive at the fourth passage mouse. In the present study, the same procedure was taken, the blind passages being made every two-week interval. In the peritoneal fluid of the sixth passage mouse *N. cuniculi* were found and this strain has been maintained in mice by subinoculations at two-week intervals.

For the detection of *N. cuniculi* in mice, the smears of the peritoneal fluid were stained either with Giemsa or with trichrome stain after Wheatley (1951). The latter method, although more complicated then the former, gave a clear view of the parasite which is stained purple red against light blue host cell protoplasm. The parasite can be found in the brain, too. In fresh smears of brain, they are usually found as a large aggregate of innumerable small refractile ovoid bodies which are probably the spores. In staind smears, it is very difficult to find out them. In the experimental works, it is often necessary to find parasites without sacrificing experimental animals. In these cases, the material to be examined must be limited to the peritoneal fluid. The same is true with the electron microscope study of the parasite. It is almost impossible to find the parasite in the ultrathinsections of the brain. It is highly desirable, therefore, to get a high population of the parasite in the peritoneal cavity. This was achieved by Y. Kaneda, our collaborator, by the injections of endoxan, an anti-tumor substance. The details of his work will soon be published on this Journal.

For the electron microscope studies, heavily infected peritoneal fluid was centrifuged, the sediment was prefixed with 2.0% glutaraldehyde for 30 minutes, fixed with 1% osmic acid solution for 60 minutes, dehydrated through graded ethanol and embedded in epoxy resin. Ultrathinsections were made with ultramicrotome Type OmU2 of Reichert and electron micrographs were taken with Hitachi HU-IIA electron microscope at the magnifications varying from 5,000 × to 30,000 ×. The pictures were enlarged two to three times for publication.

Results

Morphology of *Nosema cuniculi* as seen by light microscope has been described by several investigators. Among others, descriptions by Levabiti *et al.* (1924) and Lainson *et al.* (1964) are the most important ones. Observations with light microscope by the present author (Fig. 1) gave nothing new to add to the descriptions of previous investigators.

1) Ultrastructures of the spore. The general structures of the spore are the same with those microsporidian spores. The wall is consisted of three layers. The outer layer is electron dense and more or less corrugated (Fig. 2) and the lamellar structure as indicated in *N. locustae* by Huger (1960) could not be recognized. The middle layer is about 50 mμ thick and electron optically transparent. The inner layer is a very thin, electron dense membrane which lines the inner surface of the middle layer and is closely adjacent to the protoplasmic membrane of the sporoplasm.

The sporoplast, having a lamellar structure, occupies the anterior part of the spore and is often extended to the posterior part where a definite vacuole can be seen (Fig. 2). Sporoplasm is situated in the space between the sporoplast and the posterior vacuole having a girdle-like appearrance at the middle of the spore. Its protoplasm is granulated and extends into the space between the wall and the sporoplast or the posterior vacuole.

The nucleus is situated in the girdle-like part of the sporoplasm and is roughly navicular in shape, its concave surface being directed to the anterior pole of the spore (Figs. 3, 4 & 7). The nuclear membrane cannot be recog-

nizable, but a narrow clear zone demarcates the nucleus from the cytoplasm. The nucleoplasm is either homogeneous or finely granulated and sometimes containes electron dense bodies which may represent the chromatine granules or nucleolus.

The polar filament has its base at the anterior tip of the spore and runs backwards, obliquely to the long axis of the spore, to the posterior vacuole where it forms a coil of 4–5 turns. The base of the filament is enlarged and attached to the inner surface of the wall (Fig. 6). Complicated structures at the base as seen in *N. locustae* by Huger (1960) or in *Plistophora hyphessobriconis* by Lom & Corliss (1967) could not be demonstrated in our specimens.

The polar filament is a solid filament in appearance, circular in cross section, measuring about 100mμ in diameter. Its surface is made of a thick, electron dense membrane which looks like a sheath. Another sheath-like structure can be seen, embeded in the matrix and encircling an axial fibre (Feg. 8). Tentatively, this structure may be called "inner sheath." The axial fibre is electron dense in cross section, but is not always sharply outlined. In some specimens, a highly electron dense minute fibril can be recognized in the center of the axial fibre.

In the longitudinal sections of the polar filament, the outer and inner sheaths can be recognized, especially at the anterior straight portion of the filament (Figs. 2, 6 & 9). The axial fibre was never recognized in the longitudinal sections. In a few specimens, definite striations arranged obliquely to the long axis of the filament were demonstrated (Fig. 10). In these cases, the diameter of the filament was a little samaller then that of the filament showing outer and inner sheaths. This probably indicates that the longitudinal section was made off the axis, or between the axial fibre and outer sheath. The probability is that the striations correspond to the inner theath which is not made of a continuous membrane, but of a pressed coil of a minute fibril.

