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Morphology and Development of *Pneumocystis carinii* Observed by Phase-contrast Microscopy and Semiultrathin Section Light-microscopy

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Key words: *Pneumocystis carinii*, phase-contrast microscopy, JB-4, semiultrathin section, protozoa

Pneumocystis carinii pneumonia has been reported from all over the world in the patients who have had severe underlying diseases such as malnutrition, congenital immune deficiency, malignant neoplasms, post-organ transplantation, autoimmune diseases and so on. The occurrence of this pneumonia is apparently due to the active propagation of *P. carinii* in the lungs by strong immunosuppressive therapy against the underlying diseases mentioned above. More recently, *P. carinii* pneumonia has been noticed as the most important fatal pathogen among patients of a new disease, "acquired immune deficiency syndrome (AIDS)", mainly in the United States.

The previous studies indicate that *P. carinii* generally has 4 stages in its life cycle, i.e. trophozoite, precyst, cyst and intracystic body. Among them, the trophozoite is evidently more predominant in number than the cyst, hence more pathogenic to the host. However, the trophozoite has been little studied lightmicroscopically because it is so small in size, inconspicuous and difficult to stain, especially on paraffin sections.

The present paper describes the morphology, development and behaviour of *P. carinii*, especially the trophozoites, in the alveoli of experimental animals by using some new techniques.

Materials and Methods

In order to provocate *P. carinii* pneumonia, 40 Wistar strain rats, with body weights of approximately 200 g each, were treated subcutaneously with 25 mg of cortisone acetate twice a week. During the course of cortisone treatment, the rats were fed standard laboratory food and were allowed access *ad libitum* to drinking water supplemented with 0.05 % tetracycline to prevent bacterial infection (Frenkel *et al.*, 1966). One to five rats were sacrificed weekly for 12 weeks after initiation of the treatment.

For phase-contrast microscopy, a small piece of the infected lung was minced with scissors and rinsed in a small amount of saline on a glass slide, then pressed by cover glass. Investigation was performed mostly at room temperature, and sometimes at 28°C and 37°C in a specially designed incubator. When the phase-contrast microscopic investigation was over, a drop of Giemsa working solution was put on the margin of the cover glass. Thus the organisms, trophozoite and cyst, were investigated under both unstained and stained conditions. Other than the wet

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staining method as mentioned above, the dry method was my usual procedure. That is, an imprinted lung smear was air-dried and fixed with alcohol, then stained with Giemsa working solution for 1 to 5 hours. For the quantitative estimation of the infection intensity, the number of cysts in 1 g of the lung was counted by the cyst concentration method (Ikai *et al.*, 1977).

The author found in a preliminary experiment that the semiultrathin section made from embedded JB-4 plastic was much more useful than the ordinary paraffine-embedded section for histopathological study of *P. carinii* because the trophozoite could be detected by the former technique but not by the latter. The semiultrathin sections were prepared by the following procedures. The left lower lobe of the lung was removed and cut into small cubes about 2×2×2 mm each. They were put in a specially designed plastic syringe containing 2.5 % glutaraldehyde, and air was removed from the alveoli by the strong suction of the syringe (Ebe *et al.*, 1968; Takeuchi, 1980). Then, they were immersed in the same fixative for 3 to 5 hours, and washed with pH 7.2 phosphate buffer solution for 3 to 12 hours. In some rats, fixation was performed by infusion of the same fixative into the trachea under sodium pentobarbital anesthesia prior to removal of the lungs. The tissue was then dehydrated in serial alcohols, and embedded in JB-4 metacrylate plastic. The semiultrathin sections, 0.5 to 2 μm thick, were prepared by glass knife and stained with Giemsa. More than five hours staining in an incubator at 37°C was required to obtain satisfactory results because the section was so thin. The JB-4 embedding kit is available from Polyscience, Inc. Paul Valley Industrial Park, Warrington, PA., USA.

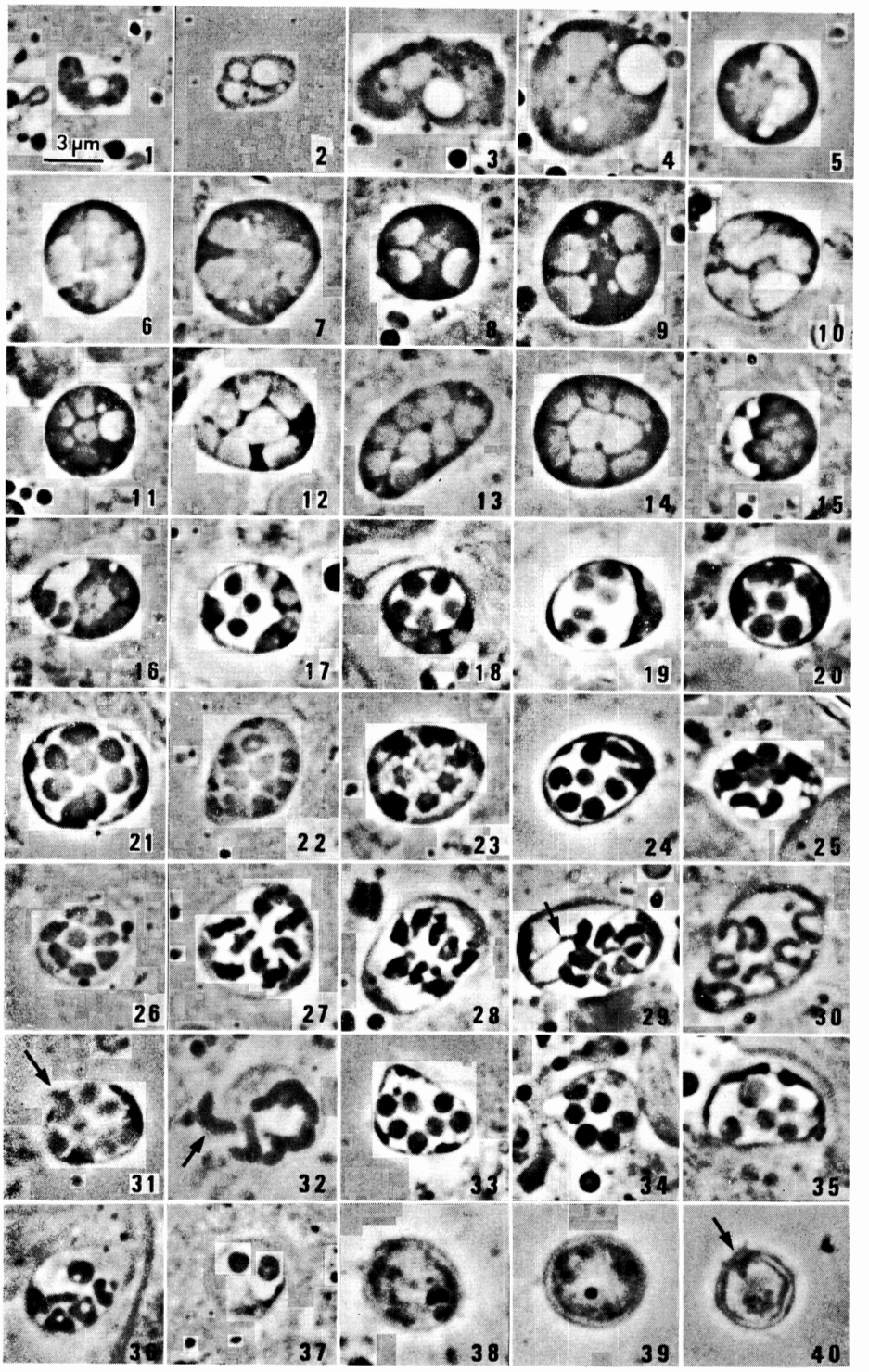
The number of trophozoites, and immature, mature and collapsed cysts were counted in 0.4 mm^2 of 2 μm JB-4 sections in order to know the ratio of those developing stages in the course of cortisone treatment.

