

Basic biology of *Pneumocystis carinii* - A Mini Review

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Basic aspects of cell biology of Pneumocystis carinii are reviewed with major emphasis on its life cycle and the structural organization of the trophozoites and cyst forms. Initially considered as a protozoan it is now established that Pneumocystis belongs to the Fungi Kingdom. Its life cycle includes two basic forms: (a) trophozoites, which are haploid cells that divide by binary fission and may conjugate with each other forming an early procyst and (b) cysts where division takes place through a meiotic process with the formation of eight nuclei followed by cytoplasmic delimitation and formation of intracystic bodies which are subsequently released and transformed into trophozoites. Basic aspects of the structure of the two developmental stages of P. carinii are reviewed.

Key words: *Pneumocystis carinii* - life cycle - trophozoites - cyst form - fine structure

At the beginning of the XX century several parasitologists dedicated most of their time looking for new parasites in the bloodstream, tissues, and faeces of normal as well as experimentally infected animals. While carrying out studies on experimentally infected guinea pigs and in humans with a disease later on designated as Chagas disease or American trypanosomiasis, Chagas (1909), using a light microscope, observed the presence of cystic forms in histological sections of lungs. It is important to remember that the description of the schizonts as intracellular dividing forms of malaria parasites had been described a few years before. This fact probably influenced Chagas to consider the cystic forms as tissular schizonts occurring during the life cycle of a new trypanosome species designated as *Schizotrypanum cruzi*. Five years later, Delanoe and Delanoe (1914) examining rats collected in Paris described the same forms and considered them as representative of a new protozoan species which was designated as *Pneumocystis carinii* as a honour to Antonio Carini, an Italian biologist who described the same microorganism in the lungs of rats collected in Brazil and which were simultaneously infected with *Trypanosoma lewisi*.

During many years *P. carinii* was considered as a protozoan without any special medical importance. However, two groups of observations were responsible for the inclusion of *P. carinii* among the most important microorganisms studied in the past years. The first one, was the conclusion based on comparative analysis of the 16 S ribosomal RNA, mitochondrial genomic gene sequences, aminoacid sequences of peptides and proteins, that *P. carinii* belongs to the Fungi Kingdom rather than to Protozoa, as considered since its original description (Ypma-Wong et al. 1992, Cushion et al. 1994). According to the

molecule used for comparative phylogenetic analysis, *P. carinii* has been included in different groups such as Chytridomycota, Zygomycota, Ascomycetons or Ustomycetons red yeasts. At present, most authors include *P. carinii* as a fungus related to ascomycetous yeasts where the well known *Sacharomyces cerevisiae* is located. Based on the analysis of the gene sequences obtained in the genome project, affinity of *P. carinii* with *Schizosaccharomyces pombe* and *Neurospora crassa* has been suggested (review in Cushion et al. 1991, Cushion 2004). However, *P. carinii* presents unique morphological and life cycle characteristics as will be described below.

Biochemical and molecular studies have shown that there is significant genetic diversity in the natural *P. carinii* population. In the same infected animal several strains may co-exist. *P. carinii* has been isolated from humans, monkeys, rats, mice, ferrets, sloths, dogs, cats, sheeps, marmosets, and voles. The available information indicates that there are distinct species of *Pneumocystis*. Analysis based on nucleotide variations of the rRNA genes revealed the existence of several genotypes (Siripattanapibong et al. 2005). During the international workshop for *Pneumocystis* held in 2001 it was suggested that the species described in humans should be named *P. jirovecii*. However, several authors did not follow this recommendation. Different host species are infected with genetically different protozoan populations indicating the existence of multiple strains and/or species. Cross infection is also observed.

The second group was the description of an interstitial plasma cell pneumonia found in premature, malnourished infants in Central and Eastern Europe during and following World War II. During the 1960s it was recognized that *P. carinii* was a major opportunistic pulmonary pathogen, causing severe pneumonia in children with primary immunodeficiency disorders and in patients using immunosuppressive drugs during treatment of cancer and organ transplantation (Walzer et al. 1974). In all cases initial treatment with pentamidine, a drug used against African trypanosomiasis, and trimethoprin-sulfamethoxazole, controlled the infection. The importance of *P. carinii* in-

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fection dramatically increased in the 1980s with the appearance of the acquired immunodeficiency syndrome (Aids) where this organism was the major cause of opportunistic infection and mortality. Indeed, up to 90% of Aids patients developed pneumocystosis, characterized by intense organism proliferation with little or no inflammatory response (reviews in Frenkel et al. 1966, Mills 1986).