2) Ultrastruture of the praliferate form. The proliferative form is irregularly elongated in shape and the size seems to be rather variable. It varies approximately from 3.0μ to 4.5μ in length and from 1.0μ to 1.5μ in breadth. The limiting membrane is distinct, being electron dense and composed of two layers (Fig. 11). The outer layer is more electron dense and thicker than the inner membrane. Protoplasm is glanulated, an electron dense mass of protoplasm can often be seen near the center of the organism (Fig. 12, 13). In all probability, this must be a nucleus, although the nuclear membrane cannot be seen. Mitochondoria, Golgi bodies and endoplasmic raticulum are not demonstrated.

The proliferative forms are located mostly in the vacuole of host cell. It is quite peculiar that a part of its surface often adheres to the host cell protoplasm as if it is absorbing nutrient from the cell (Fig. 12). On its surface adjacent to the host cell protoplasm, the limiting membrane of the organism becomes indistinct, although a demarcation line between host cell protoplasm and the parasite can be recognized somehow (Fig. 14).

3) Ultrastructure of the intermediate stages between the proliferative from and the spore. A number of micrographs showing several developmental stages of the spore were obtained. The earliest stage we ever met is an organism which has no morphological differences from the proliferative form except that the primordium of the polar filament can be seen in the cytoplasm (Fig. 15). The sections of the coiled filament are located in vacuoles and the filament itself has yet developed no definite internal structures. Fig. 16 indicates a longitudinal section of the filament in an early developmental stage. This filament is not smooth in outline, but shows indistinct helical structures. Fig. 17 shows an organism which is highly vacuolated and may be degenerated. Its polar filament has developed to the normal size and its internal stuructures have become visible. This organism is not a mature spore yet, because its outer wall is not completed. Fig. 18 shows an organism which has a polar filament showing definite internal structure, but the organism itself is irregular in shapes as is the case in the proliferative form and has not developed the spore wall