Results

1. Phase-contrast microscopic investigation

From the thousands of specimens observed, some trophozoites and cysts are presented in Figs. 1 to 40, presuming the development of this organism. Trophozoites (Figs. 1-3) are ameboid in external appearance and 2 to 8 μm in diameter. Usually they have one or more lucid spherical vacuoles and one less lucid nucleus in the cytoplasm. The author has not found any actually moving trophozoite throughout the present investigation. An organism in Fig. 4 seems to be the precystic stage because it is spherical in shape although the nucleus and vacuoles are still similar to those of the trophozoite. The organisms shown in Figs. 5-12 are considered to be immature cysts in which the intracystic bodies are forming. Some workers consider this stage to still be precystic. Figures 13 and 14 indicate 8 intracystic bodies which almost fill up the cyst. Therefore, the cyst looks like it has 8 small rooms. Figures 15 to 20 show the course of separation and independence of the intracystic bodies in the cyst. Figures 21-29 indicate the polymorphism of the intracystic bodies such as spherical (Fig. 21), partially angular (Fig. 22), having short filopodia (Fig. 23), both spherical and elongate (Figs. 24, 25), ameboid with smooth surface (Fig. 26), ameboid with irregular surface (Figs. 27, 28), ameboid with long filopodia (arrow) which has a small swelling on its end (Fig. 29), and banana shaped (Fig. 30).

