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Electronmicroscopic Observation of *Pneumocystis carinii*

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Pneumocystis carinii is the causative organism of fatal pneumonia encountered in the patients with congenital immune deficiency disorder, or in the recipients of the immunosuppressive therapy for malignant neoplasm, organ transplantation, or other conditions. It also occurs in the premature or malnourished infants (Burke and Good, 1973). Above all, in developed countries, *P. carinii* pneumonia is most frequently seen in the patients suffering from leukemia (Walzer *et al.*, 1974). Simone *et al.* (1972) also stated that *P. carinii* was the most frequent specific cause of death during the remission of childhood leukemia. Furthermore, the incidence of *P. carinii* pneumonia in acute lymphatic leukemia can be related to the intensity of chemotherapy (Hughes *et al.*, 1975). Perera *et al.* (1970) suggested that more intensive chemotherapy caused the outbreak of *P. carinii* pneumonia by activating the inapparent *P. carinii* infection commonly associated with neoplastic diseases. Therefore, it is expected that the cases of *P. carinii* pneumonia will be found more frequently in future, because the therapy for malignant neoplasms tends to become more intensive. Indeed, the number of cases has recently increased also in Japan, and *P. carinii* pneumonia has come to be one of the most important complications in the treatment of the immunosuppressed patients (Yoshida *et al.*, 1978).

On the other hand, Hughes *et al.* (1977) reported that the preventive administration of trimethoprim-sulfamethoxazole to the patients with cancer who were at high risk for *P. carinii* pneumonia was successfully performed over a two-year period. In the pediatric department of our university hospital, we have also attempted the chemoprophylaxis by this drug combination for about a year, and obtained the excellent results (Arakawa *et al.*, 1979).

It is important to comprehend the life cycle and the taxonomic position of *P. carinii* for the improvement of treatment and prophylaxis. However, these problems have not yet been confirmed, because the attempt to cultivate the organism *in vitro* has not completely succeeded, though some investigators reported that *P. carinii* propagated on the cell culture for a while (Pifer *et al.*, 1977; Latorre *et al.*, 1977). Many investigators have discussed the life cycle and the taxonomic position of the organism

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mostly on the basis of their electronmicroscopic observations. However, it is still at issue, whether *P. carinii* is a protozoan or a fungus, and whether trophozoites multiply by binary fission, budding, or endogeny (Seifert and Pliess, 1960; Barton and Campbell, 1969; Vavra and Kučera, 1970; Campbell, 1972; Vossen *et al.*, 1978).

In the present investigation, attempts were made to find out suitable fixation and preparation method of material for electron microscopy as the first step. Then, the ultrastructure of *P. carinii* was studied to reveal the life cycle, especially the development of intracystic bodies and the multiplication of trophozoites.

Materials and Methods

The Wistar rats weighing approximately 200 g were injected subcutaneously with 25 mg cortisone acetate twice a week to provoke *P. carinii* pneumonia. All the rats were provided *ad libitum* with the drinking water containing 500 mg of tetracycline per liter to prevent bacterial infection (Frenkel *et al.*, 1966). These rats were sacrificed 8–14 weeks after the initiation of the treatment. The lungs were removed and cut into small pieces. They were transferred into the plastic syringe described later, and immersed completely in a fixative for 10–15 minutes. When they hardened and became solid, they were minced into strips about 1×1×3 mm, and transferred again into the syringe with the fresh fixative.

The tip of the plastic syringe was melted down by heat, and a pinhole was made at the scale of 3–4 ml by a redhot pin. The plunger was slowly pushed until air in the syringe was completely extruded through the pinhole. Then, occluding the pinhole with a thumb, the plunger was strongly pushed and pulled several times to eliminate the air from the alveolar lumen (Ebe *et al.*, 1968). When the strips went to the bottom, they were transferred and immersed into the fresh fixative for 2 hours. The fixative was cooled in cracked ice.

Some lungs of rats were utilized to prepare a pellet specimen by the application of the cyst concentration method (Ikai *et al.*, 1977.) In brief, the lung tissue was homogenized and centrifuged. The sediment was digested with 0.1% collagenase-physiological saline solution and incubated at 37 C for 2 hours. The digested fluid was filtered through a sheet of gauze and centrifuged. The sediment was fixed in buffered glutaraldehyde for 2 hours. After three times repetition of centrifugation and washing with buffer solution, the last sediment was embedded in 2% agar to make a pellet.

The strips and the pellets were fixed in several solutions as follows: (a) 0.6%–3% buffered potassium permanganate (This fixative was not applied to the pellet specimen.) (b) 2.5% buffered glutaraldehyde followed by 0.6% buffered potassium permanganate (c) 2.5% buffered glutaraldehyde followed by 1% buffered osmium tetroxide. The buffer was prepared according to Millonig (1962) and adjusted to pH 7.4. For practical usage, 2% osmium tetroxide stock solution was diluted with the equal quantity of the double concentrated buffer solution. In the case of glutaraldehyde and potassium permanganate, 5% glutaraldehyde and 1.2%–6% potassium permanganate were also diluted with the equal quantity of the double concentrated buffer solution. All the fixation processes were performed in cracked ice.

After the fixation, the tissue was dehydrated in a graded ethanol series followed by two changes of propylene oxide, and embedded in epoxy resin. The blocks were cut on Porter-Blum I microtome with glass knives. The ultrathin sections were picked up on uncoated copper grids, and stained with uranyl acetate and Reynolds' lead citrate (Watson, 1958; Reynolds, 1963).

Other portions of the lungs were fixed in 10% formalin and embedded in paraffin.

Sections were stained with hematoxylin and eosin, toluidine blue 0 (Chalvardjian and Grawe, 1963), and Gomori's methenamine silver nitrate (Grocott, 1955). Impression smears were also stained with Giemsa stain and toluidine blue 0. I have confirmed light-microscopically the existence of *P. carinii*, and excluded the samples with the infection of other causes.

Results

I. Fixation and pellet preparation procedures

At first, the above-mentioned fixation methods were attempted to find the most appropriate method for the various organelles of the organism. The tissue strips were fixed for 15 minutes to 2 hours in the buffered permanganate solution of which the concentration was varied from 0.6% to 3%. The contents of *P. carinii* leached out extremely during fixation and dehydration. In spite of thickness of the pellicle, the components of the organism tended to dissolve out more intensively than those of host cells. The best preservation within the range tested was accomplished, when the tissue was fixed in 0.6% buffered permanganate for 30 minutes. Even in this condition, all the organelles but a nuclear mass and several granules were not recognized. Furthermore, the unit membrane of pellicle was not observed.

Subsequently, it was attempted to fix the tissue in 2.5% buffered glutaraldehyde, followed by 0.6% buffered permanganate. The good preservation was provided, when the tissue was fixed in the glutaraldehyde for 2 hours, and postfixed in the permanganate for 20–30 minutes. The plasmalemma was distinctly observed. The contour of pellicle was frequently delineated clearly. It is also the merit of this fixation method that cristae of mitochondria are definitely recognized, although distinct cristae are scarcely observed in glutaraldehyde-osmium fixation. For example, the cristae of mitochondria in intracystic bodies are visible in this fixation (Fig. 2 C), although those are not found in the case of glutaraldehyde-osmium fixation (Fig. 16). Fig. 1 A and Fig. 2 B show the mitochondria of trophozoites and cysts fixed in the glutaraldehyde-permanganate solutions.

As mentioned above, the glutaraldehyde-permanganate fixation brings remarkable preservation of the organism, especially of membrane system. But it cannot supplant glutaraldehyde-osmium fixation, because the former has the fault that some parts of the organism are preserved well but the others are not. For example, pellicles of intracystic bodies and cysts were not recognized so clearly as cristae of mitochondria in intracystic bodies, as shown in Fig. 2. It seems due to intensive dissolution of the organism during fixation and dehydration. Furthermore, host cells and the organisms showed somewhat granular texture in comparison with those obtained by glutaraldehyde-osmium fixation. The tissue density and contrast were also deficient. Therefore, it was attempted to immerse the tissue in aqueous uranyl acetate solution after the fixation for enhancement of the tissue density and contrast (Ishizaki, 1970). This procedure, however, proved inappropriate, because it facilitated dissolution of the organism.

When the tissue was fixed in the buffered glutaraldehyde solution for 2 hours and postfixed in the buffered osmium solution for 2 hours, satisfactory preservation was obtained. Although negative and positive pressures were applied to sink the tissue strips, no ultrastructural change was found both on the organisms and on the host cells.