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Plate 1

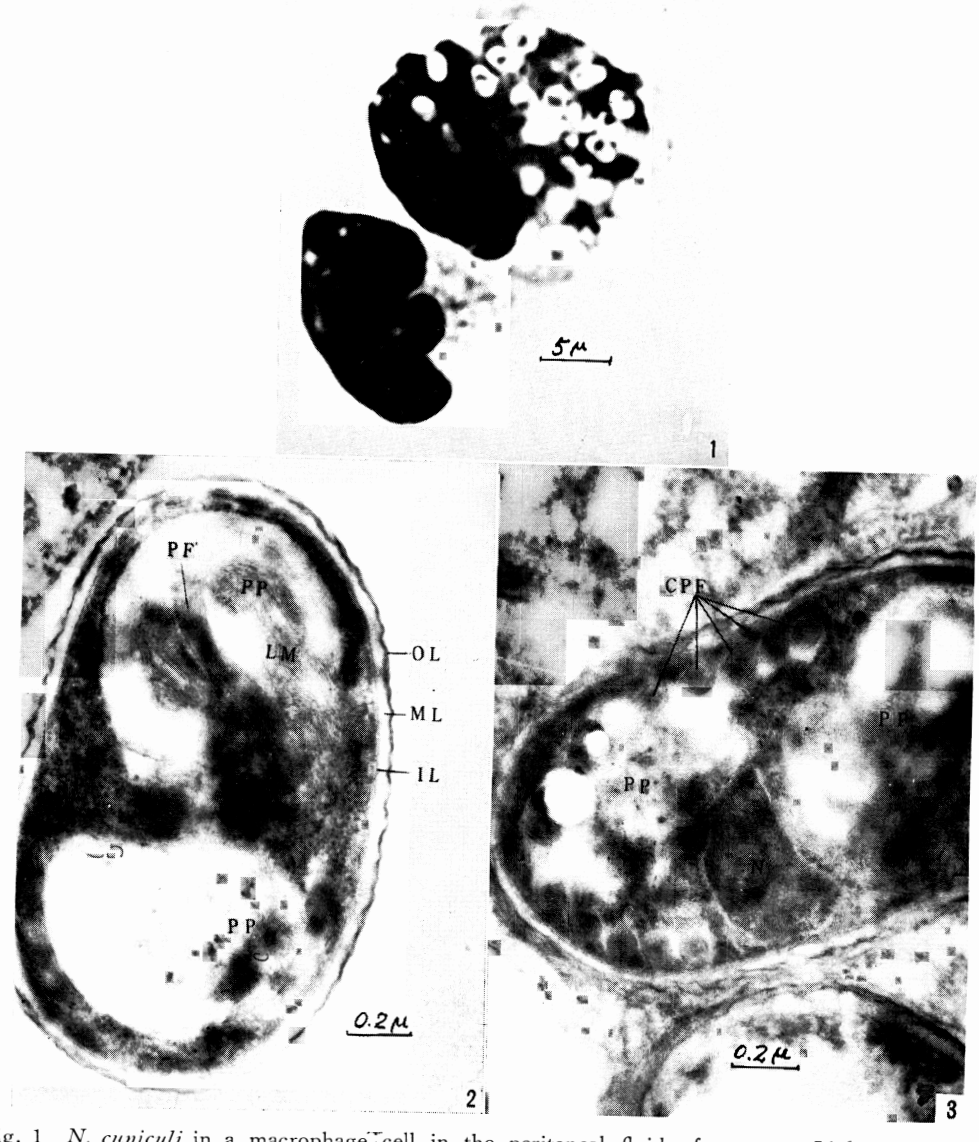

Fig. 1 *N. cuniculi* in a macrophage cell in the peritoneal fluid of mouse. Light microscope picture. about 2,000×.

Fig. 2 Longitudinal section of a spore. Outer (OL), middle (ML) and inner (IL) layers of the wall are discernible. Polaroplast (PP) containing polar filament (PF) and lamellar structure (LS) is a round clear area limited by the granulated protoplasm of the sporoplasm (SP). Polaroplast extends posteriorly in to the posterior (PP). 50,000×.

Fig. 3 Longitudinal section of a spore. Nucleus (N), coil of the polar filament (CPF) and polaroplast (PP) in the anterior and posterior part of the spore are demonstrated. 50,000×.