The intracystic body itself and its filopodia often showed active movement, which stopped when they were heated. Figures 31-37 show the course of excystation of the intracystic bodies. In Figs. 31 and 32, ameboid and banana shaped intracystic bodies are escaping through a pore of the cyst wall (arrow). The cysts shown in Figs. 33-37 have 2 to 7 intracystic bodies suggesting they are in the course of excystation. An arrow in Fig. 40 indicates a ruptured part of the cyst wall. Small dense spherical granules recognized in the cytoplasm of the cysts shown in Figs.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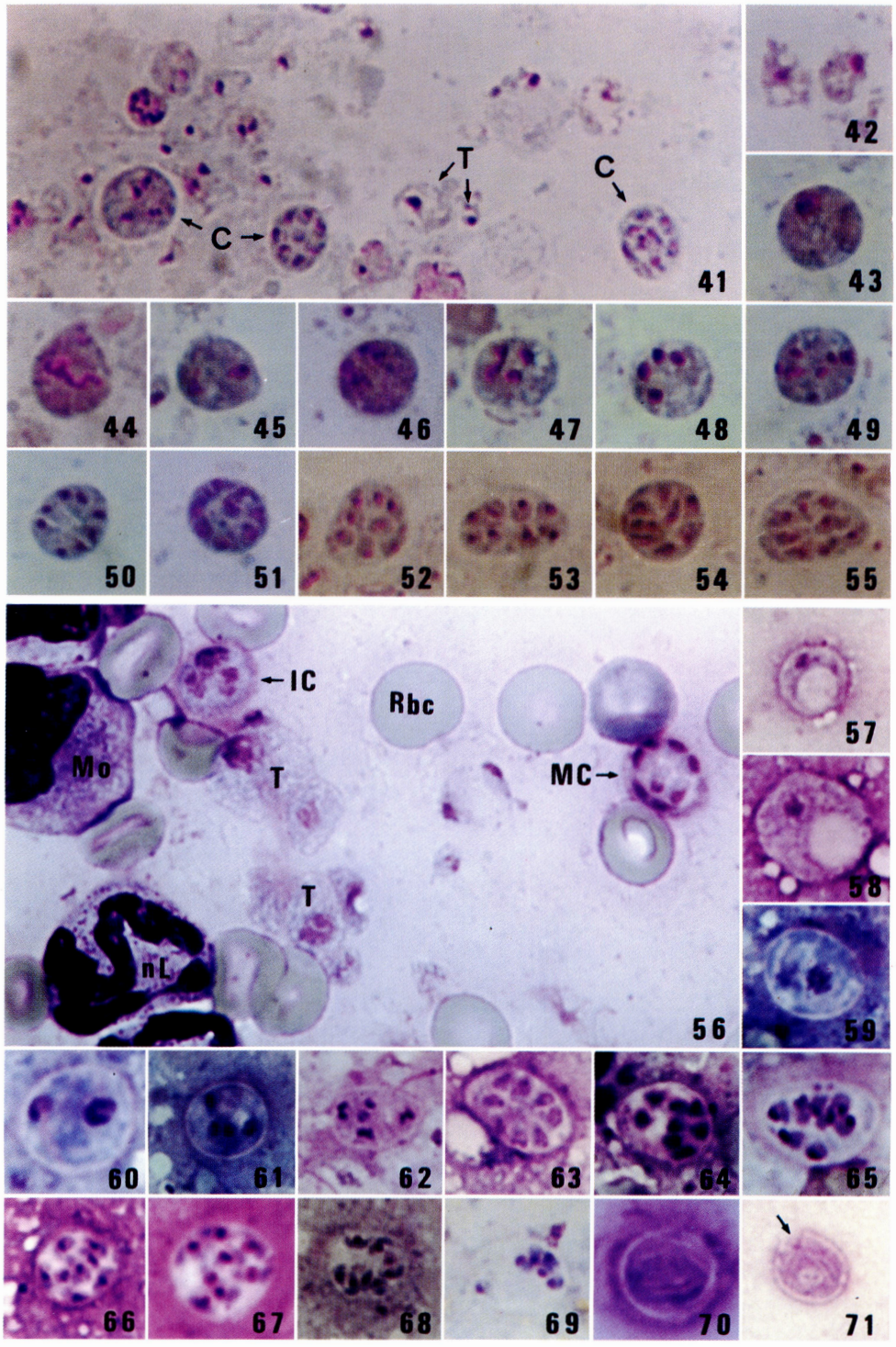
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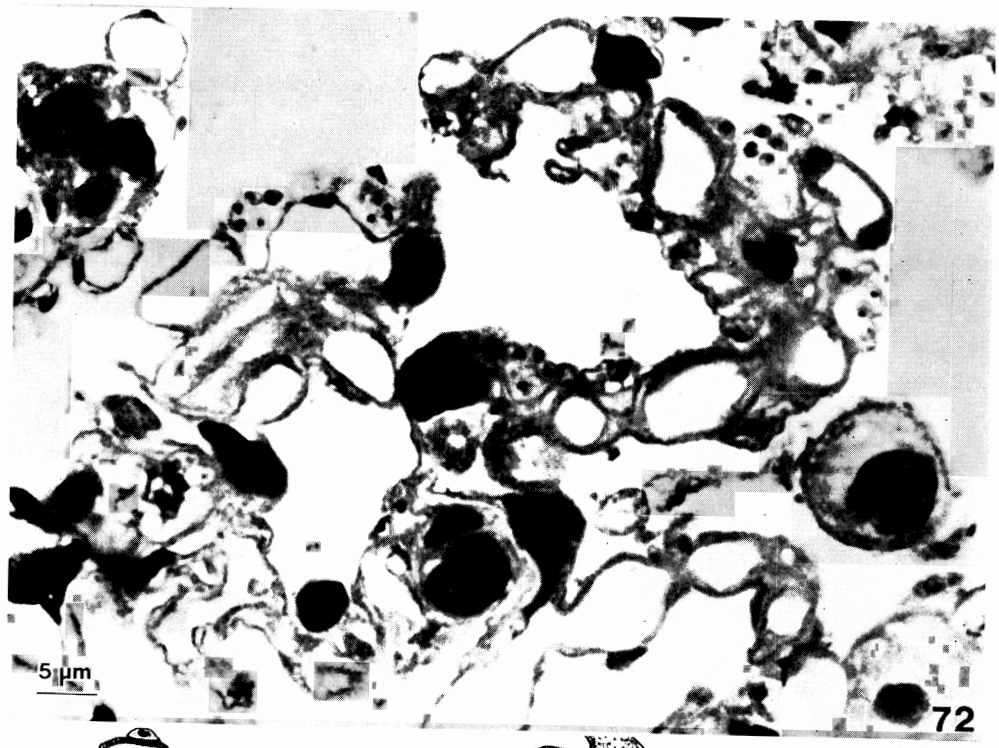
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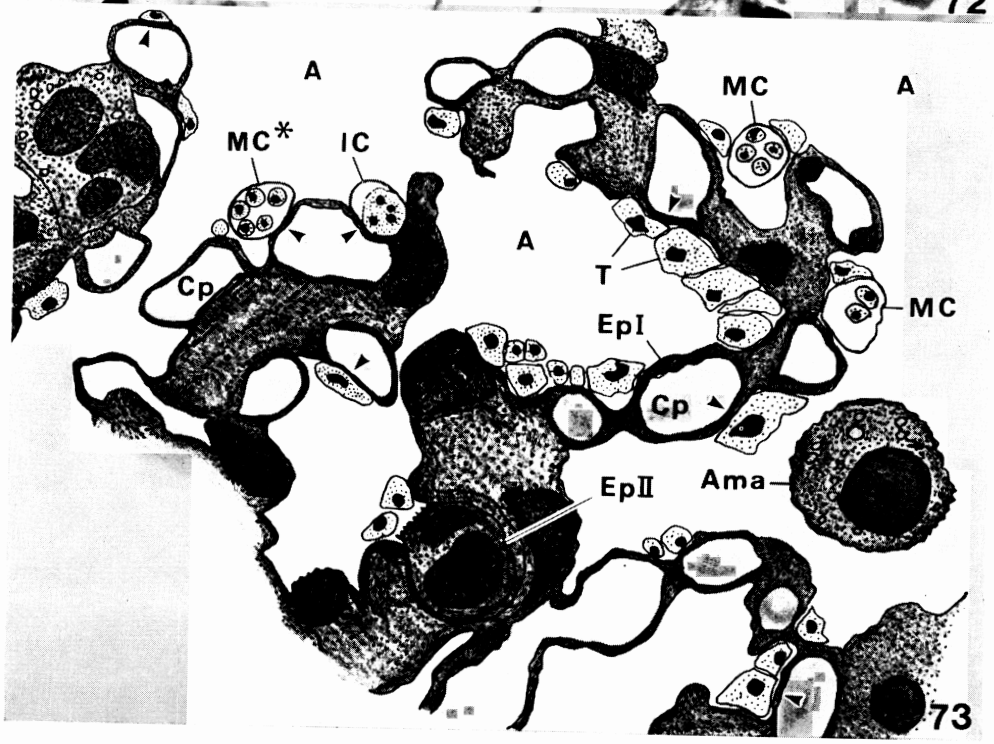
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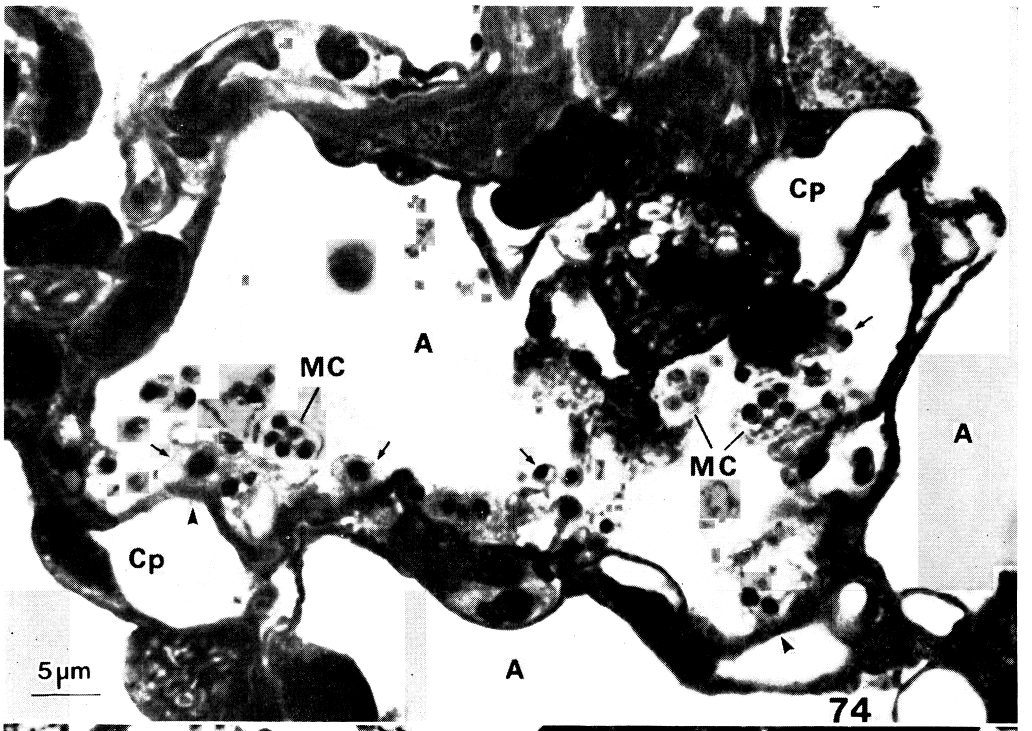
Ama
73