Due to all these factors the interest in studying *P. carinii* increased and the Society of Protozoologists organizes every two years a special scientific meeting on opportunistic organisms where papers dealing with *P. carinii* and *Toxoplasma gondii* predominate. The papers presented in these meetings are published in special issues of the *Journal of Eukaryotic Microbiology* (<http://www.blackwellpublishing.com/journals>).

In this short review we intend to analyze some basic aspects of the biology of *P. carinii* emphasizing its life cycle, and its cell biology, with special emphasis on its morphology, as observed by electron microscopy.

The life cycle

There are several reports on the life cycle of *P. carinii*, each one presenting different views and various developmental stages. We will consider here a life cycle accepted by most of the authors and which incorporates data obtained using electron microscopy (Fig. 1).

It is not yet clear which is the main infective form responsible for the primary infection. However, it is well established that airborne transmission is the most important one. For instance, corticosteroid-treated rats develop *P. carinii* infection when housed with infected rats. Infection by drinking water or food was excluded in these experiments. One way to start working with *P. carinii* is to administer corticosteroids to normal laboratory rodents, thus indicating that highly infective forms are present in the environment.

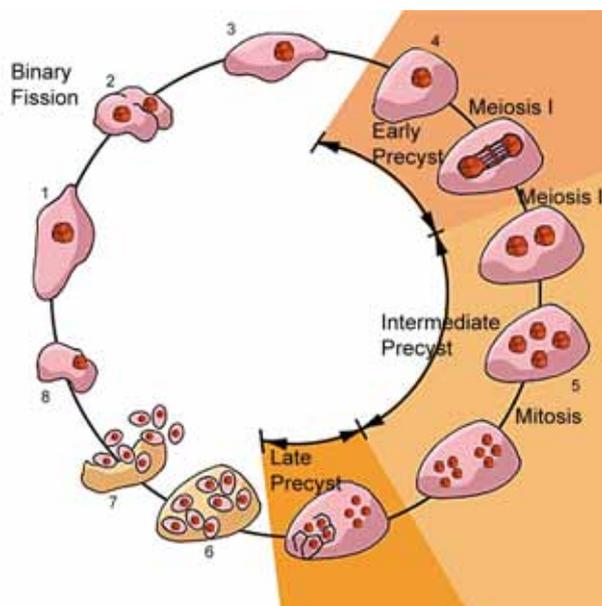


Fig. 1: diagram summarizing the present knowledge on the life cycle of *Pneumocystis carinii* as explained in the text.

Fig. 1 shows a schematic view of the life cycle of *P. carinii*. Two developmental stages are well characterized: the mature cyst and the trophozoite. The trophozoite form is variable in shape, measuring about 0.3 μm in diameter, and they usually form clusters. Some authors consider them to have ameoboid characteristic, with the presence of cytoplasmic projections similar to filopodia. However, no such type of cell motility has been reported in living samples.

Trophozoites originate directly from the cyst. Each mature cyst may contain up to eight spherical intracystic bodies, which give rise to eight trophozoites. It has been reported that trophozoites may originate from cysts containing spherical, banana-shaped or ameoboid intracystic bodies. The initial trophozoite is haploid and divides by binary fission or endogeny. Two trophozoites may conjugate giving rise to a diploid cell which then divides, as described above for the haploid trophozoites or begin a meiotic process of division with two meiotic cycles where three nuclear divisions takes place, forming a large spherical cell with eight nuclei. Subsequently, there is a process of cell delimitation, forming eight intracystic bodies. Important evidence for the presence of meiosis during the *P. carinii* life cycle is the presence of a synaptonemal complex (Matsumoto & Yoshida 1984). Preliminary analysis of the partial genome of *P. carinii* genes coding for proteins similar to those involved in the mating process in other fungi have been detected (Smulian et al. 2001).