Plate 2

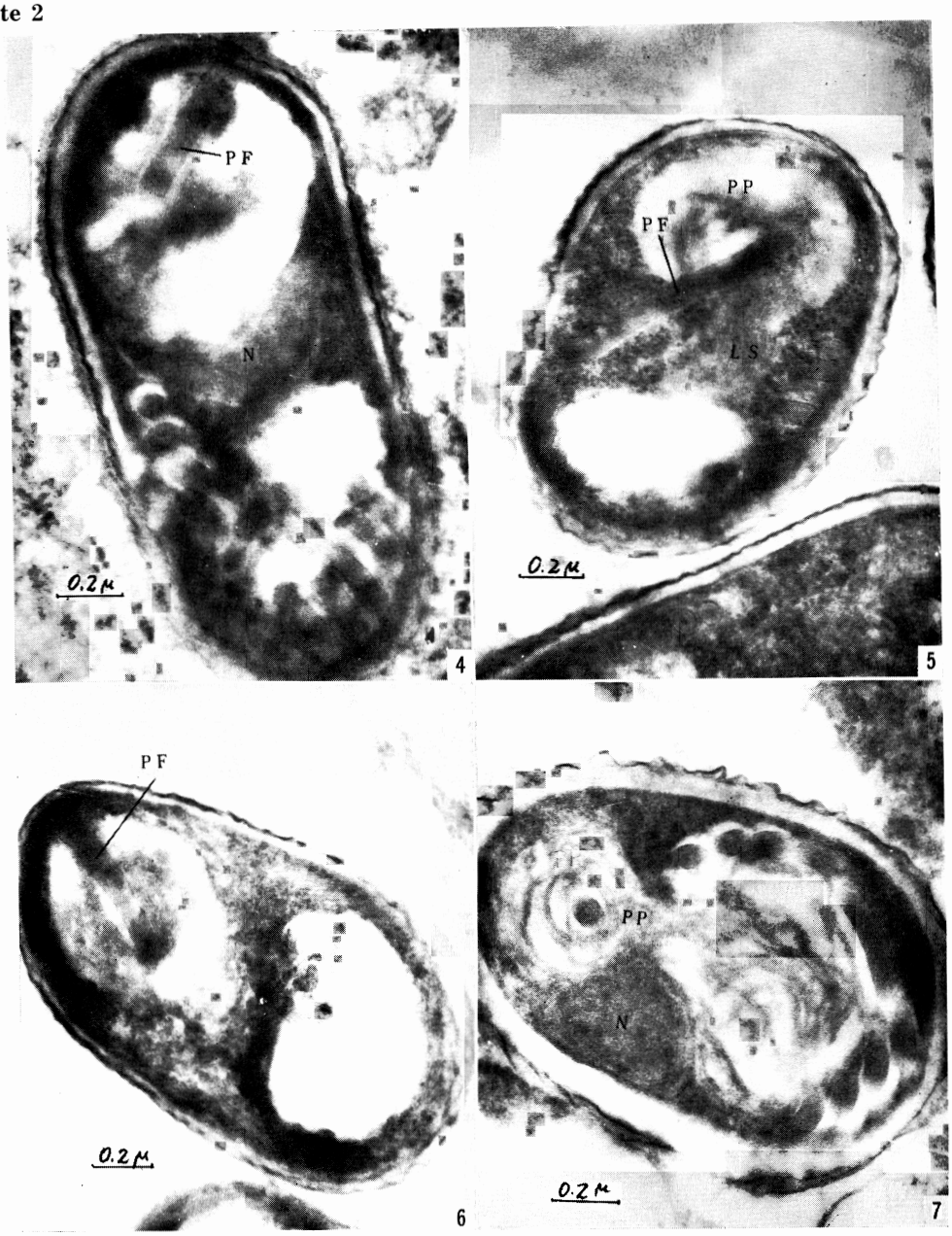

Fig. 4 Longitudinal section of the spore. Polar filament (PF) close to its base is demonstrated. The nucleus (N) is navicular in shape with its almost homogenous protoplasm. The coil of the filament is clearly seen. 50,000×.

Fig. 5 Oblique section of the spore. The polar filament extends obliquely through the girdle-part of the sporoplasm. The lamellar structure (LS) of plaroplast extends backwards through the sporoplasm. 50,000×.

Fig. 6 Longitudinal section of a spore. The base of polar filament is demonstrated. Enlargement of the outer sheath at the base is demonstrated. The inner sheath is also discernible. 50,000×.

Fig. 7 Longitudinal section of the spore. The lamellar structure of the polaroplast (PP) extends into the posterior vacuole. The nucleus (N) and the coil of filament are seen. 50,000×.