Cp
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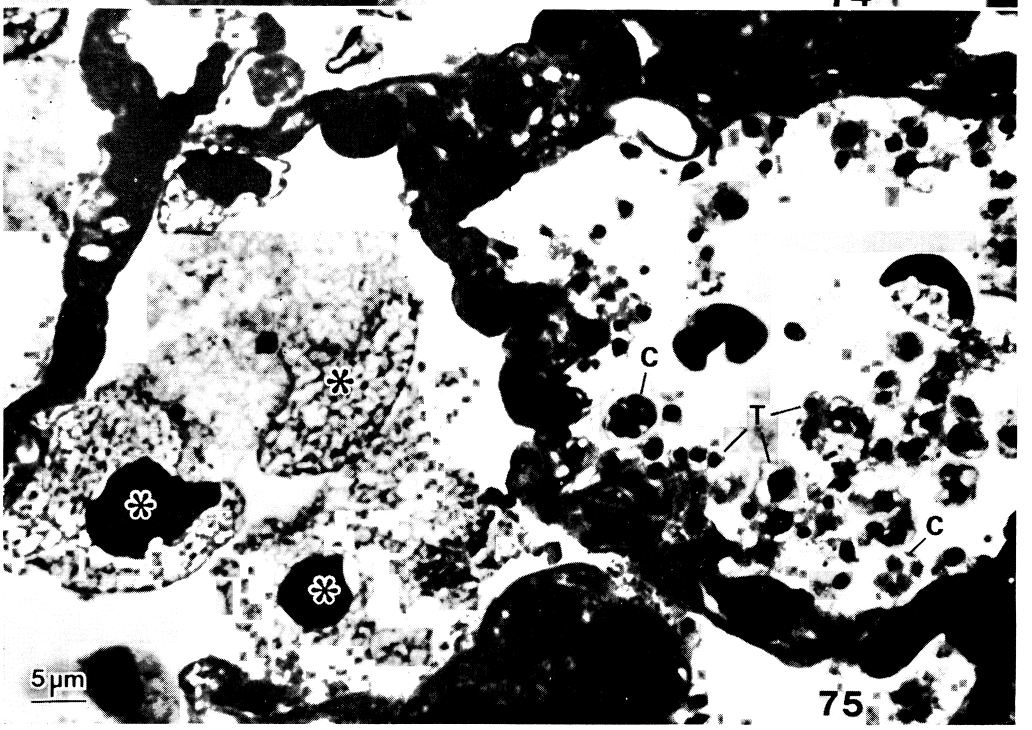
MC
A
Cp
5μm
A
74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C
5μm
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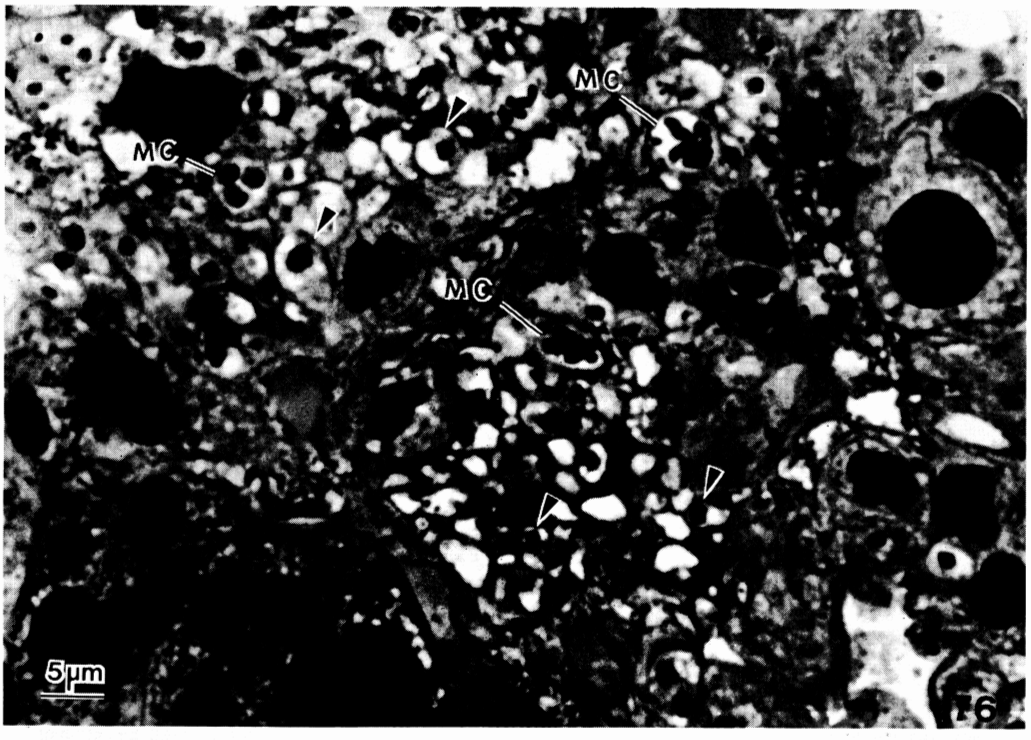
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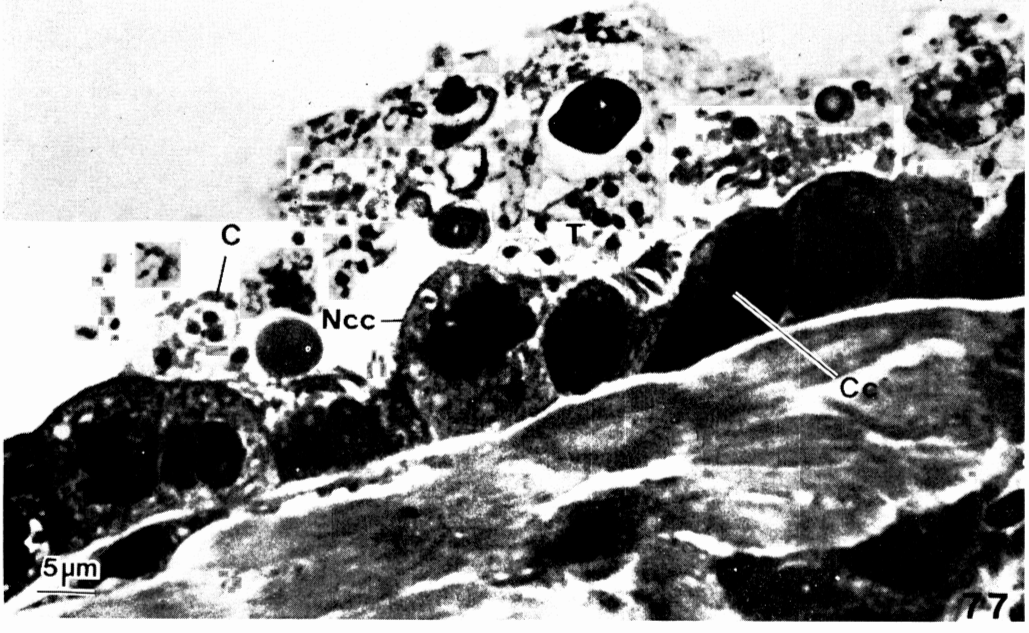
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Figs. 1-40. The development of *Pneumocystis carinii* as observed by phase-contrast microscope. Figs. 1-3 Small to large trophozoites which have polymorphic shapes, one nucleus and some vacuoles. Fig. 4 Precyst. Figs. 5-12 Immature cysts in which the intracystic bodies are forming. Figs. 13-14 Cysts containing 8 immature intracystic bodies. Figs. 15-20 Maturation and independence of intracystic bodies in the cysts. Figs. 21-29 Mature cysts having polymorphic intracystic bodies such as spherical (Fig. 21), partially angular (Fig. 22), irregular with short filopodia (Fig. 23), both spherical and elongated (Figs. 24, 25), ameboid with smooth surface (Fig. 26), ameboid with irregular surface (Figs. 27, 28) and ameboid with long filopodia (arrow) which have small swellings at the middle (Fig. 29). Fig. 30 A cyst which has typical banana shaped intracystic bodies. Figs. 31-37 Course of excystation of intracystic bodies. Figs. 31 and 32 An intracystic body is coming out from the cyst through a pore of the cyst wall (arrow). Figs. 33-37 The cysts have 2-7 intracystic bodies suggesting they are in the course of excystation. Figs. 38-40 Empty cysts after excystation. In Fig. 40, the arrow indicates a ruptured part of the cyst wall (The scale in Fig. 1 is applicable to all figures).