It is important to point out that all these forms have been identified based on the observation of infections in animals and, in a few cases, in cell cultures. Further studies are still necessary to reproduce in vitro the complete life cycle of *P. carinii*.

Attempts have been made to cultivate the protozoan in different cell lines, using an approach typical for protozoa. Samples isolated from the lung of infected animals were inoculated into cell cultures in which they proliferated. Cysts and trophozoites have been observed although the latter predominates, growing as clusters in the supernatant. However, many organisms attached to portions of the cells. In some cases the morphology of the attachment resembles that observed in vivo where *P. carinii* attaches to type I pneumocytes (Bartlett et al. 1994). Attempts to cultivate the organism in cell free media, as usual for fungi, succeeded in ten-fold amplifications of the number of cells. However, continuous axenic cultivation of *P. carinii* is difficult, and has been obtained only by few groups (Merali et al. 1999).

Cell biology of the trophozoite

Trophozoites are pleomorphic and usually associate with each other forming clusters. They are easily identified in the lung of infected animals due to their irregular shape and close association with pneumocytes. Due to its pleomorphism the association of the trophozoites to each other is very irregular, forming complex aggregates with many interdigitations, sometimes making it difficult to distinguish each individual cell (Figs 2-4).

The cytoplasm of the trophozoite is poor in organelles (Vavra & Kucera 1970). Free ribosomes predominate. Glycogen particles are also seen. Tubular and ramified struc-

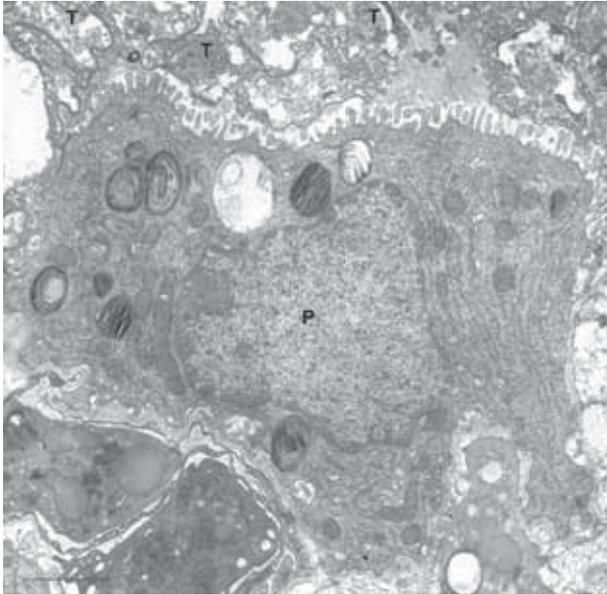


Fig. 2: thin section showing the presence of a large number of trophozoites (T) in the apical portion of a pneumocyte (P). Bar = 2 μ m.

tures, resembling the endoplasmic reticulum are observed. A small nucleus is seen in variable positions. Usually it appears homogeneous, with an electron density similar to that of the cytoplasm. A nucleolus is evident and located centrally or peripherally to the nucleus. Dividing nuclei have been observed. The mitotic process occurs within an intact nuclear membrane and there are mitochondria with short cristae. Osmiophilic bodies, vacuolar spaces, microtubules and an incipient Golgi complex were also reported, although not frequently seen (Dei-Cas et al. 1989).

For some authors the trophozoite is limited by two unit membranes spanning approximately 20-30 nm. The outer membrane is decorated with an electron dense layer and separated from the inner membrane by a thin electron lucent space (Vossen et al. 1978, Hughes 1987). According to our view, one characteristic feature of the trophozoite is the presence of a 20-30 nm thick and dense coat, which is found in the whole surface of the cell, including the regions of interdigitations (Figs 2-4). At high magnification it is clear that the coat is not homogeneous, leaving small electron translucent areas (Fig. 4). Cross sections of filopodium-like regions give the impression that some periodicity exists.

In heavily infected animals areas are found where portions of the *P. carinii* surface were released as a shedding-like process. Hundreds of longitudinally and transversally sectioned small tubules are observed, always containing the plasma membrane and the characteristic surface coat (Figs 3-4).

