The *Pneumocystis* life cycle

Cécile-Marie Aliouat-Denis[1,2], Anna Martinez[1,2], El Moukhtar Aliouat[1,2], Muriel Pottier[1], Nausicaa Gantois[2], Eduardo Dei-Cas[2,3]/+

[3]Department of Parasitology-Mycology, Faculty of Medicine, University Hospital Center [1]Department of Parasitology, Faculty of Biological and Pharmaceutical Sciences, University of Lille Nord de France, 3 rue du Pr. Laguesse, BP83, 59006 Lille, France [2]EA3609, IFR142, Pasteur Institute of Lille, Lille, France

First recognised as "schizonts" of Trypanosoma cruzi, Pneumocystis *organisms are now considered as part of an early-diverging lineage of Ascomycetes. As no robust long-term culture model is available, most data on the* Pneumocystis *cell cycle have stemmed from ultrastructural images of infected mammalian lungs. Although most fungi developing in animals do not complete a sexual cycle in vivo,* Pneumocystis *species constitute one of a few exceptions. Recently, the molecular identification of several key players in the fungal mating pathway has provided further evidence for the existence of conjugation and meiosis in* Pneumocystis *organisms. Dynamic follow-up of stage-to-stage transition as well as studies of stage-specific proteins and/or genes would provide a better understanding of the still hypothetical* Pneumocystis *life cycle. Although difficult to achieve, stage purification seems a reasonable way forward in the absence of efficient culture systems. This mini-review provides a comprehensive overview of the historical milestones leading to the current knowledge available on the* Pneumocystis *life cycle.*

Key words: *Pneumocystis* - cell sorting - life cycle stages - ploidy - mating

History of research on the *Pneumocystis* life cycle

The history of the *Pneumocystis* genus, which was reviewed recently (Calderón-Sandubete et al. 2002, Redhead et al. 2006), begins in Brazil, where Carlos Chagas (1879-1934) first discovered *Pneumocystis* cystic forms in 1909 while he was assessing malaria prophylaxis measures in the state of Minas Gerais (Delaporte 2003). Chagas observed such forms in the lungs of guinea pigs inoculated with the blood of two children with trypanosomiasis and in the lungs of the first human case of acute American trypanosomiasis (Chagas 1911, Delaporte 2003). He wrongly thought that such forms revealed the occurrence of a schizogonic process in *Trypanosoma cruzi*, and for this reason he proposed to name the genus *Schizotrypanum* (Chagas 1909). In 1910, Antonio Carini (1872-1950), while director of the São Paulo Pasteur Institute (Brazil), found similar cysts in the lungs of rats (*Rattus norvegicus*) infected by *Trypanosoma lewisi* (Carini 1910). He sent tissue samples to Alphonse Laveran at the Pasteur Institute of Paris, where two Laveran's fellows, Mr. and Mrs. Delanoë, observed similar pulmonary cysts in sewer rats (*R. norvegicus*) from Paris that were not infected by trypanosomes. They concluded that the pulmonary cystic bodies reported by Chagas and Carini were indeed a new biological entity unrelated to trypanosomes (Delanoë & Delanoë 1912, Hughes 1987, Calderón-Sandubete et al. 2002) and they speculated about its potential relationship with coccidian protozoa. They suggested naming it *Pneumocystis carinii*: "Pneumo" in relation with lung tropism, "cystis" because of its typical shape and "*carinii*" to honour Dr. Antonio Carini (Delanoë & Delanoë 1912). Delanoë and Delanoë (1912) only mentioned cystic forms and no reference was made to non-cystic forms of *Pneumocystis* for a long time. In 1942, van der Meer and Brug published the first light microscopy photographs of *Pneumocystis* trophic and cystic forms in human beings [accompanied by concise and aesthetic drawings' reproduced in the book by Hughes (1987)].

Although many authors (for instance, Jirovec 1964, Kim et al. 1972) suggested hypotheses regarding the *Pneumocystis* life cycle, the best ones were based on ultrastructural observations (Vavra & Kucera 1970, Campbell 1972, Vossen et al. 1978, Matsumoto & Yoshida 1984, Yoshida 1989, Dei-Cas 2000). Thus, a major event in the history of *Pneumocystis* life cycle research was undoubtedly the finding of synaptonemal complexes in the nucleus of early sporocytes, indicating the occurrence of a meiotic division, i.e., of a sexual cycle (Matsumoto & Yoshida 1984). Thus, these authors proposed an original *Pneumocystis* life cycle hypothesis with a high heuristic value (Dei-Cas et al. 2004, 2006, Aliouat-Denis et al. 2008) that associates both sexual and asexual modes of multiplication (Matsumoto & Yoshida 1984, Yoshida 1989).

The fungal nature of *Pneumocystis* was suggested by Vavra and Kucera (1970) on the basis of ultrastructural studies. It was further strengthened by Ruffolo (1994) when he proposed to rename the *Pneumocystis* stages according to fungal terminology. The controversy about the fungal versus protozoan nature of *Pneumocystis* remained until the end of the 1980s when the first definite proof of its fungal nature was provided (Edman et al.

Financial support: ANR-ERA-NET "Pneumocystis" PathoGenoMics Program, ANR-06-PATHO-009-01, French Ministry of Research and Education (EA3609, University of Lille 2, Quadrennial Contract, 2006-2009)

+ Corresponding author: eduardo.dei-cas@pasteur-lille.fr

Received 24 July 2008
Accepted 10 March 2009

1988, Stringer et al. 1989). The genus *Pneumocystis* has its own designated Pneumocystidaceae family (Eriksson 1994), which belongs to the subphylum "Taphrinomycotina" *sensu* Eriksson and Winka 1997 or "Archiascomycetes" *sensu* Nishida and Sugiyama 1994. The monophyletic taxon "Taphrinomycotina" includes ecologically and morphologically diverse fungi that diverged early from Ascomycota (Sugiyama et al. 2006).