In addition, it was attempted to fix the tissue in hypotonic buffered permanganate solution. In this fixation, trophozoites usually swelled out as balloons, whereas cysts were scarcely transformed.

The method to prepare a pellet specimen

didn't affect the ultrastructure of precysts, cysts and host cells. The precyst shown in Fig. 11 is derived from a pellet and the precyst in Fig. 10 from a tissue strip. Both precysts have the thin pellicle which is identical with that of trophozoites, and therefore they are expected to be affected more easily than cysts by various factors. Judging from these figures, it is clear that precyst is not affected by this method. With regard to trophozoites, this method has also no affection on the ultrastructure of their pellicle and their organelles, though it may affect the shape of pleomorphic trophozoites to some extent.

II. The ultrastructure of *P. carinii*

P. carinii has several structurally distinctive forms, and each of them is called by various terms. The nomenclature reflects authors' view on the taxonomic position of *P. carinii*. In the present paper, those have been and will be tentatively called trophozoites, precysts, cysts, and intracystic bodies, because those are concise and most frequently applied. By the way, trophozoites have been called trophic stages (Vavra and Kučera, 1970), thin-walled cysts (Vossen *et al.*, 1978), t-forms (Price and Hughes, 1974), dünnwandige Pneumocysten (Seifert and Pliess, 1960), or zartwandige Cysten (Wessel and Ricken, 1958). Cysts have been termed thick-walled cysts (Barton and Campbell, 1969), c-forms (Price and Hughes, 1974), dickwandige Pneumocysten (Seifert and Pliess, 1960), or derbwandige Cysten (Wessel and Ricken, 1958). Intracystic bodies have the synonyms: daughter cells (Vossen *et al.*, 1978),sporozoites (Murphy *et al.*, 1977), and Cystenkörperchen (Wessel and Ricken, 1958; Seifert and Pliess, 1960).

(A) Trophozoites

Among the various forms, trophozoites are most frequently found in the alveoli of infected rats. They form an aggregated mass along the alveolar wall (Fig. 3) and sometimes fill up the alveolar lumen. The pellicle of trophozoites measures 20–30 nm across, and consists of an outer electron-dense layer and an inner unit membrane. They contain a nucleus with a diameter of 0.5–1.5 μm. The nucleus has a nucleolus and a nuclear membrane (Figs. 1 B, 4, 5). The nuclear membrane is often fragmentary. Rough-surfaced endoplasmic reticulum, mitochondria, granules, and vacuoles are found in the cytoplasm. Rough-surfaced endoplasmic reticulum is rarely found, and demonstrates direct continuity with a nuclear membrane (Fig. 5). A mitochondrion is usually round or oval, and its matrix is dense. Cristae of mitochondria are scarce and appear to be lamellar in glutaraldehyde-permanganate fixation (Fig. 1 A). Two kinds of granules are found in the cytoplasm (Fig. 4). One is the small, round, and electron-dense granule which is often aggregated. It is thought to be a glycogen granule. The other is the medium-sized, round, and moderately electron-dense granule supposed to be a lipid globule. Vacuoles are recognized especially in large trophozoites, and are at times delineated by a membrane (Figs. 1 B, 3, 5).

Trophozoites can be subdivided into two types on the basis of their shape and size. One type is the small and round, or oval, organism with a rather smooth pellicle. It usually adheres to the alveolar wall closely. It is also found to exist in the concave of collapsed and crescent cysts (Figs. 1 B, 4, 25). Judging from these features, it is conjectured that this small type has been just released from the cysts. The other type is large, up to 6 μm in the maximum diameter, and very irregular in shape (Figs. 3, 6, 7, 8). In this type, the contents of cytoplasm are lost in various degrees, and then vacuoles are formed. In the case of extreme vacuolation, the contents are completely deprived, and the trophozoite appears to be a ghost (Figs. 6, 9).

The large trophozoites have infoldings or invaginations. The gross folds are called

pseudopodia. The small protrusions are termed tubular expansions by Vavra and Kučera (1970) or filopodia by Barton and Campbell (1969). Tubular expansions are shown as ring-like cross sections (Figs. 3, 6). Their diameters are mostly in the range of 80–100 nm, although they have wide range of size. Their pellicle consists of an inner unit membrane and an outer electron-dense layer. This construction is identical with that of trophozoites' pellicle. The inside of tubular expansions is usually empty, although electron-dense granules are sometimes contained (Fig. 3). As shown in Fig. 7, tubular expansions are directly connected with trophozoites. Accordingly, they prove to be the extending parts of trophozoites. They are almost exclusively found around the large trophozoites, especially on the side of the parasites facing the epithelium or the other parasites (Figs. 3, 6). Occasionally, they, as well as pseudopodia, invaginate into the cytoplasm of host cells (Fig. 8). In almost all cases, both the pellicle of the organisms and the cytoplasmic membrane of host cells are intact. The gap was observed between them.

By contrast, trophozoites have sometimes the deep and narrow subsidence of pellicle supposed to be a groove or a well on the surface of the organism (Figs. 3, 9). But the structures which imply pinocytosis, binary fission, or conjugation are not found in the vicinity of the subsidence.

(B) Precysts

Precysts are defined as the intermediate form between trophozoites and cysts. Trophozoites and cysts are defined distinctly from the structure of their pellicles. I think a pellicle is the most suitable and reasonable feature to discriminate them. But precysts have not so obvious features as trophozoites and cysts. The most remarkable characteristic of precysts is aggregated mitochondria (Figs. 10, 11, 12). The mitochondria have dense matrix and vesicular cristae, while the mitochondria of trophozoites fixed in the glutaraldehyde-permanganate solutions have lamellar cristae, as mentioned above (Fig. 1). But this difference seems due to fixation method, because the mitochondria of trophozoites fixed in the glutaraldehyde-osmium solutions have also vesicular cristae (Fig. 3).

Precysts are not irregular-shaped but frequently ovoid, measuring 4–5 μm in diameter. They still have some tubular expansions. No nucleus is found in precysts so far as I have examined. The cytoplasm contains glycogen granules and ribosomes, and it is rather dense. Furthermore, membranous structures are frequently recognized. They may serve as a reservoir of the membranous component which is utilized in intracystic body formation (Figs. 11, 12).

Precysts have two kinds of pellicles. One is the same pellicle as that of trophozoites which consists of an inner unit membrane and an outer electron-dense layer (Figs. 10, 11). The other is composed of three layers and is identical with the pellicle of cysts, as discussed later (Fig. 12). The middle electron-lucent layer may appear between the unit membrane and the outer electron-dense layer, and consequently the pellicle becomes of cyst type.

(C) Cysts

Cysts are subdivided into two forms: the cyst containing intracystic bodies, and the collapsed cyst from which intracystic bodies have already escaped. Cysts are oval or crescent, and smooth in contour. They measure 3–5 μm across (Figs. 2, 15, 20–25). They still have a few tubular expansions. The pellicle of cysts consists of three layers: an outermost electron-dense layer, a middle electron-lucent layer, and an innermost unit membrane. The pellicle is 70–140 nm thick as a whole. The outermost layer measures 30–60 nm across and the middle layer 50–100 nm. Thickness of the pellicle and irregularity of the contour may be related to the maturity of cysts. As shown in Fig. 20, a mature cyst filled up completely with the

intracystic bodies has a rather thick and irregular-shaped cyst wall with a remarkably thickened middle electron-lucent layer.

Occasionally, the thickened portion of pellicle is observed as shown in Figs. 21, 22, and 24. It is approximately twice as thick as the other portion. This is due to the thickening of a middle electron-lucent layer. By the way, darkly-stained spots are often found light-microscopically in the cysts stained with Gomori's methenamine silver nitrate, which impregnates cyst walls with silver particles. The darkly-stained spots are discoid, opposed commas-like, or parentheses-like, as shown in Fig. 13. In faintly-stained cysts, both sides of the opposed commas are occasionally connected end to end by thin delicate strands. In heavily-stained cysts, these spots appear to be discoid. It is assumed that the thickened portion of pellicle corresponds to the darkly-stained spot.