Freeze-fracture analysis revealed the presence of only one unit membrane displaying a large number of randomly distributed intramembranous particles on both protoplasmic and extracellular faces (Yoneda et al. 1982). The particle density was higher on the P than on the E fracture face (Yoshida 1989).

Fig. 5 shows a schematic view of the trophozoite forms displaying various organelles.

What is the nature of the thick surface coat seen only in the trophozoites surface? There are some suggestions that it anchors the organism or is involved in nutrient uptake. Studies using labeled lectins showed that residues of mannose, N-acetyl-glucosamine and galactose/N-acetyl-galactosamine are exposed on the cell surface (Yoshikawa et al. 1987, Cushion et al. 1988, Pesanti & Stanley 1988). Labeling with lectins recognizing fucose or sialic acids was very light or even absent (De Stefano et al. 1992).

One important surface antigen of *P. carinii* has 116 kDa in polyacrilamide gel electrophoresis (SDS-PAGE). Under non-reducing conditions it appears to exist as an

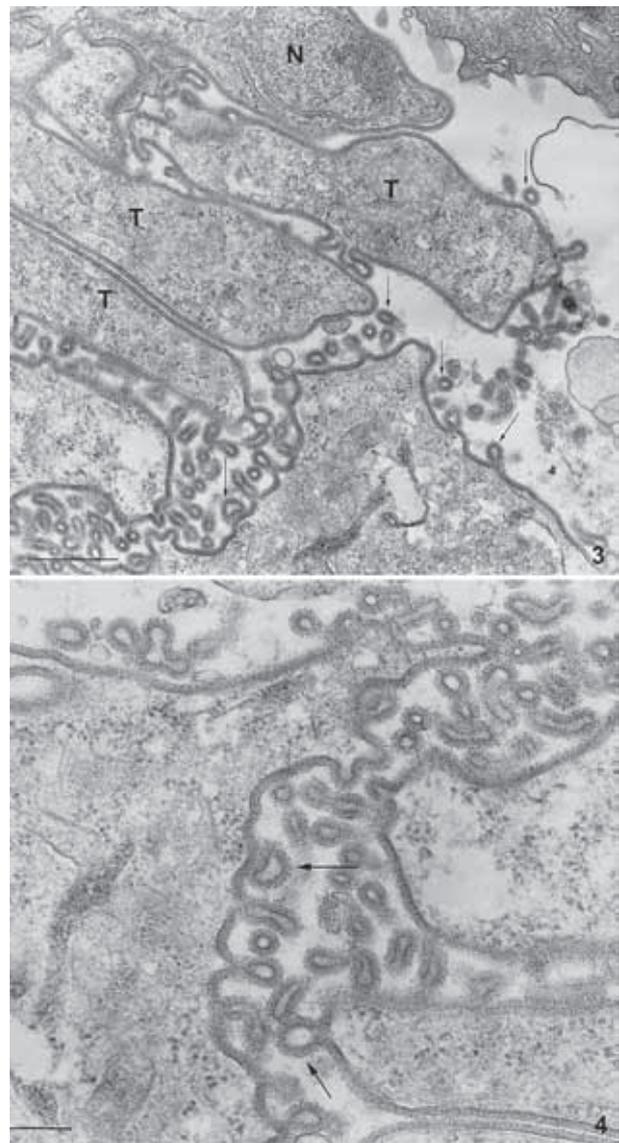


Fig. 3: thin section showing several trophozoites. A thick surface coat is evident, especially in cross sections of the surface projections (arrows). N, nucleus; T, trophozoite. Bar = 200 nm. Fig. 4: higher magnification of a trophozoite form showing the plasma membrane and the thick surface coat. A sub-structure of the coat can be seen (arrows). Bar = 400 nm.