Figs. 41-55 *Pneumocystis carinii* observed by wet Giemsa staining. Fig. 41 Some trophozoites (T) and cysts (C). Fig. 42 Two trophozoites. Fig. 43 Precyst. Figs. 44-49 Immature cysts in which the nuclear division and the intracystic body formation are occurring. Figs. 50-55 Mature cysts containing ameboid (50 and 51), spherical (52 and 53), and banana shaped (54 and 55) intracystic bodies.

Figs. 56-71 *Pneumocystis carinii* observed by dry Giemsa staining. Fig. 56 Trophozoites (T), immature cyst (IC), mature cyst (MC), neutrophils (nL), monocyte (Mo), and red blood cells (Rbc) are seen. Figs. 57 and 58 Trophozoites. Figs. 59-62 Immature cysts in which the nuclear division and intracystic body formation are occurring. Figs. 63-68 Mature cysts containing 8 intracystic bodies of various shapes such as round to ameboid (63-65), and crescent to banana shaped (66-68). Fig. 69 A cyst from which the intracystic bodies are excysting. Fig. 70 Empty cyst. Fig. 71 Empty cyst with ruptured (arrow) cyst wall.

Figs. 72, 73 A photograph and its drawing of a histological section of rat lung lightly infected with *Pneumocystis carinii*. The specimen is a semiultrathin section made from JB-4 plastic embedded material and stained with Giemsa. About 28 trophozoites (T), 1 immature cyst (IC) and 3 mature cysts (MC) can be seen. The host tissue is almost normal, and air spaces of the alveoli are well preserved. In this field, about 20 % of the alveolar-capillary barriers were covered with the organisms (arrowhead for some) when measuring the barrier as an extended line. Cp: Capillary blood vessel. A: Alveolar sac. Ama: Alveolar macrophage. EpI: Type I epithelial cell. EpII: Type II epithelial cell. MC*: Mature cyst adhering to the alveolar capillary barrier.

Fig. 74 JB-4 embedded semiultrathin section of moderately infected alveoli with *Pneumocystis carinii*. The tissue is affected by the infection to some extent, and the number of blood capillaries (Cp) have considerably decreased even though the alveolar air space (A) is still preserved. In this alveolus (A), about 30 trophozoites (small arrows for some) and 3 mature cysts (MC) can be seen. Most of the trophozoites seem to be closely attached to the epithelial cells, and some of these are situated on the blood-air barrier (arrowhead).
Fig. 75 The late stage of infection showing many trophozoites (T for some) and 2 cysts (C) in an alveolus, and some large foamy alveolar macrophages (*) in the next alveolus. These macrophages are different in size and in cytoplasmic appearance from the normal macrophage. The tissue is considerably damaged, and air space and the number of blood capillaries are markedly decreased.

Fig. 76 A heavily infected late stage alveolus. An alveolus is completely filled with trophozoites (arrowhead) and cysts (MC). The alveolar tissue is heavily damaged, and no air space is seen.
Fig. 77 A semiultrathin section of the terminal bronchiole showing a cyst (C) and many trophozoites (T) on the ciliated cells (Cc) and Clara cells (Ncc). These findings suggest the continuous elimination of the organism through the trachea; hence the importance of examination of sputum or bronchial lavage for the diagnosis.

13, 22, 23, 26, 33, 38-40 are considered to be the mitochondria which commonly seen by the electron microscopy.

2. Giemsa staining

1) Wet method

When the phase-contrast microscopic investigation was over, a drop of Giemsa working solution was put on the margin of the cover glass. Thus, the organisms could be examined under both unstained and stained conditions. Figure 41 shows some trophozoites and cysts obtained by the wet staining method mentioned above. Two trophozoites shown in Fig. 42 are ameboid in shape and they have one nucleus and some vacuoles. They seemed to correspond with Figs. 1 and 2 taken by phase-contrast microscope. The organism shown in Fig. 43 is supposed to be a precystic stage from its spherical appearance and concentrated single nucleus. Figures 44-49 show immature cysts in which the intracystic bodies are forming. Figures 50-55 are mature cysts whose intracystic bodies are 8 in number and almost fill up the cysts. The shape of the intracystic body varies as ameboid (Figs. 50, 51), spherical (Figs. 52, 53) and banana shaped (Figs. 54, 55).

2) Dry method

The imprinted lung smears were air-dried and fixed with alcohol, then stained with Giemsa. In Fig. 56, one mature cyst (MC), one immature cyst (IC), some trophozoites (T), two neutrophils (nL), one monocyte (Mo) and some red blood cells (Rbc) are seen. The organisms shown in Figs. 57 and 58 are considered to be the trophozoites from the reasons that they vary in size and shape, and have a single nucleus and a thin pellicle. The organisms in Figs. 59-71 are all in the cystic stages. They have thick cyst walls which are not stained by Giemsa (Figs. 59-62, 64, 65, 70, 71). The process of nuclear division is also shown in Figs. 59-62. It is probable that binary fission is repeated three times finally forming 8 nuclei. Figures 63-68 show mature cysts containing polymorphic intracystic bodies. For example, round to ameboid in Figs. 63-65, and crescent to banana shaped in Figs. 66-68. The organism in Fig. 69 is presumably in the course of excystation which resembles the cyst shown in Fig. 36 by phase-contrast microscopy. Figures 70 and 71 are the empty cysts after excystation of the intracystic bodies. The feature of an empty cyst with a ruptured cyst wall (arrow) corresponds to that of Fig. 40. There are some small dense granules at the inner surface of the cyst walls shown in Figs. 64 and 65. They are probably the mitochondria which are also observed in Figs. 22, 23, 26, 33, 38-40 by phase-contrast microscopy.

3. Semiultrathin section embedded in JB-4 plastic

In order to investigate the morphology and parasitizing behavior of trophozoites and cysts as well as the response of the alveolar tissue in more detail, semiultrathin sections of 0.5-2.0 μm thick were made from the materials embedded in JB-4 plastic. The present author divided the alveoli into 3 types, i.e. lightly infected alveoli, moderately infected alveoli and heavily infected alveoli according to the intensity of *P. carinii* infection and the response or damage of the alveolar tissue. Although the lightly infected alveoli were most common in the initial stage of cortisone treatment, some were also found in the middle stage and less frequently seen in the terminal stage.