In young cysts with unaccomplished intracystics bodies, the cytoplasm is as rich in mitochondria and granules as in precysts (Figs. 14, 15). With the maturation of intracystic bodies, cyst's organelles disappear and then vacuolated area increases. Occasionally, degenerated mitochondria persist in the cyst cavity (Fig. 19). When intracystic bodies leave the cysts, they collapse and come to be cup-shaped or crescent. In a crescent cyst, as shown in Fig. 23, the cytoplasmic debris is enclosed with the membrane which is supposed to be a contracted innermost unit membrane of cyst wall. Although the outermost electron-dense layer remains to be intact, the inner boundary of the middle electron-lucent layer comes to be unclear. The middle layer appears to be granular in parts. The collapsed cyst is shown also as a ring-like cross section (Fig. 24). The concave cavity in Fig. 23 corresponds to the central area surrounded by the cyst wall in Fig. 24. A crack of the cyst wall is found in the center of the thickened portion, and it exists on the convex of the cyst. The crack runs from the cyst cavity through the cyst wall to the outside. Intracystic bodies must have left the cyst through the crack.

(D) Intracystic bodies

A cyst contains up to 8 intracystic bodies which paly a role in reproduction. Intracystic bodies measure 1–2 μm across. They have the nucleus with a nucleolus and a nuclear membrane. They have also rough-surfaced endoplasmic reticulum and a mitochondrion, though they have not glycogen granules and lipid globules (Figs. 2 C, 14–22). Rough-surfaced endoplasmic reticulum is connected with the nuclear membrane (Figs. 2 C, 16–18). Mitochondria have several cristae and dense matrix. The cristae are lamellar in glutaraldehyde-permanganate fixation. Mitochondria are sometimes denser than other portion of the cytoplasm (Figs. 2 C, 16).

Intracystic bodies are subdivided into 5 types, based on the structure of pellicle and the figure as a whole. The first type, which is the most primitive, is surrounded with a single membrane. In this stage, the cytoplasm of cysts remains still in plenty (Figs. 14–17). The cyst in Fig. 14 has aggregated mitochondria and two masses supposed to be intracystic bodies. They are delineated with the membrane which appears to be a fine line. Each of them has the relatively electron-dense mass which may be a nuclear mass, though the boundary of the mass is unclear. The right intracystic body has an extremely electron-dense mass in the nuclear-like mass. The similar extremely electron-dense mass is also found in the intracystic body-like mass, the pellicle of which is not clearly recognized. Those are considered to be nucleoli. Five intracystic bodies and scattered mitochondria are found in the cyst shown in Fig. 15. Each intracystic body has a nucleus, though a nuclear envelope is not recognized. Its pellicle is more prominent than that of intracystic bodies in Fig. 14. In the central intracystic body,

its pellicle is partially formed and it seems to be at the midst of pellicle formation. Although the intracystic body in Fig. 16 has the similar pellicle to that of the intracystic bodies in Fig. 15, the former demonstrates a more definite nucleus with a nucleolus, rough-surfaced endoplasmic reticulum, and a round moderately electron-dense body. Judging from the feature of mitochondrion in Fig. 2 C, this round body is also supposed to be the mitochondrion, the crista of which is not recognized. Intracystic body shown in Fig. 17 has an apparent single unit membrane on its surface. However, the age of this cyst is not much different from that of the above-mentioned cysts, because much cytoplasmic substance still remains.

By the way, the pellicle of intracystic bodies in Fig. 14 and 16 is not thicker than the unit membrane of cyst wall. However, the pellicle of intracystic bodies is thicker than the unit membrane of cyst wall in the case of Fig. 15. Therefore, these intracystic bodies might begin the transition to the second type. In addition, the fact that five intracystic bodies in Fig. 15 are nearly in the same stage suggests that intracystic bodies in a certain cyst develop almost simultaneously.

The second type of intracystic bodies is found in the cyst where vacuolation has considerably progressed. Its pellicle is composed of an outermost unit membrane, a middle electron-dense layer, and an innermost unit membrane (Fig. 18).

The third type is usually seen in highly vacuolated cysts. This type is characterized by its pellicle composed of an inner unit membrane and an outer electron-dense layer (Fig. 19). This pellicular structure is identical with that of trophozoites. The intracystic bodies of type 1–3 are all sperical in shape and measure 1–1.5 μm across.

Fig. 20 shows the cyst completely filled with intracystic bodies. They are of the fourth type. The intracystic bodies of this type are relatively irregular in shape and larger than the third type, though they have the same pellicle as the third type.

The fifth type is very electron-dense and elongate or banana-shaped (Fig. 21). This type is also found in highly vacuolated cysts. It has a nucleus, and rough-surfaced endoplasmic reticulum. Although the pellicle seems to be similar in appearance to that of trophozoites, it is frequently detouched from the cytoplasm. While dense intracystic bodies are at times found among the other types, the detouchment of pellicle is not observed among them. Therefore, this type is considered to be a degenerative form.

The third and the fourth types have a strong resemblance to small free trophozoites which are frequently found in the concavity of collapsed cysts (Figs. 1 A, 25). Accordingly, they must be expelled from cysts through the crack of cyst wall, though the crack doesn't necessarily exist on the concave of collapsed cysts, as shown in Fig. 24. In addition, intracystic bodies are sometimes attached by a strand to the cyst wall or the cytoplasm adhering to the cyst wall (Fig. 22). The intracystic bodies in Fig. 22 seem to be degenerative.

(E) Other findings

The existence of cell division and propagation of the organism in the stage of trophozoites is a matter of interest. In this study, the trophozoite containing a daughter trophozoite is found in the alveolar lumen, though it is not so frequently found (Fig. 26). Although the inner unit membrane is not recognized in the pellicle of a mother trophozoite, its pellicle is as thick as that of the daughter trophozoite. The mother trophozoite is irregular in shape and has several tubular expansions. It also contains much cytoplasm, though the definite organelles are not found. On the other hand, the daughter trophozoite has a typical pellicle and the electron-dense mass which is suggestive of a nucleus.

In addition, the mother trophozoite may include more than one daughter trophozoite,

because the shape of the daughter trophozoite is complicated. Although the number of daughter trophozoites cannot be exactly estimated, these findings strongly support the multiplication of the organism in the stage of trophozoites as well as in the stage of cysts.

Multiple macrophages with foamy cytoplasm are often found in the alveolar lumen. They usually have the phagosomes including amorphous material. The organisms of distinct figure are scarcely found in phagosomes, and therefore it is supposed that the ingested organisms are rapidly digested. However, the intact organisms are occasionally found in phagosomes. In Fig. 27, the trophozoite which still has tubular expansions and a typical pellicle is found in a phagosome. An electron-lucent layer is partially recognized between an inner unit membrane and an outer electron-dense layer of its pellicle. It may imply either the beginning of transformation into cyst stage or the degenerative change caused by phagocytosis.

Discussion

I. Fixation and pellet preparation

Permanganate fixation and glutaraldehyde-permanganate fixation were used in the present study. Permanganate was introduced, for the first time, as a new fixative for electron microscopy by Luft (1956). He applied 0.6% buffered permanganate solution to animal tissues. Thereafter, Mollenhauer (1959) utilized 2–5% buffered or unbuffered permanganate solution for plant cells, and he and his colleague utilized 3% buffered or unbuffered permanganate solution for animal tissue (Zebrun and Mollenhauer, 1960; Mollenhauer and Zebrun, 1960). Seifert and Pliess (1960) utilized permanganate as a fixative for *P. carinii* and stated that the figure of mitochondria and nuclei in this fixation was better than that in osmium fixation. They also mentioned that the halo around the unit membrane of tubular expansions showed more enhanced contrast in permanganate fixation.

Vavra and Kučra (1970) also reported as follows: *P. carinii* is usually sensitive to slight variation in technical procedures, especially fixation. Trophozoite is not well preserved even when fixed in the osmotically adjusted glutaraldehyde-osmium fixative. Only the rapid perfusion of the alveoli with buffered and isoosmotic permanganate fixative yields relatively good results. However, they described neither concentration nor duration of this fixation. Accordingly, permanganate fixation was attempted under the various conditions, but it proved inferior to glutaraldehyde-osmium fixation.

As the next step, glutaraldehyde-permanganate fixation was tried. This procedure has currently been applied to fungi. Egashira *et al.* (1970) indicated that this procedure was superior to permanganate fixation because of better preservation of organelles, especially of storage organelles. Osumi (1967, 1970) stated that *Saccharomyces* fixed in glutaraldehyde-permanganate solutions showed smooth texture in comparison with permanganate fixation, although tissue density and contrast were deficient and membrane systems were somewhat unclear. The present study indicated that *P. carinii* is well preserved, when it is fixed in 2.5% buffered glutaraldehyde for 2 hours and postfixed in 0.6% buffered permanganate for 20–30 minutes. With regard to membrane systems, this fixation is superior to glutaraldehyde-osmium fixation. This characteristic coincides with that of permanganate fixation stated by Luft (1956). Besides, the cytoplasm in this fixation is not so granular as that in permanganate fixation in accordance with the description of Osumi (1967, 1970).