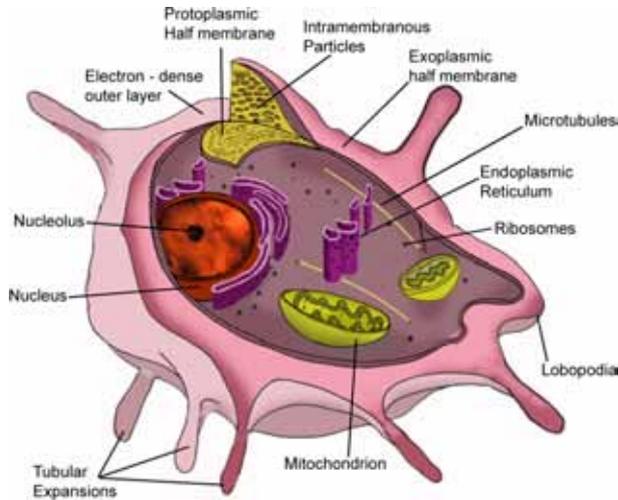


Fig. 5: schematic view of the trophozoite form where the main structures described in the text are indicated.

aggregated form with a molecular weight of $> 2 \cdot 10^6$ kDa. This protein is localized in the surface coat and has similarities with mucin-type glycoproteins (Radding et al. 1989). Based on the fact that administration of monoclonal antibodies recognizing this protein had a beneficial effect on the course of the experimental pneumonia, it has been suggested that it plays some role in pathogenesis (Gigliotti & Hughes 1988). Indeed, the surface of trophozoites is covered by the major surface glycoprotein or glycoprotein A which is a family of protein encoded by up to one hundred heterogeneous genes. These genes are localized at the ends, upstream of the subtelomeric and telomeric repeats of all chromosomes. Transcription is limited to a single gene each time and is involved in a process that resembles the antigenic variation that has been well characterized in *Trypanosma brucei* (Stringer & Keely 2001).

Trophozoites interact with the surface of pneumocytes (Fig. 2). In most of the cases such interaction occurred through the surface coat that established contact with the microvilli of the epithelial cells. At some points such contact was done through filopodium-like structures. There are evidences that fibronectin is a mediator of the *P. carinii* attachment to pneumocytes with the participation of fibronectin-binding receptor on the fungus surface and a fibronectin-binding integrin of the host cell surface (Pottratz et al. 1991, 1994, Aliouat et al. 1993).

Part of the *P. carinii* population is ingested by alveolar macrophages in a process mediated by the Dectinn-1 β -glucan receptor, with production of hydrogen peroxide and subsequent killing of the organism (Steele et al. 2003).

Cell biology of the cystic form

The cysts are easily identified due to their typical morphology, being spherical structures with a mean diameter of 5-8 μ m, containing up to eight intracystic bodies (Figs 6-12). Each intracystic body has a mean diameter of 1.2 μ m.