Plate 3

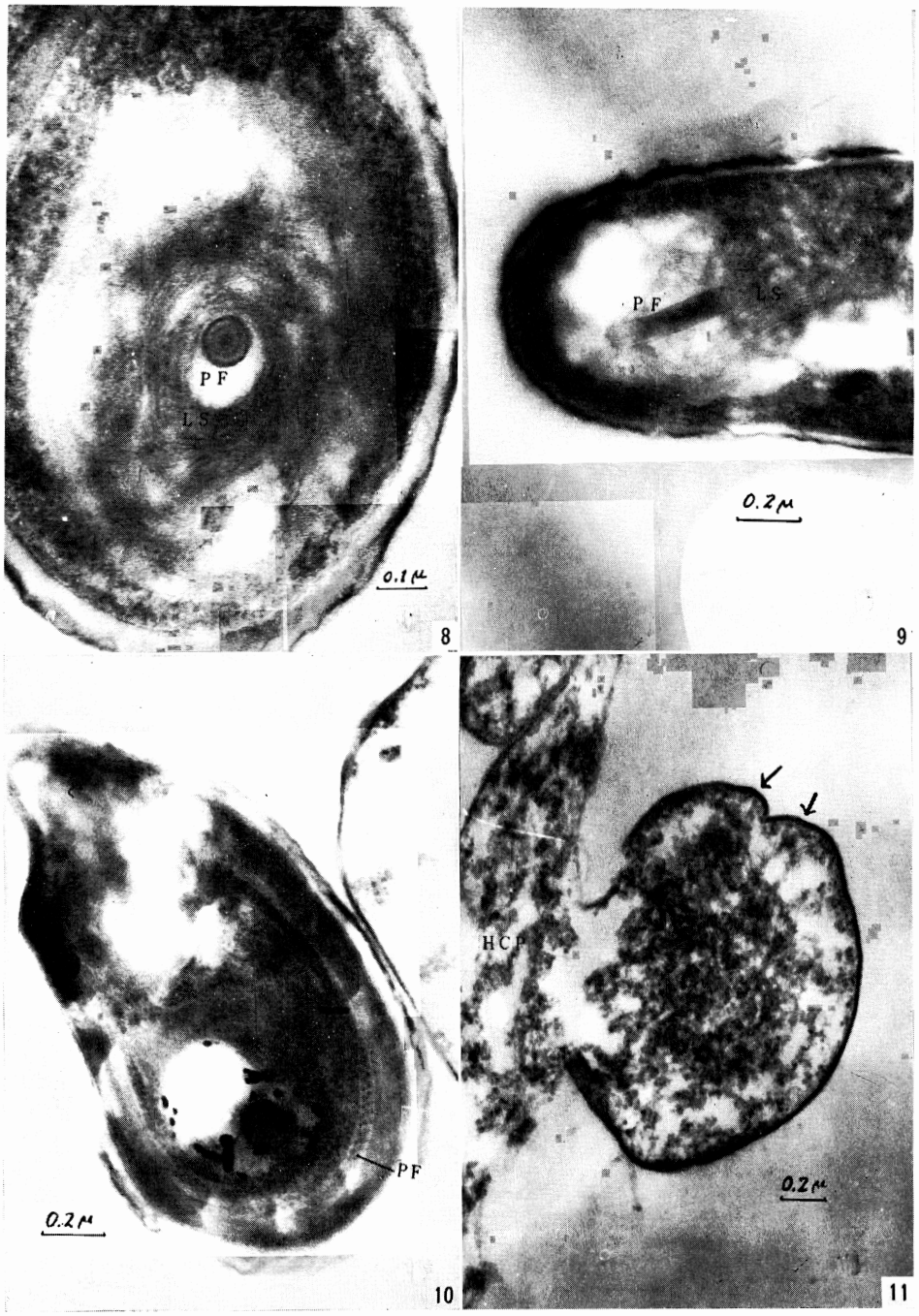

Fig. 8 Cross section of a spore. Polar filament (PF) with its outer and inner sheaths and the axial fibre is clearly demonstrated. A minute fibril in the center of axial fibre can be recognizable. Lamellar structure (LS) surrounds the polar filament. 70,000×.

Fig. 9 Longitudinal section of a spore. Outer and inner sheaths of the polar filament (PF) are discernible. The lamellar structure (LS) extends into the sporoplasm. 50,000×.

Fig. 10 Longitudinal section of a spore. This spore is partly destroyed. The helical structure of of the polar filament (PF) is demonstrated. 50,000×.

Fig. 11 Section of a proliferative form which adheres to the host cell protoplasm (HCP). Double layers of the plasma membrane are discernible (arrows). 50,000×.