Fungi are frequently fixed in aqueous permanganate solution alone, or preceded by aqueous glutaraldehyde solution (Ega-

shira *et al.*, 1970). In the present study *P. carinii* was also attempted to fix in hypotonic buffered permanganate solution. Although cyst didn't show any apparent affection of the hypotonic solution, trophozoites swelled like balloons. Then, trophozoites proved susceptible to osmotic pressure in agreement with the report of Vavra and Kučera (1970). In addition, they insisted that *P. carinii* reacted to buffered or unbuffered permanganate fixation in the same way as fungi, and adopted this fact as one of the reasons why *P. carinii* was classified as a fungus rather than as a protozoan. However, I have failed to preserve *P. carinii* well even in buffered permanganate.

It became evident that the pellet specimen was very useful in observasion of the organism and host cells. The cyst concentration method (Ikai *et al.*, 1977) was applied to the pellet preparation. It turned out that this procedure had not affected the ultrastructure of host cells and various stages of the organism. Recently, Walzer *et al.* (1979) reported a new separation method of *P. carinii* from lung tissue. According to their method, the lung pieces were ground through a wire mesh screen with a Teflon pestle, washed with Hank's balanced salt solution, and centrifuged. Then the sediment was digested with collagenase and hyaluronidase. In their electron microscopic examination after the digestion, few affection by this method was found on the ultrastructure of each stage of *P. carinii*. Their results are in agreement with those of the present study. Therefore, *P. carinii*, even in the stage of trophozoites, seems to be resistant to a certain degree of mechanical and enzymatic treatment.

II. Ultrastructure of *P. carinii*

In this report, I will deal with *P. carinii* derived from both human and rat as the identical species, although it is still at issue whether the organisms of both origins are identical. Seifert and Pliess (1960) suggested morphological differences between them. However, Barton and Campbell (1967), Vavra and Kučera (1970), Huang and Marshall (1970), and Campbell (1972) regarded them as the identical species. I suppose that the morphological differences may be caused for the most part by the poor preservation of human material, because none of the previous authors examined fresh human material obtained by biopsy. Even Frenkel (1976), who has insisted that the organisms of both origins are quite different species and has proposed that the organism of human origin should be designated *Pneumocystis jiroveci* and the name of *P. carinii* should be restricted to the organism from rats, has accepted that significant morphological differences are not found.

(A) Trophozoites

Trophozoites have a nucleus with a nucleolus and a nuclear membrane, although the nuclear membrane is frequently fragmentary. Rough-surfaced endoplasmic reticulum, mitochondria, glycogen granules, and lipid globules are also found in their cytoplasm. I could confirm all the organelles described by other authors (Seifert and Pliess, 1960; Barton and Campbell, 1969; Vavra and Kučera, 1970; Campbell, 1972; Vossen *et al.*, 1978), except the round body which is also contained in intracystic bodies, according to Barton and Campbell (1969).

The pellicle of trophozoites, including tubular expansions or filopodia, was the subject of much controversy for many years, and the controversy sometimes lapsed into confused interpretations. For examples, Vavra and Kučera (1970) stated as follows: Trophozoites are covered completely by a multilayered pellicle 300 A wide. Particularly in glutaraldehyde-osmium fixed parasites it can be further resolved into a unit membrane which limited the cytoplasm of the parasite and several, usually 4, less conspicuous layers of alternating density. When osmium alone is used for fixation, the pellicle looks like a single membrane

covered externally by a thick electron-dense layer. Permanganate fixation shows the pellicle as one dense layer. In the present investigation, however, the pellicle consists of a unit membrane and an electron-dense layer in both glutaraldehyde-osmium fixation and glutaraldehyde-permanganate fixation. This result accords with the report of Vossen *et al.* (1978), though they utilized glutaraldehyde-osmium fixation only.

Trophozoites usually have complicated fine projections designated tubular expansions (Vavra and Kučera, 1970) or filopodia (Barton and Campbell, 1969). They were also termed fein tubulaere Strukturen (Seifert and Pliess, 1960), villous-like projections (Huang and Marshall, 1970), or microvillous tubular structures (Ham *et al.*, 1971). By many transmission and scanning electron microscopic studies (Huang and Marshall, 1970; Murphy *et al.*, 1977; Sueishi *et al.*, 1977; Tamura *et al.*, 1978; Uni *et al.*, 1978), it is generally accepted that the tubular expansions are the projected parts of the organism, although Price and Hughes (1974) interpreted them as reactive alveolar macrophage plasma membranes. Fig. 7 in the present study also shows the evidence clearly.

With regard to the significance and the function of tubular expansions, several interpretations have been proposed up to now. First, Seifert and Pliess (1960) stated as follows: The pellicle of tubular expansions has the same structure as that of trophozoites. The tubular expansions usually appear with the degeneration of trophozoites, and have a strong resemblance to myxoviruses, especially influenza viruses, in their size, distribution, and structure. They must have believed that the formation of tubular expansions implicated the degeneration of virus-infected trophozoites and the multiplication of viruses. After that, two reports concerning the concurrent infection of *P. carinii* and virus were published. Wang *et al.* (1970) reported two human cases of combined *P. carinii* and cytomegalovirus infection. They showed three possible explanations of the combined infection: First, *P. carinii* and cytomegalovirus may be symbiotic; secondly, *P. carinii* may play the role of an intermediate host; thirdly, both human tissue and *P. carinii* may be infected at the same time with cytomegalovirus. Vawter *et al.* (1970) also reported two human cases of *P. carinii* and respiratory syncytial virus infection, and suggested the symbiosis or the parasitism between the two pathogens. However, Price and Hughes (1974) mentioned that the presence of one or more infectious agents in addition to *P. carinii* was associated with host immunological and nutritional deficiencies, and that this fact didn't necessarily reflect parasitic symbiosis. In any case, these reports may be favorable to the interpretation of Seifert and Pliess (1960).

On the other hand, the hypothesis of Brzosko and Wojewódzka (1969) is as follows: The emergence of tubular expansions is an expression of adaptive mechanisms of the parasite related to unfavorable environmental conditions. Namely, the immunological phenomena occuring on the parasite membranes and damaging them may stimulate their compensative increase of the surface for nutrient intake. It is not excluded, however, that in unfavorable conditions tubular expansions may arise as a result of the rejection of membrane fragments which have been blocked with antibodies and thus have become functionally less valuable.

All the above-mentioned authors seem to recognize tubular expansions as a sign of degeneration. Judging from the fact that the tubular expansions invaginate deeply and firmly into the host cells as shown in Fig. 8, those must have aggressive and active functions. Vavra *et al.* (1968, 1970) explained that tubular expansions contributed to increase the absorptive surface of the parasite, and to anchor the parasite in the alveolar space either to the alveolar epi-

thelium or to other parasites. Besides, Murphy *et al.* (1977) examined the organism cultivated *in vitro* on monolayers of primary embryonic chick epithelial lung cells by scanning electron microscopy, and showed three kinds of tubular expansions: first, interparasitic fibers; secondly, fibers which appear to anchor the parasite to host cells; thirdly, fine fibrils common to both the parasite and a host cell. Although their interpretation on the functions of these structures was almost the same as Vavra and Kučera's (1970), they additionally suggested the new function that the interparasitic fibers might offer a conduit for interparasitic exchange of protoplasm. As described by Barton and Campbell (1969), *P. carinii* seems to metabolize principally low-molecular-weight substances, because the parasite lacks the organelles usually associated with phagocytosis or hydrolytic processes. Therefore, it is soon convinced that tubular expansions may play a role in exchange or procurement of material as well as in anchorage. However, the question of interparasitic exchange of protoplasm has to remain open, until the further investigation concerning the interparasitic fibers is performed.

By the way, Barton and Campbell (1967), as well as Ham *et al.* (1971), indicated the existence of "a complex system of anastomosing membranes". The former authors described that these membranes were typically composed of five layers including outer electron dense limiting layers, subjacent granular layers, and a central electron lucent layer. They also stated that these interlacing membranes formed irregular compartments containing electron dense glycogen-like particles, poorly defined aggregates of finely granular material, and other structures that could not be identified. But these structures are interpreted in the present author's opinion as follows: The irregular compartments are equivalent to trophozoites with a small quantity of the cytoplasm itself. The outer electron dense limiting layers and the subjacent granular layers correspond to the pellicles of two trophozoites which are parallel to each other. The central electron lucent layer represents the gap between two closely neighboring trophozoites.