1) Lightly infected alveoli

Figure 72 shows a 2 μm thick section of the alveoli lightly infected with *P. carinii* after 5 weeks of cortisone treatment. Figure 73 is a sketch of Fig. 72 photograph with explanatory headings. The host tissue is almost normal, and air spaces (A) of all alveoli are plentiful enough. Type I epithelial cells (Ep I), type II epithelial cells (Ep II) and capillary blood vessels (Cp) are well preserved. In this figure, about 28 trophozoites, 1 immature cyst (IC) and 3 mature cysts (MC) can be distinguished firmly adhering to type I epithelial cells. Some of them are on the alveolar-capillary barrier as indicated by arrow heads. The measurement of the length of the alveolar-capillary barrier covered with

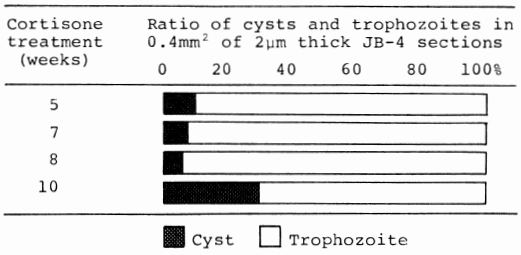

Fig. 78 Ratio of cysts and trophozoites in the course of cortisone treatment.

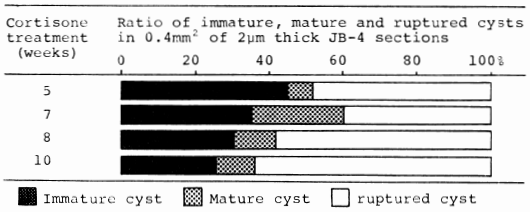

Fig. 79 Ratio of immature, mature and ruptured cysts in the course of cortisone treatment.

the organisms showed about 20 % of the entire barrier in this field.

2) Moderately infected alveoli

Figure 74 is a 2 μm thick section of the alveoli of a rat treated with cortisone for 8 weeks, showing moderate infection with *P. carinii*. Although the alveolar air space is still enough, the arrangement of the alveolar septum is irregular, and the number of blood capillaries considerably decreased. In this figure, 3 mature cysts (MC), and about 30 trophozoites (arrows for some) can be seen.

3) Heavily infected alveoli

With the progress of infection (Fig. 75), almost all alveoli are filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophages (asterisks), which differ in size and in cytoplasmic appearance from the normal macrophage. The alveoli shown in Fig. 76 are filled almost completely with trophozoites and cysts of various stages of development. Those substances are the elements of so-called honeycombed material in paraffin sections. Figure 77 shows a cyst and some trophozoites on the ciliated cells and Clara cells of the terminal bronchiole.

4. Ratio of cysts and trophozoites

The ratios of cysts and trophozoites in 0.4 mm² of 2 μm thick JB-4 sections in the course of cortisone treatment are shown in Fig. 78. In this measurements, the immature, mature and ruptured cysts are counted as the cyst, and the precyst as the trophozoite. The cysts constituted only 6 to 10% of the *P. carinii* organism at the fifth, seventh and eighth weeks of cortisone treatment, but they increased to 32 % at the tenth week. The amount of this increase may depend upon how much the encystation increases at the terminal stage of infection.

Figure 79 presents the ratios of immature, mature and ruptured cysts in the course of cortisone treatment. The immature cysts were 45 % of the total number of cysts at the fifth week, and their number decreased gradually to 26 % at the tenth week. On the other hand, the ruptured cysts reciprocally increased from 48 % to 64 % except in the seventh week, in which the mature cysts increased greatly.

Discussion

Morphological study on *P. carinii* by phase-contrast microscopy has been done by some authors. Fingerland and Vortel (1954), and Pliess (1957), stressed the value and usefulness of phase-contrast microscopy of fresh material of the lungs in identifying the *P. carinii*. Bommer (1961) found cyst-like orgaisms by phase-contrast microscopy in the lungs of premature babies who died from interstitial plasma cell pneumonia, and stated that *P. carinii* must be a sporozoan-like organism rather than the yeast or degenerated nuclei of the host cells. Recently, Pifer *et al.* (1977) and Masur and Jones (1978) examined the trophozoites and cysts of *P. carinii* in culture using phase-contrast microscopy. However, they did not describe the life cycle of the organism. The present study disclosed a part of the life cycle, from young trophozoite to mature cyst and excystation, by phase-contrast microscopy under the living condition of the organism.

It became evident that the wet Giemsa stain, which was performed by adding a drop

of Giemsa working solution on the margin of cover glass when the phase-contrast microscopic observation ended, was quite useful to examine the trophozoites and cysts under both stained and unstained conditions.

As is commonly known, it is difficult to see the trophozoites in usual paraffin embedded lung sections with any kind of staining, whereas it is easy to see the cysts. Therefore, a better method to reveal the trophozoites light microscopically in the histopathological sections has been expected. Ham *et al.* (1971) used, for the first time, the plastic embedded semiultrathin sections (1 μm) with Paragon multiple staining in examining the *P. carinii* pneumonia in man. Although they demonstrated the pathological changes of the lung tissue, their only comment on the pathogen was that "scattered within the alveolar spaces were small clumps of material suggestive, but not confirmatory, of the *Pneumocystis carinii*". In Japan, Nagai and Kamata (1974) and Nagai *et al.* (1978) observed light microscopically *P. carinii* in semiultrathin sections (500 mμ) which were made from Epoxy resin embedded lung tissues then stained with toluidine blue 0, azur II-methylene blue, tribasic, Paragon and Giemsa. They demonstrated thin-walled pneumocysts and thick-walled pneumocysts separately, and the organisms parasitizing in layers on the surface of the epithelial lining cells. Yoshida and the present author (1981) preliminarily repored on the morphology and life cycle of *P. carinii* using phase-contrast microscopy and semiultrathin sections in JB-4 embedded lung material.