Plate 4

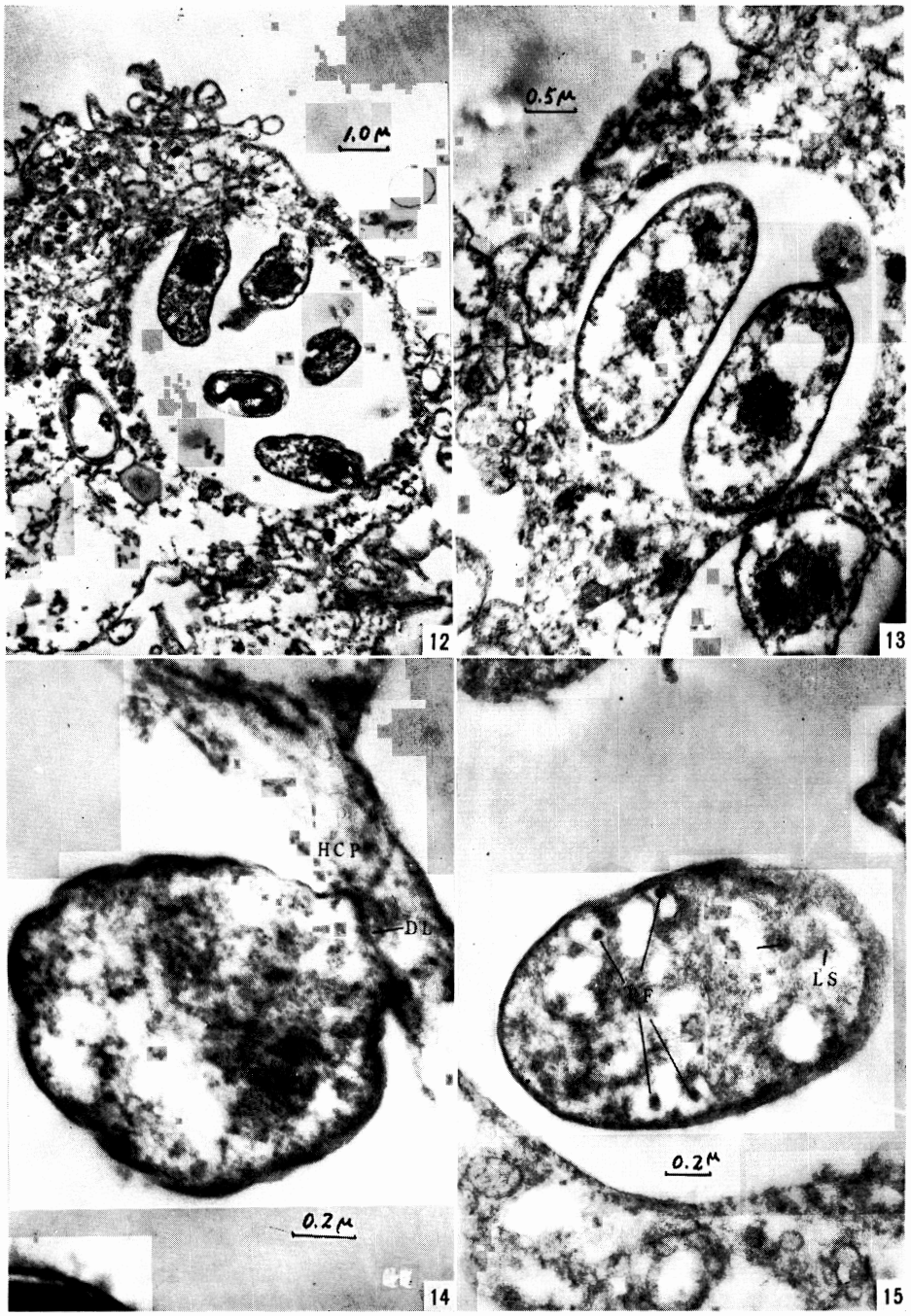

Fig. 12 Proliferative forms in a vacuole of host cell. The oval one at the center is a spore. Three adhere to the host cell protoplasm. The nucleus can be seen in those three parasites. 8,000×.

Fig. 13 Two proliferative forms in a vacuole. The nucleus is visible. 17,000×.

Fig. 14 A proliferative form adhering to the host cell protoplasm (HCP). The demarcation line (DL) is definitely visible. 50,000×.

Fig. 15 The early stage of the spore formation. The internal structure of the polar filament (PF) is not yet differentiated. 30,000×.