Besides, Barton and Campbell (1967) demonstrated "polar membranous caps" as the characteristic structures for crescent cysts, and Shively *et al.* (1974) confirmed them as well. However, these structures are not specific ones for crescent cysts and consist of tubular expansions and pellicles of neighboring trophozoites in the present author's opinion.

(B) Precysts

Precysts are the transitional forms from trophozoites to cysts. Kim *et al.* (1972) demonstrated precysts by light microscopy. They stained an impression smear at first with polychrome methylene blue or Giemsa stain, and then stained the same organisms with methenamine silver nitrate. They observed, by the former stain, the large organisms with a thick wall, darkly stained cytoplasm, and one nuclear body. These organisms proved to have some capsular material by methenamine silver nitrate stain. Some of these organisms also appeared to be in nuclear division.

By electron microscopy, Campbell (1972) and Vossen *et al.* (1978) described precysts. Barton and Campbell (1969) reported also a transforming stage as the largest trophozoite. However, the descriptions of the above authors seem not to be sufficient and comprehensive. Indeed, it is difficult to give precise definition to precysts according to ultrastructural features, while trophozoites and cysts are distinctly defined on the basis of pellicular structure.

The interpretation of Vavra and Kučera (1968, 1970) seems to be most appropriate. Their view, as well as mine, is as follows: Precysts are the organisms with a few of tubular expansions, and are characterized

by a dense clump of aggregated mitochondria. Their pellicle, which is composed of two layers like trophozoites' at first, is transformed into three-layered one with the appearance of a middle electron-lucent layer. Then several nuclear masses appear in the precysts, together with the transformation of pellicle into three-layered one, or cyst wall. With the appearance of pellicle of intracystic bodies, precysts become cysts.

(C) Cysts

Cysts are characterized by the three-layered pellicle as mentioned above. Barton and Campbell (1967) once reported that cyst wall consisted of six layers and revised later to be composed of three layers (Barton and Campbell, 1969; Campbell, 1972). Occasionally, the inner unit membrane is not observed, and so the middle electron-lucent layer appears to blend with the internal structure of a cyst as shown in Fig. 24 (Shively, 1974).

In methenamine silver stained specimens, discoid or parentheses-shaped darkly stained spots are often observed in cysts as shown in Fig. 13. These spots are also recognized in toluidine blue 0 stain (Chalvardjian and Grawe, 1963). Up to now, these spots have been noticed by many investigators, and have been termed dark-staining ring-like structures, darkly staining ovoid intracystic bodies, parentheses-like structures, and so on (McNeal and Yaeger, 1960; Barton and Campbell, 1967; Kim and Hughes, 1973; Nagai *et al.*, 1978; Pintozzi *et al.*, 1979). Judging from their expression, some of them seem to have considered that the spots are related to a certain structure in the cytoplasm (Barton and Campbell, 1967; Nagai *et al.*, 1978). However, the present author thinks that the spots are correspond to the focal thickening of cyst wall from the following reasons (Figs. 21, 22, 24). The spots are found in both ovoid cyst and crescent cyst as shown in Fig. 13. The crescent cysts have already expelled intracystic bodies and they contain the cytoplasmic debris only. Furthermore, the cyst wall is mainly composed of neutral mucopolysaccharide, or chitin (Bruns and Boettger, 1955). Methenamine silver stain demonstrates mainly cellulose-chitin aggregate (Grocott, 1955). Therefore, this stain is thought to exhibit the cyst wall rather than the cytoplasmic debris. In addition, Kim *et al.* (1972) reported that by treating cysts with acid alcohol, the silver impregnation onto the cyst wall was inhibited, and the staining of these parentheses-like structures was blocked.

By electron microscopy, Huang and Marshall (1970) showed that the electron-dense layer of pellicle of both cysts and trophozoites, as well as that of tubular expansions, was stained strongly with methenamine silver. On the contrary, Huneycutt *et al.* (1964) stated that the silver had an affinity for central structures as well as pellicles. These reports are inconsistent mutually. However, it is impossible to mention the other structure which seems to be responsible for the spots on electron microscopic level, as indicated Vavra and Kučera (1970).

Bommer (1962) and Ham *et al.* (1971) reported that crescent cysts seemed to be characteristic stage of human *Pneumocystis* organism, because the crescent cysts were rarely found in rats. In the present study, however, the crescent cysts were frequently found. They are the most predominant form among various forms of cysts.

(D) Intracystic bodies

In the present study, the author proposed to subdivide intracystic bodies into five types mainly based on their pellicular structures and configurations as follows: (1) The pellicle is recognized as a single membrane at first, and later it exhibits the character of a unit membrane distinctly; (2) The pellicle is composed of an outermost and an innermost unit membrane and a middle electron-dense layer; (3) The pellicle is identical with that of trophozoites, and the configuration as a whole is round. This type is much the

same as so-called small trophozoites; (4) The intracystic bodies of this type have the same pellicle as type (3), but those are irregular in shape and fill the cyst cavity completely; (5) This type is elongate or banana-shaped, and extraordinarily electron-dense.

On the other hand, Barton and Campbell (1967, 1969) reported only one type of intracystic bodies the pellicle of which is identical with that of type (2), according to the present classification (Campbell, 1972). Vavra and Kučera (1970) reported 4 types of intracystic bodies. Three of them correspond to type (1), (3), and (4) of the present classification. Another type is described as follows: Although intracystic bodies are only slightly more electron-dense than the cyst cytoplasm in the beginning of their development, their opacity increases until they are nearly impenetrable for the electrons. Also their shape changes from round to elongate or banana-shaped. The thin original double membrane covering the young intracystic bodies become more prominent and is later covered by a thick layer of granular, electron-dense material limited outside by another double membrane which is, however, rather inconspicuous. It seems that they didn't observe the intracystic body of type (2) which is round and rather electron-lucent. Vossen *et al.* (1978) also didn't confirm the type (2). On the other hand, type (5) seems to have a thick pellicle as described by Vavra and Kučera (1970), though the outermost unit membrane of the pellicle has not been confirmed clearly in the present experiment. Therefore, it seems that the above-mentioned dense intracystic body corresponds to almost exactly type (5), and that it is appropriate to distinguish type (2) from type (5).

The intracystic bodies of type (2), (3), and (4) are in the stage ready for excystation in the author's opinion. However, Wessel and Ricken (1958), Seifert and Pliess (1960), and Vavra and Kučera (1970) considered that only type (4) could leave a cyst, and Vossen *et al.* (1978), type (3) and (4) could. On the other hand, Barton and Campbell (1969) described the trophozoites, the pellicle of which had focally the external membrane similar to the internal unit membrane (Campbell, 1972). These trophozoites remind the author of the intracystic bodies of type (2). Besides, Vavra and Kučera (1970) demonstrated the moment when the intracystic bodies of type (4) were just escaping from a cyst through a crack of the cyst wall. In addition, it seems that intracystic bodies grow and develop in the sequence from type (1) to type (4). Because the cytoplasm of cysts becomes progressively more vacuolar and more degenerative, accompanied with the development of intracystic bodies in the above-mentioned sequence, and finally the intracystic bodies of type (4) fill the vacuolated cyst cavity completely. Therefore, type (2) and (4) can leave cysts without doubt, and type (3), which is the intermediate form between type (2) and type (4), also must leave cysts as Vossen *et al.* (1978) suggested.

Furthermore, Vavra and Kučera (1970) supposed as follows: The stages leaving a cyst are larger than the younger free stages. This may suggest that the intracystic bodies divide soon after they have been expelled from the cyst. However, it is rather unnatural that intracystic bodies divide soon after their excystation. According to their description, the stages leaving a cyst correspond to the intracystic bodies of type (4) which are the largest of all types and the younger free stages correspond to the small trophozoites which are similar to the intracystic bodies of type (2) and (3). If type (2), (3), and (4) can leave a cyst as suggested by the present author, their observation can be explained simply and reasonably without the hypothesis that intracystic bodies devide soon after their excystation. It is natural that some trophozoites are smaller than the intracystic body of type (4), because the intracystic bodies of type (2) and (3), which are smaller than the intracystic body of type

(4), also leave a cyst.