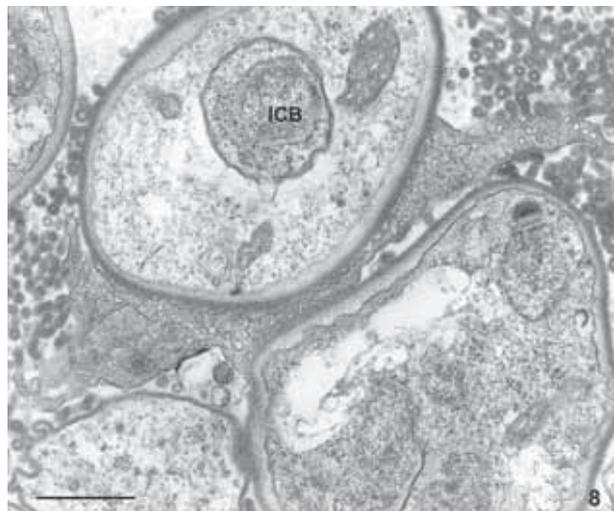
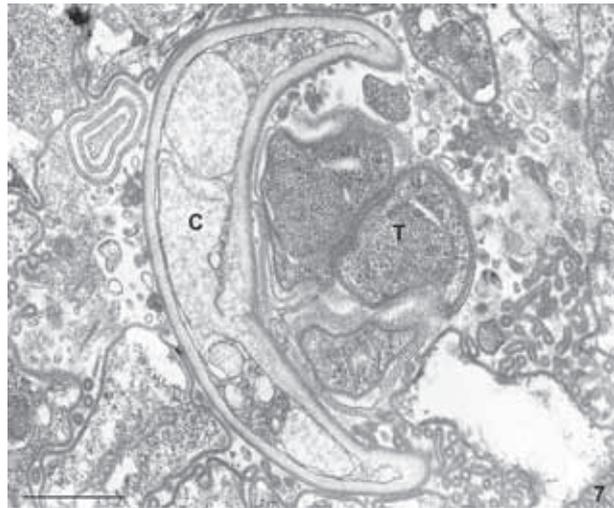
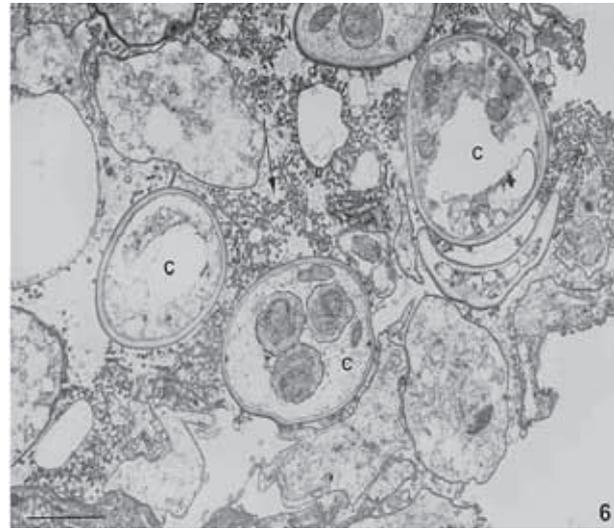
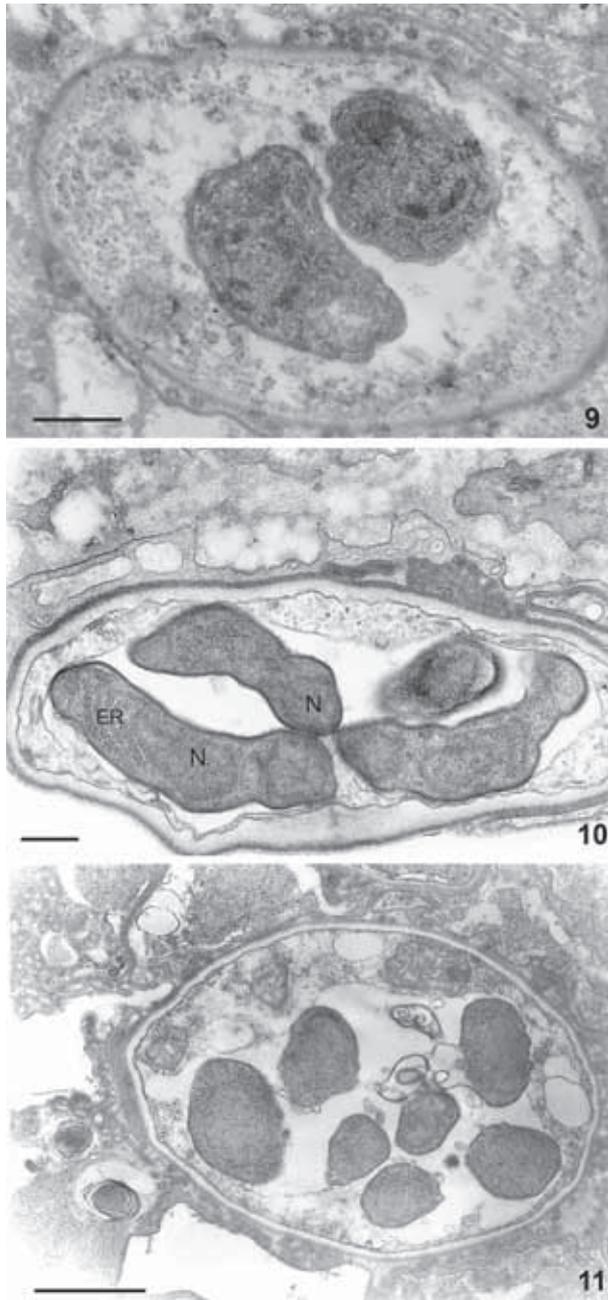


Fig. 6: thin section showing the presence of several cyst forms (C) in the lung of an infected rat. A large number of cross sections of the trophozoite surface projections are also seen (arrow). Bar = 2 μ m. Figs 7-8: thin sections showing structural features of the cyst forms. Structures such as the nucleus (N), mitochondrion (M), vacuoles (V), ribosomes and profiles of the endoplasmic reticulum can be seen. In the cyst shown in the upper portion of figure 8 one intracystic body (ICB) is seen. Bars: Fig. 7 = 2 μ m; Fig. 8 = 1 μ m.