Plate 5

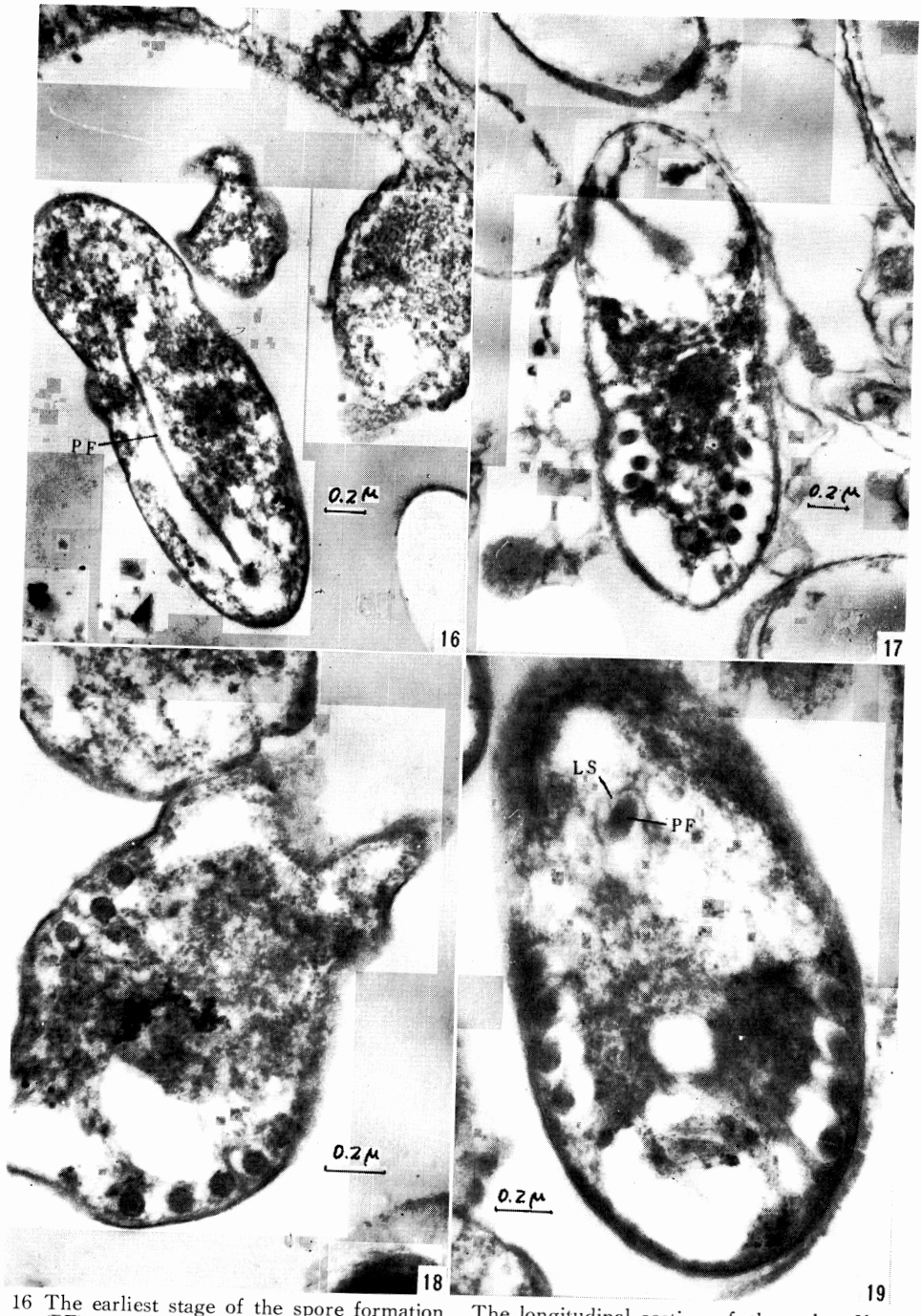

Fig. 16 The earliest stage of the spore formation. The longitudinal section of the polar filament (PF) is demonstrated. It is still very thin. 30,000×.

Fig. 17 An advanced stage of spore formation. The polar filament has developed its internal structure. The surface membrane of the cell is composed of an electron dense substance on its inner and outer surface. 30,000×.

Fig. 18 The polar filament is much developed, but no indication of the spore wall formation can be recognizable. 50,000×.

Fig. 19 A stage close to the mature spore. The polar filament is almost completed. Only a part of the lamellar structure (LS) is formed. Clear areas in the anterior and posterior pole are being differentiated: the sporoplasm is taking a girdle-like position. 50,000×.