The intracystic body of type (5) has been observed by Seifert and Pliess (1960), Vavra and Kučera (1970), and Vossen *et al.* (1978). Vavra and Kučera regarded it as the organism ready for a period of dormancy and as the resistant stage designed perhaps for dissemination of the parasite. They also supposed that it grew and developed inside a cyst into the intracystic body resembling young trophozoites. By contrast, Seifert and Pliess (1960) supposed that it might be a degenerative form, because it exhibited irregularity of size, vacuolization, and detachment of pellicle from the cytoplasm. The present author also regards it as a degenerative form, because the detachment of pellicle has never been observed among the other types, though rather electron-dense variants of other types are sometimes seen.

As to the origin of pellicle of intracystic bodies, two interpretations have been proposed. Many authors have observed that the pellicle was connected with the plasmalemma of cyst wall, as shown in Fig. 22 (Wessel and Ricken, 1958; Seifert and Pliess, 1960; Barton and Campbell, 1967, 1969; Campbell, 1972). Bommer (1962) and Vossen *et al.* (1978) supposed that the pellicle was formed by the invagination of the plasmalemma of cyst wall. On the other hand, Campbell (1972) indicated that the pellicle appeared to be derived from membranous material within the cyst and that it never appeared to be derived from the unit membrane of cyst wall. Vavra and Kučera (1970) suggested that the pellicle was formed at least partly *de novo* by synthesis inside the cyst cytoplasm, and that the invagination of the cyst plasma membrane might perhaps contribute to the formation of the pellicle. The present author's opinion is that the pellicle is formed *de novo*. Because the invaginating plasmalemma ought to become duplicated or to form a vacuole and unfold later in order to surround a nucleus and the adjacent cytoplasm, if the pellicle is derived from the invaginating plasmalemma of cyst wall and if the plasmalemma is intact and unruptured during the invagination. In fact a plasmalemma proved intact, even when coccidians enter into host cells (Bannister, 1977). Therefore, the latter assumption seems to be true and proper. By the way, the intracystic bodies of type (2) have a middle electron-dense layer of various thickness. Therefore, the middle electron-dense layer is supposed to be formed between two unit membranes. Then it comes into question how the second unit membrane is formed. If the pellicle of intracystic bodies is formed *de novo*, the processes to form the pellicle of type (2) seems to be too complicated. On the contrary it is explained simply, if the pellicle is derived from the invaginating plasma membrane. Accordingly further investigations will be needed to settle this problem.

In addition, round bodies and subpellicular microtubules were reported by other authors, although they could not be confirmed in the present study (Barton and Campbell, 1967, 1969; Vossen *et al.*, 1976).

(E) Host-parasite relationship

The phagocytized *P. carinii*, as shown in Fig. 27, has been observed by many authors (Seifert and Pliess, 1960; Price and Hughes, 1974; Tamura *et al.*, 1978; Vossen *et al.*, 1978). The parasite proved *in vitro* to be promptly digested after the interiorization by macrophages, even when they were under the influence of corticosteroid (Masur and Jones, 1978; Behren and Pesanti, 1978). These results may well correspond to the present investigation that the macrophages containing intact parasites in their phagosomes were hardly seen.

Behren and Pesanti (1978) indicated that *P. carinii* couldn't be an intracellular parasite, because they did not find the replication of the organism within macrophages in contrast to *Toxoplasma gondii*. On the other hand, Price and Hughes (1974) re-

ferred to the possibility of cyst accumulation and trophozoite replication in alveolar cells. In addition, Shively *et al.* (1974) showed the existence of trophozoites and cysts in the alveolar epithelium. Vossen *et al.* (1977, 1978) also demonstrated that trophozoites existed in the interstitial space, the capillary lumen, and the cytoplasm of epithelial cells, and that the trophozoites in the cytoplasm lay free or were surrounded by a parasitophorous vacuole membrane. Indeed, the parasite lying free may be intracellular, but more careful examination seems to be necessary to answer the question whether the parasite surrounded by the membrane is actually intracellular or merely invaginates into the epithelium, because the parasite frequently invaginates deeply as shown in Fig. 8. Furthermore, the latter authors showed a group of trophozoites in the frame work of the alveolar septum and suggested these trophozoites had been liberated from the epithelial cells. However, these trophozoites are quite similar to a group of trophozoites in the alveolar pore presented by Nagai and Kamata (1974). Consequently, the questions as to the intracellular development of the parasite and the deterioration of epithelial cells by the parasite seem to remain still open.

(F) Life cycle and Taxonomic position

Up to now, several views on life cycle of *P. carinii* have been proposed, based on electron microscopic observations (Wessel and Ricken, 1958; Seifert and Pliess, 1960; Vavra and Kučera, 1970; Campbell, 1972; Vossen *et al.*, 1978). All the views reported previously, as well as the author's view, coincide each other in the following points that trophozoites develop into cysts and that intracystic bodies formed within a cyst leave the cyst and become trophozoites again. However, there are some differences among these views in detail. For example, Wessel and Ricken (1958) indicated that intracystic bodies were formed in Napfcysts by constriction of the dense cytoplasm adhering to the cyst wall. With regard to trophozoites, Vavra and Kučera (1970) and Campbell (1972) suggested the possibility of binary fission, budding, or conjugation. On the other hand, Vossen *et al.* (1978) demonstrated that daughter cells were formed by endogeny in a trophozoite and that no difference was found between the daughter cells in a cyst and those in a trophozoite. The present findings, as shown in Fig. 26, are favorable to the latter view.

P. carinii is currently accepted to be a protozoan, although its exact taxonomic position is not confirmed yet. Wessel and Ricken (1958), Barton and Campbell (1969), Campbell (1972), Ham *et al.* (1971) and Vossen *et al.* (1976 and 1977) suggested it was a protozoan. Their views were based on the following features: First, the structures of mitochondria and pellicle of *P. carinii* resemble those of sporozoa; Secondly, subpellicular microtubules and pseudopodia are found in *P. carinii*, as well as in sporozoa. These organelles suggest that *P. carinii* is motile.

On the other hand, Seifert and Pliess (1960), and Vavra and Kučera (1970) proposed that *P. carinii* might be a kind of fungi. The former authors pointed out the intracystic body formation was similar to the spore formation in *Saccharomyces*. They also showed a similarity between the trophozoite and the thin-walled form of the yeast *Rhodotorula*. The later authors suggested that *P. carinii* might be a dimorphic fungus in which the mycelium was reduced to a unicellular state but in which the ability to sporulate was preserved.

However, it seems that the authors of two groups mentioned above gave different interpretations to the same structures or the same organelles. For instance, Barton and Campbell (1969) pointed out that the mitochondria of *P. carinii* having tubular and vesicular cristae were of the common protozoan type, while Vavra and Kučera (1970) stated that the mitochondria of many fungi

were relatively poorly developed and, as in *Pneumocystis*, had only a few lamellar cristae. Generally speaking, trophozoites seem to resemble rather protozoa, and cysts have a resemblance to fungi. However, the conclusive evidences have not yet been found to resolve whether *P. carinii* is a protozoan or a fungus. Therefore, it may be appropriate to interpret *P. carinii* as a bridge between protozoa and fungi for the present, as proposed by Hughes (1978). Because it is decisive that *P. carinii* is a unicellular and eukaryotic organism, namely, a member of the Protista.

Summary

P. carinii occured in cortisone-treated rats was investigated by electron microscopy. Several fixation methods and the pellet preparation by the application of the cyst concentration method were attempted. The ultrastructure of *P. carinii* and its life cycle from trophozoites through precysts to cysts were confirmed. Furthermore, I presented my own views concerned with development of intracystic bodies and multiplication in the stage of trophozoites. The results are summarized as follows:

(1) Glutaraldehyde-permanganate fixation is superior to glutaraldehyde-osmium fixation as to the preservation of membrane systems, especially of cristae of mitochondria.

(2) The pellet specimen was of great benefit in order to observe the organisms and host cells efficiently. Precysts, cysts, and host cells are not affected by the application of the cyst concentration method.

(3) According to pellicular structures and configurations as a whole, intracystic bodies are subdivided into 5 types as follows:

(i) The intracystic body surrounded with a single membrane which acquires later the character of a unit membrane.

(ii) The intracystic body with the pellicle composed of an outermost and an innermost unit membrane and a middle electron-dense layer.

(iii) The intracystic body with the pellicle composed of an inner unit membrane and an outer electron-dense layer. It resembles small trophozoites.

(iv) The intracystic body surrounded with the same pellicle as that of type (iii). They fill up the cyst cavity completely and each of them has a rather irregular shape in contrast with type (i)–(iii).

(v) The extremely electron-dense and banana-shaped intracystic body.

The intracystic bodies may develop in the sequence from type (i) to type (iv). Type (ii), (iii), and (iv) may leave the cyst through the crack of cyst wall. Type (v) may be a degenerative form.