The cyst wall has two layers and is approximately 50 nm thick (Figs 6-8). The outer layer, with a thickness of about 15 nm, is more electron-dense. The inner layer, with a thickness of 35 nm, is less dense and is in contact with the plasma membrane. Some authors described the presence of a membrane-like structure on the outer electron-dense layer (DeStefano et al. 1990a). However, this structure was not seen in most of the published electron micrographs. A general schematic view of the cystic form is shown in Fig. 12.



Figs 9-11: evolution of the cyst form. Cysts containing two, four, and eight intracystic bodies are shown. Each intracystic body contains all components characteristic of a eukaryotic cell such as the nucleus, endoplasmic reticulum and other organelles. Bars: Fig. 9 = 1.5 μ m; Fig. 10 = 250 nm; Fig. 11 = 1 μ m.

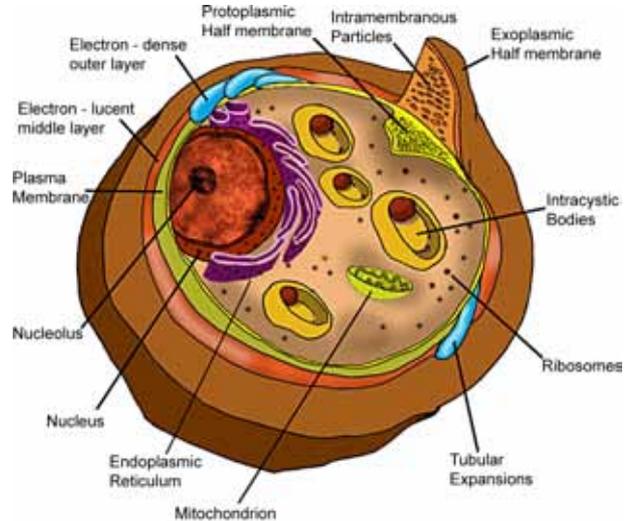


Fig. 12: schematic view of a cyst form where the main structures are indicated.

Freeze-fracture studies show the presence of intramembranous particles on the fracture faces of the cyst membrane (Yoneda et al. 1982). This number, however, was smaller than that observed in the membrane of the trophozoites.

Biochemical analysis has shown that the cyst wall is rich in glucosyl/mannosyl and galactose/N-acetyl-D-galactosamine residues (DeStefano et al. 1990b). The inner portion of the cyst contains two components: a matrix and the intracystic bodies. The matrix contains mitochondria, ribosomes, empty vacuoles and membrane debris. Its dimension varies according to the size of the cyst and the number of intracystic bodies. The intracystic body is a spherical to oval shape, with a centrally located nucleus, endoplasmic reticulum, free ribosomes, mitochondria, and glycogen particles.

It is very common to observe cystic bodies with a banana-like shape. These forms only show the matrix content, but no intracystic bodies. They are considered as remnants of the ruptured cysts which have released the intracystic bodies.

The use of the Thiéry technique, which reveals the presence of carbohydrates, showed the presence of glycogen particles in the trophozoites, the intracystic bodies and in the cyst matrix. The trophozoite's plasma membrane, the membrane lining the cyst and the intracystic bodies was also labeled, but the trophozoite's surface coat and the cyst wall were not labeled.

Perspectives

It is clear from the results described above that we know little about the cell biology of *Pneumocystis*. This is because there is no experimental system in which the organisms can be easily maintained *in vitro*, that would enable to its life cycle be studied in detail. Therefore, it is important that such a system is found to open up new possibilities for further biochemical and molecular studies.

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