Plate 6

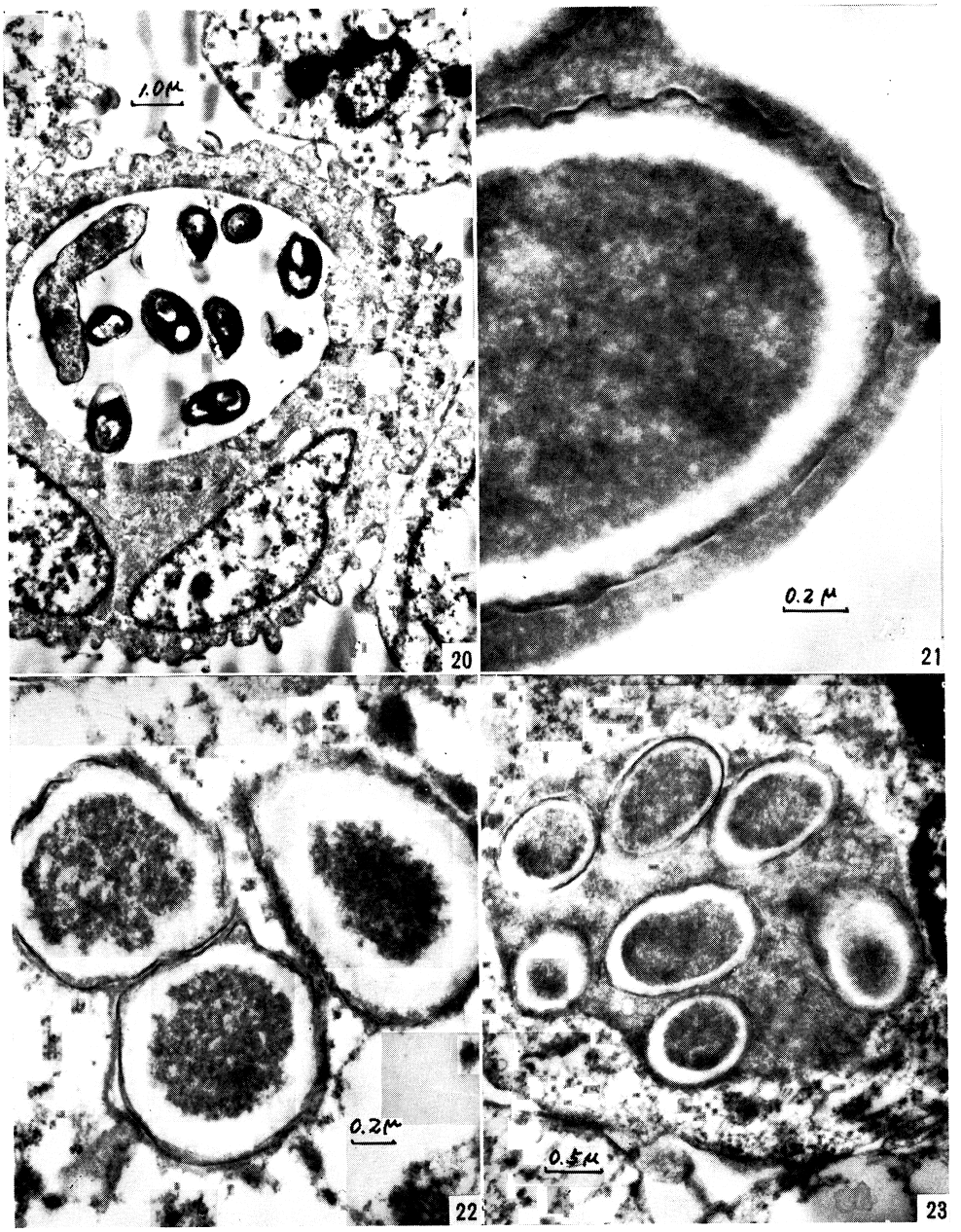

Fig. 20 A dividing form and many spores in a host cell vacuole. Each daughter cell has its own nucleus. 8,000×.

Fig. 21 A spore-like body with its corrugated outer membrane and a clear zone which has no inner limiting membrane. Protoplasm is almost homogenous with no internal structures. The plasma membrane is also lacking. 50,000×.

Fig. 22 Spore-like bodies with granulated protoplasm which seems to have undergone much shrinkage. The clear zone under the corrugated membrane is wide and uneven. 30,000×.

Fig. 23 An aggregation of spore-like bodies. It is surrounded by electron dense protoplasm which has a definite demarcation from the rest of host cell protoplasm. 17,000×.

Nosema cuniculi の微細構造に関する研究

赤　尾　信　吉
(慶応大学医学部寄生虫学教室)

Nosema cuniculi は Wright & Craighead (1922) により初めて報告され，その大きさは縦 4.0 μ, 横 1.5 μ の原虫である．寄生部位としては脳，脊髄，腎臓等に好んで増殖し，感染動物は脳炎様症状を呈するものである．1942 年に Levaditi はこの虫体を *Encephalitozoon cuniculi* と命名し，最近までこの名称が用いられていた．併し Nelson (1962) および Lainson *et al.* (1964) の研究により本虫体は Microsporidia に属することが確認され，Lainson *et al.* はこれを *Nosema cuniculi* と呼ぶべきものであることを提唱した．1966 年には Petri が *N. cuniculi* を吉田 Sarcoma 中より発見，これについて電顕的な報告が為されている．本研究ではマウスに自然感染している虫体を Endoxan 注射により多数に増殖せしめたものについて電顕的に観察し，栄養型，spore およびその中間の発育形態，構造を明らかにした．spore の形態構造は基本的には他種のものにおけると同一であり，最外層には unitmembrane が存在し中間層はかなり厚いオスミウム好染性の物質がとりまき最内層は sporoplasm を包括している．spore の前半には層状構造をもつ polaroplast が存在しその中心に polar filament が貫いて後走し，この線維は spore の後半部でコイル状に彎曲して，後部までのびている polaroplast をとりまいている．このpolar filament の構造は内部が更に微小 coil から形成されるものと観察された．spore の核は sporoplasm の中心に存在しているが明瞭な核膜は観察できなかつた栄養型は宿主細胞質内では殆んど楕円形であるが，宿主細胞の vacuole 内に存在する場合にはアメーバ状の種々の形に変化している．アメーバ様形態の栄養型は宿主細胞の vacuole の limitting membrane に接着し恰も栄養を細胞側より吸収しているかの像が多数観察された．

この栄養型から spore に至る発育の種々になる階程が見出され，*Nosema cuniculi* の life cycle を一応形態学的に完成することができた．