(4) The trophozoite supposed to contain a daughter trophozoite was found. This observation is favorable to the view that trophozoites multiply by endogeny.

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Pneumocystis carinii の電子顕微鏡的観察

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Pneumocystis carinii は主として免疫不全状態の患者に肺炎を惹起する病原体であるが，悪性腫瘍に対する治療成績の向上と共にその対策は重要なものとなって来た．しかし *P. carinii* の分類学上の位置や生活史など，依然として不明な問題が多い．これらの問題はこれ迄主に電子顕微鏡による観察結果に基づいて論じられて来たが，未だに定説がない．例えば，分類学上の位置については原虫説と真菌説とがあり未だ結論に達していない．また，生活史でも未解明な部分が多く trophozoite が発育して cyst となり増殖する事について疑いはないが，その他にも二分裂，出芽または内生（endogeny）によって増殖する可能性が指摘されている．著者はこれらの問題点について更に検討を行う為コーチゾン投与によりラットに発生させた *P. carinii* を用いて電子顕微鏡的観察を行った．まず著者は *P. carinii* の観察に最も適した固定法や試料作製法を見出すための検討を行った．従来，グルタルアルデヒド・オスミウム酸二重固定が *P. carinii* の固定に広く用いられてきたが，今回の研究によるとミトコンドリアのクリスタなどの膜構造の観察にはグルタルアルデヒド・過マンガン酸カリウム二重固定が適している事が判った．Vavra and Kučera (1970) は過マンガン酸カリウム固定を賞用したが，今回の実験では溶出が激しく良い結果を得る事はできなかった．

また，集シスト法（猪飼ら，1977）を応用してペレットを作製し材料としたところ precyst, cyst およびラットの細胞は良好な状態に保たれ効率的に観察する事が出来た．

P. carinii の微細構造や生活史に関しては，従来の報告とほぼ一致する結果を得たが，intracystic body の発育と trophozoite 段階での増殖についいては新たな所見を得た．すなわち intracystic body はこれ迄の報告では1ないし4種類認められていたが，著者は外皮の構造や形態に基づいて次の様に5種類に区分することを提唱した．

（1） 外皮が極めて薄い一層の膜から成るもの．後にこの膜は単位膜である事が明瞭となる．

（2） 外層と内層は単位膜で，中間層が電子密度の高い層からなり，全体の形がほぼ円形のもの．

（3） 外皮は単位膜と電子密度の高い層からなり，外形はほぼ球状を呈し，小型の trophozoite に類似しているもの．

（4） 外皮の構造は (3) と同一であるが，cyst 内腔に充満しやや複雑な外形を示すもの．

（5） 半月状で電子密度が高く，しばしば外皮の剝離を伴うもの．

cyst の細胞質の変化の過程から判断すると，(1) から (4) の順に発育し，(2)～(4) の各型が脱嚢し得るものと考える．また，(5) は変性型であると考えられる．

daughter trophozoite を内部に持つ trophozoite を観察した．これは trophozoite 内でも cyst 内での intracystic body 形成と同じ方法で増殖が行なわれているのではないかという Vossen *et al* (1978) の説を支持する所見である．

Explanation of Figures

Fig. 1A A trophozoite with a mitochondrion (M) showing definite laminar cristae. The trophozoite exists in the concave of a collapsed cyst (C) and contains another mass supposed to be a nucleus. Its pellicle consists of a unit membrane and an electron-dense layer. Tubular expansions (arrow head). Glutaraldehyde-Permanganate. ×42,000.

Fig. 1B A trophozoite. Cytoplasm of the organism contains a nucleus (N), vacuoles, and the small mass (arrow) which may correspond to a so-called round body. One of vacuoles (V) is partially surrounded with a membrane and contains several granules. The plasmalemma is clearly shown, but the nuclear membrane is not. The texture is somewhat granular. Glutaraldehyde-Permanganate. ×67,000.

Fig. 2A A mature cyst with three intracystic bodies (ICB), three mitochondria (M), and a small amount of cytoplasmic debris. Two mitochondria are degenerative. A nucleus, rough-surfaced endoplasmic reticulum, and a mitochondrion in the intracystic bodies are well preserved, but the pellicular unit membranes of cyst and intracystic bodies are not. Glutaraldehyde-Permanaganate. ×15,000.

Fig. 2B Mitochondria with their membranes, lamellar cristae (asterisk), and dense granules. Cyst wall (CW). Glutaraldehyde-Permanganate. ×66,000.

Fig. 2C An intracystic body with a mitochondrion showing their membranes and lamellar cristae (M). The cytoplasmic debris adheres to the cyst wall (CW) of which the inner unit membrane is not visible. A nuclear membrane demonstrates direct continuity with rough-surfaced endoplasmic reticulum (ER). Nucleus (N). Glutaraldehyde-Permanganate. ×39,000.

Fig. 3 A group of trophozoites along the alveolar wall. The trophozoites adhere closely to the alveolar wall and have tubular expansions with various diameters among them. Mitochondria with vesicular cristae (M); Tubular expansions containing granules (asterisk); A membrane-bound vacuole with granules (V); A deep subsidence of pellicle (arrow). Glutaraldehyde-Osmium. ×13,000.

Fig. 4 Two small trophozoites adhering to the alveolar wall. The left one has a nucleus (N) with a nucleolus and a nuclear membrane. Lipid globule (arrow); A cluster of glycogen granules (arrow head). Glutaraldehyde-Osmium. ×12,000.

Fig. 5 A trophozoite of which the rough-surfaced endoplasmic reticulum (ER) demonstrates direct continuity with a nucleus (N). The pellicle of the trophozoite, including tubular expansions, consists of a unit membrane and an electron-dense layer. Vacuole (V). Glutaraldehyde-Osmium. ×30,000.

Fig. 6 Vacuolated trophozoites (T) have tubular expansions on the side facing to the alveolar wall or to each other. Glutaraldehyde-Osmium. ×12,000.

Fig. 7 A large trophozoite, the cytoplasm of which is partially compressed, has tubular expansions (arrow). Glutaraldehyde-Osmium. ×14,000. *Inset*; Detail of the tubular expansions. They are continuous with the cytoplasm. ×31,000.

Fig. 8A Pseudopodia and tubular expansions of trophozoites (T) invaginate into an alveolar cell. Nucleus (N). Glutaraldehyde-Osmium. ×11,000.

Fig. 8B Detail of the invaginating part. The pellicle of pseudopodia (asterisk) and tubular expansions (arrow head) is composed of a unit membrane and an electron-dense layer. They are surrounded with the cytoplasmic membrane of the host cell. ×32,000.

Fig. 9 A trophozoite (T) with a deep subsidence of pellicle (arrow) and vacuolated trophozoites (T). The one surrounded with tubular expansions in the lowest part appears as a ghost. Glutaraldehyde-Osmium. ×13,000.

Fig. 10 A precyst characterized by a dense clump of mitochondria (M) with vesicular cristae. Note that it has lost almost all the tubular expansions. Glutaraldehyde-Osmium. ×14,000. *Inset*; Detail of its pellicle. It consists of a unit membrane and an electron-dense layer. This structure is identical with that of trophozoites' pellicle. ×72,000.

Fig. 11 A precyst derived from a pellet. The cytoplasm contains a clump of mitochondria (M) and membranes. Glutaraldehyde-Osmium. ×14,000. *Inset*; Detail of its pellicle. It is of trophozoite-type. ×71,000.

Fig. 12 A precyst derived from a pellet. The cytoplasm contains a clump of mitochondria (M) and membranes. Note that the contents of this precyst is identical with those of the precysts in Fig. 10 and 11, but this precyst has the pellicle of cyst-type. Glutaraldehyde-Osmium. ×18,000. *Inset*; Detail of its pellicle. It is composed of three layers: a unit membrane, an electron-lucent layer, and an electron-dense layer. ×50,000.

Fig. 13 Cysts stained with methenamine silver nitrate. Cup-shaped cysts have discoid or parentheses-shaped darkly-stained spots. ×1,400.

Fig. 14 A young cyst with two intracystic bodies covered with a single membrane (arrow). The cyst has a clump of mitochondria (M) and a few tubular expansions. The intracystic bodies have a rather dense mass supposed to be a nucleus (N). The extremely electron-dense mass exists in the right intracystic body and in the intracystic body-like mass which lies just below the right one, and of which the pellicle is not recognized. This mass may be a nucleolus. Derived from a pellet. Glutaraldehyde-Osmium. ×17,000.