In the present study, the semiultrathin sections disclosed that the trophozoites, sometimes the cysts also, closely attached to the type I epithelial cells covering the blood-air barriers. This phenomenon increased with the term of cortisone treatment, suggesting the progress of alveolar-capillary block.

The presence of *P. carinii* cysts in the trachea and bronchus of cortisone treated rats was already pointed out by Yoshida *et al.* (1978). They also showed several clinical cases of *P. carinii* pneumonia diagnosed by detection of the cysts in their sputa. The present author found not only the cysts in the airway but also the trophozoites, and emphasizes that the detection of the trophozoite in sputum is very helpful for diagnosis of this pneumonia. Moreover, the trophozoite, as well as the cyst, may play an important role in the transmission of *P. carinii*.

Summary

The morphology, development and behaviour of *P. carinii*, especially the trophozoites in the alveoli of cortisone-treated rats, were investigated by phase-contrast microscopy and semiultrathin section embedded in JB-4 plastic. The results are summarized as follows:

1. By phase-contrast microscopy, trophozoites were ameboid in external appearance and 2 to 8 μm in diameter. Usually they have one or more lucid spherical vacuoles and one less lucid nucleus in the cytoplasm. Maturation and independence of intracystic bodies were observed in the developing cysts. The intracystic bodies are polymorphic, i.e. spherical, ameboid or banana shaped. The intracystic bodies sometimes have filopodia which have small swellings at the middle or at the end. The intracystic body itself and the filopodia often showed active movement, which stopped when they were heated.

2. When the phase-contrast microscopic observation was over, the same sample was immediately stained with Giemsa. Thus, the organisms could be examined under both unstained and stained conditions. The probable process of nuclear division is that binary fission is repeated three time finally forming 8 nuclei.

3. In order to investigate the morphology and parasitizing behavior of the trophozoites and cysts as well as the response of the alveolar tissue in more detail, semiultrathin sections of 0.5–2 μm thick were made from the materials embedded in JB-4 plastic and stained with Giemsa. According to the intensity of *P. carinii* infection and the response or damage of the alveolar tissue, the alveoli are divided into 3 categories as follows:

(1) In lightly infected alveoli, some troph-

ozoites and cysts are found to be closely attached to the type I epithelial cells, sometimes covering the blood-air barriers. This suggests that an alveolar-capillary block may occur when the organisms have increased in number.

(2) In moderately infected alveoli, the number of *P. carinii* increases, the tissue is affected by the infection to some extent, and the number of blood capillaries considerably decreases.

(3) In heavily infected alveoli, almost all alveoli are filled with trophozoites, cysts, debris of the host cells and occasionally with foamy macrophages, which differ in size and in cytoplasmic appearance from the normal macrophage. These substances are the elements of so-called honeycombed material in paraffin sections. The alveolar tissue is heavily damaged, no air spaces can be seen, and the number of blood capillaries are markedly decreased.

4. Semiultrathin sections of the terminal bronchiole often showed many trophozoites and some cysts on the ciliated cells and Clara cells. These findings suggest the continuous elimination of the organism through the trachea. Hence the examination of sputum or bronchial lavage will be valuable for diagnosis of this pneumonia. Moreover, the trophozoite, as well as the cyst, may play an important role in the transmission of *P. carinii*.

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位相差顕微鏡ならびに準超薄切片光顕的観察による *Pneumocystis carinii* の形態と発育の研究

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ニューモシスチス・カリニ（以下 *Pc* と略）はヒトならびに他の動物の肺に広く潜在的に感染しており，それら宿主が何らかの原因により免疫不全に陥つた場合，肺胞内ではげしく増殖して致死的な肺炎を起こす．

Pc はその生活史上，栄養型，前嚢子，嚢子およびその中に生ずる嚢子内小体の4つの stage をもつている．その中で栄養型は最も数が多く，従つて宿主への病原性も強いと思われるが，光学顕微鏡的検索では，形態の小さいこと，難染色性であることからパラフィン切片上では確認し難い．

本研究の目的は光顕的に栄養型を確認する方法を確立し，それによつて *Pc* の発育経過を観察することにある．コーチゾン処理したラットを用いての研究の結果，位相差顕微鏡によつて *Pc* を生きたまま観察し，同じ標本にギムザ液を加えて染色し観察する．また切片標本は JB-4 樹脂包埋を行い，0.5～2μm の準超薄切片を作りギムザ強染色を施す．これらの方法により栄養型をはじめ，ほぼ全発育段階の *Pc* を光顕下に観察することができた．

位相差顕微鏡下では栄養型は大小のアメーバ状を示し，1核と若干の小胞がみられたが運動するものはなかつた．つづいて前嚢子，未熟嚢子，その中に形成されてゆく嚢子内小体，成熟嚢子，脱嚢中の嚢子および空の嚢子などが観察された．また成熟した嚢子内小体は糸状突起をもつものや運動するものも認められた．これら標本にギムザ染色を施すことによりさらに細胞小器官がよく観察され，発育経過の理解を助けた．

JB-4 樹脂包埋準超薄切片は従来のパラフィン切片に比し，とくに栄養型が明確に識別でき，その数を数えることができた．まず軽度 *Pc* 感染肺では少数の栄養型および嚢子がI型肺胞上皮細胞に接着して寄生し，とくに alveolar-capillary barrier の上に存在するものがかなりあり，感染が進めばガズ交換の障害に関与することが容易に推定された．

次第に感染の程度が進むと *Pc* の数が増し，組織の変化，とくに肺胞毛細血管の減少が認められた．重度感染肺では肺胞は多数の栄養型および嚢子，宿主細胞の残屑，肺胞マクロファージなどで埋めつくされ，air-space はほとんど見られず，毛細血管も著しく減少し，ガス交換の著明な低下が明らかであつた．

一方，毛細気管枝の切片上にしばしば栄養型や嚢子が検出されたことから，本肺炎の診断において喀痰や気管粘液について嚢子のみならず栄養型も検索することが有用であると考えられた．さらに *Pc* の直接伝播に際し，嚢子のみならず栄養型も重要な役割をもつているものと考えられた．