Fig. 15 A young cyst with five intracystic bodies and many round mitochondria (M). Intracystic bodies have a nucleus (N) of which the envelope is not recognized. The pellicle of them consists of a single membrane which is somewhat thicker than the unit membrane of cyst wall. The central one is in the midst of pellicle formation. Derived from a pellet. Glutaraldehyde-Osmium. ×15,000. *Inset*; Detail of its three-layered cyst wall. ×92,000.

Fig. 16 An intracystic body covered with a single membrane. The cyst is still rich in the cytoplasm. The intracystic body contains a nucleus (N) with a nucleolus, a rough-surfaced endoplasmic reticulum (ER), and a mitochondrion (M) the cristae of which are not recognized. Its pellicle is as thick as the unit membrane of cyst wall (CW). Glutaraldehyde-Osmium. ×42,000.

Fig. 17 An intracystic body covered with a unit membrane. The cytoplasm contains a nucleus (N) of which the envelope is continuous with rough-surfaced endoplasmic reticulum (ER). Cytoplasmic debris adheres to the cyst wall (CW). Glutaraldehyde-Osmium. ×41,000. *Inset*; Detail of the intracystic body's pellicle showing the structure of unit membrane. ×108,000.

Fig. 18 An intracystic body covered with two unit membranes. Nucleus (N); Rough-surfaced endoplasmic reticulum (ER); Cyst wall (CW). Glutaraldehyde-Osmium. ×46,000. *Inset*; Detail of its pellicle. It is composed of an inner and an outer unit membrane and a middle electron-dense layer. ×106,000.

Fig. 19 An intracystic body covered with a unit membrane and an electron-dense layer. This pellicular structure is identical with that of trophozoites. Cyst wall (CW). Glutaraldehyde-Osmium. ×55,000. *Inset*; Low power picture of this cyst. The intracystic body exists below the thickened portion of cyst wall. The cyst contains a degenerative mitochondrion (M). ×13,000.

Fig. 20 A cyst filled with intracystic bodies which have the pellicle of trophozoite-type and a rather irregular contour. A small amount of the cyst's cytoplasm is still recognized in its left upper corner. The intracystic body in the lowest part may be degenerative. Nucleus (N). Glutaraldehyde-Osmium. ×21,000. *Inset*; Detail of the cyst wall. A middle-electron lucent layer increases its thickness and an outer electron-dense layer decreases. The intracystic body with the pellicle of trophozoite-type adheres closely to the cyst wall. ×33,000.

Fig. 21 A cyst with extremely electron-dense crescent or elongate intracystic bodies. The intracystic bodies and most of the remaining cytoplasmic debris adhere to the thickened portion of cyst wall (bold arrow). The pellicle of intracystic bodies is detouched from the cytoplasm (arrow). Glutaraldehyde-Permanganate. ×13,000. *Inset*; An extremely electron-dense intracystic body with a rather thick pellicle. On the left side of the intracystic body, the pellicle is detouched. Glutaraldehyde-Permanganate. ×23,000.

Fig. 22 A cyst with intracystic bodies attached by a strand (arrow head) to the cyst wall or to the cytoplasm adhering to the cyst wall. All the intracystic bodies are degenerative. There exists a whirlpool-like structure. The thickened portion of cyst wall (arrow). Derived from a pellet. Glutaraldehyde-Osmium. ×15,000.

Fig. 23 A crescent collapsed cyst. The cell debris is wrapped in a contracted unit membrane

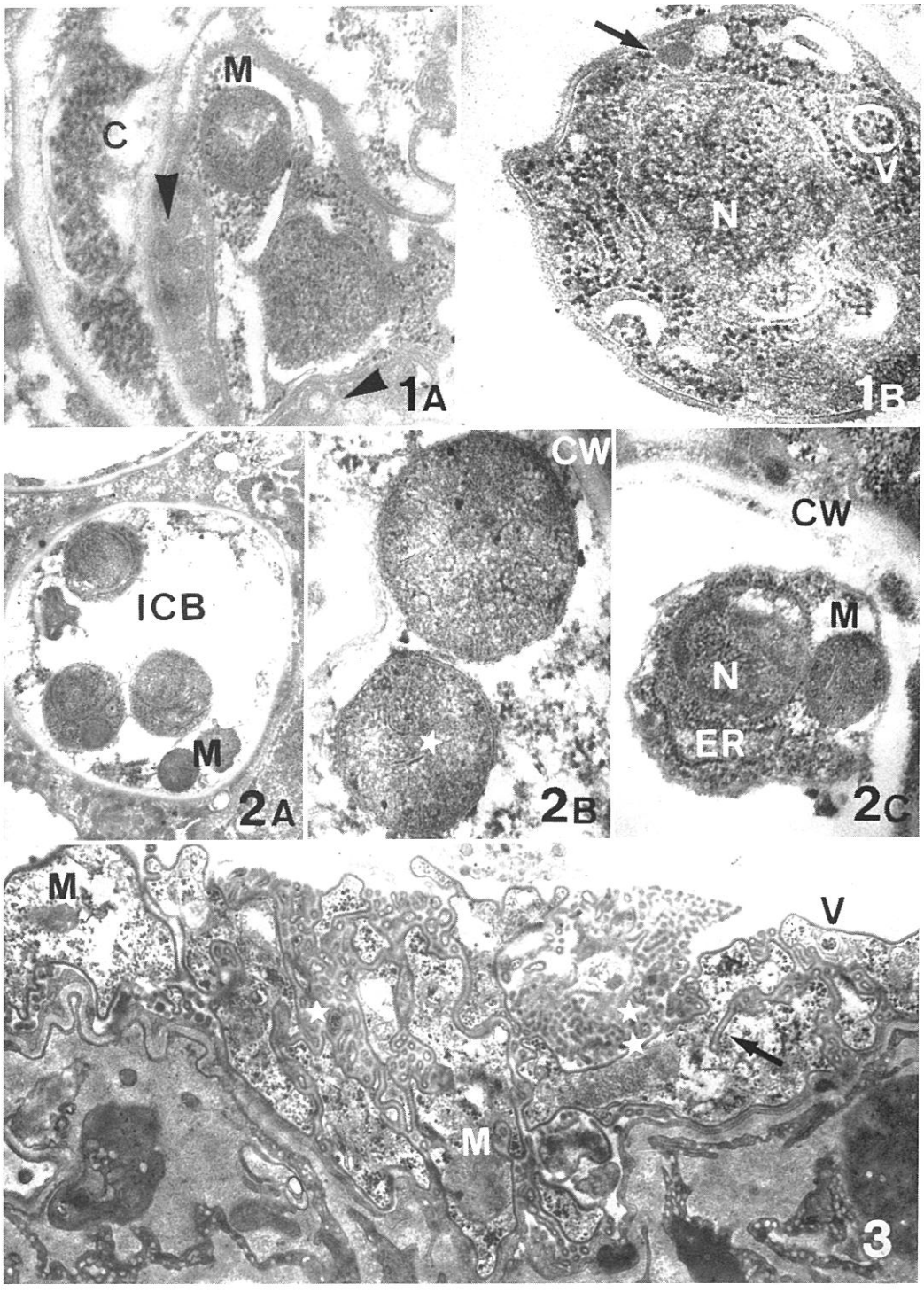
of cyst wall. The middle electron-lucent layer of cyst wall may be disintegrated. Derived from a pellet. Glutaraldehyde-Osmium. ×15,000.

Fig. 24 A ring-shaped collapsed cyst. The central area surrounded with the cyst (asterisk) corresponds to the concave of a crescent cyst. Note the crack of cyst wall (arrow) which exists on the center of the thickened portion facing to the convex of the cyst. Derived from a pellet. Glutaraldehyde-Osmium. ×16,000.

Fig. 25 Small trophozoites in the concave of a collapsed cyst (C). Trophozoite (T); Nucleus (N); Vacuole (V). Glutaraldehyde-Osmium. ×15,000.

Fig. 26 A trophozoite containing a daughter trophozoite. The mother trophozoite exists free in the alveolar lumen and contains also the organelle which cannot be identified. The daughter trophozoite has a nucleus-like mass (N) and several branching parts (asterisk). Glutaraldehyde-Osmium. ×24,000.

Fig. 27 A phagocytized trophozoite (T) with tubular expansions (arrow). Derived from a pellet. Glutaraldehyde-Osmium. ×4,800. *Inset*; Detail of its pellicle. An electron-lucent layer is partially recognized between a unit membrane and an electron-dense layer. ×16,000.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