Pneumocystis life cycle stages

Hypotheses on the *Pneumocystis* life cycle stem from transmission electron microscopy (TEM) micrographs and three-dimensional reconstructions of *Pneumocystis* organisms as well as recent molecular data. All known life cycle stages of *Pneumocystis* have been found in the lungs of infected mammals and, more rarely, in extrapulmonary locations primarily in severely immunocompromised hosts (Ng et al. 1997, Anuradha & Sinha 2007). Several authors have drawn slightly different hypothetical life cycle schemes in which both sexual and asexual reproduction alternate in vivo (Yoshida et al. 1984, Yoshida 1989, Cushion 2004, Dei-Cas et al. 2004, De Souza & Benchimol 2005, Thomas & Limper 2007, Aliouat-Denis et al. 2008). Lack of robust, long-term culture has prevented detailed dynamic follow-up of differentiation of the *Pneumocystis* life cycle stages. Thus, it was formerly impossible to test hypotheses on the life cycle.

Trophic forms, sporocytes and mature cysts are usually considered as the three main morphological forms involved in the *Pneumocystis* life cycle. Trophic forms are the most abundant of all *Pneumocystis* life cycle stages, representing 90-95% of the total population in the lungs of hosts with pneumocystosis. These vegetative forms appear as mononuclear, 2-8 µm in diameter, mostly haploid eukaryotic cells (Cornillot et al. 2002, Dei-Cas et al. 2004) presenting a thin cell wall consisting of an electron-dense single layer (Table). Ameboid in shape, trophic forms display cytoplasmic projections known as filopodia, which allow them to attach closely to type I pneumocytes (Aliouat-Denis et al. 2008). Ultrastructural studies as well as the re-

TABLE

Life cycle parasite stages of *Pneumocystis* organisms

A: Light microscopy

	Trophic form	Early sporocyte	Intermediate sporocyte	Late sporocyte	Cyst
TBO	-	-	+	+	+
Giemsa	+	+	+	+	+

TBO: Toluidine Blue O; -: unstained; +: stained (modified from Dei-Cas et al. 2004).

B: Transmission electron microscopy

	Trophic form	Early sporocyte	Intermediate sporocyte	Late sporocyte	Cyst	Spore
Shape	Irregular	Ovoid	Ovoid	Ovoid	Ovoid	Ovoid, amoeboid or crescent-shaped
Diameter (µm)	2-8	3.5-4.5	4-6	4-6	4-7	1-2
Number of nucleus	1	1	2-8	8	8[a]	1
Ploidy[b]	n	2n[c]	n	n	N[a]	n
Synaptonemal complexes	No	Yes	No	No	No	No
NAO[d]	No	No	Yes	Yes	No	No
Nuclear spindle	No	Yes	Yes	No	No	No
Mitochondrion[e]	Elongated, little ramified	Ramified	Dendritic	Split	Ovoid, dense	Ovoid, dense
Cell wall (diameter in nm)	Electron-dense layer (20-25 nm)	Electron-dense layer (35-45 nm)	Electron-dense + inner electron-lucent layers (80-100 nm)	Electron-dense + inner electron-lucent layers (80-120 nm)	Electron-dense + electron-lucent layers (80-120 nm)	Electron - dense layer (20-23 nm)
Filopodia	numerous	poorly developed	scarce	scarce	scarce	none

a: one nucleus in each one of the eight spores; *b*: hypothetical ploidy considering the report by Matsumoto & Yoshida (1984) and our own observations (modified from Dei-Cas et al. 2004); *c*: diploid early sporocyte results probably from conjugation of haploid trophic forms; *d*: nuclear-associated organelles; *e*: these terms refer to detailed computer-aided 3D-ultrastructural reconstruction of *Pneumocystis* mitochondrion as described by Palluault et al. (1991a, b).

cent discovery of the *Ste3* pheromone receptor gene and the *Ste2*-like homologue within the *Pneumocystis* genome suggest the existence of mating types and the probable conjugation of *Pneumocystis* trophic forms in vivo (Itatani 1996, Smulian et al. 2001, Cushion 2004). Following the fusion of mating types, a round, thin-walled, mononuclear and probably diploid early sporocyte is produced and shortly thereafter enters a meiotic division process. Ultrastructural images of a synaptonemal complex reported within the nucleus of early sporocytes support the occurrence of meiosis in *Pneumocystis* (Matsumoto & Yoshida 1984, Peters et al. 2001). Meiosis is followed by an additional mitotic replication resulting in eight nuclei in the late sporocyte stage (Table, Fig. 1). While nuclear division and organelle segregation proceed, an electron-lucent layer appears between the electron-dense layer and the plasma membrane of the intermediate sporocyte. It further thickens at the late sporocyte stage to finally produce a thick-walled mature cyst that measures 4-7 µm in diameter (Dei-Cas et al. 2004). Its surface is rather smooth, with few filopodia. In the mature cyst, eight individual spores are clearly delineated and are protected by a cell wall that appears as thick as the cell wall of the trophic form (Table). Spores consist of a single nucleus and a fairly dense cytoplasm with a rounded, well-defined electron-dense mitochondrion, a well-developed rough endoplasmic reticulum and numerous ribosomes

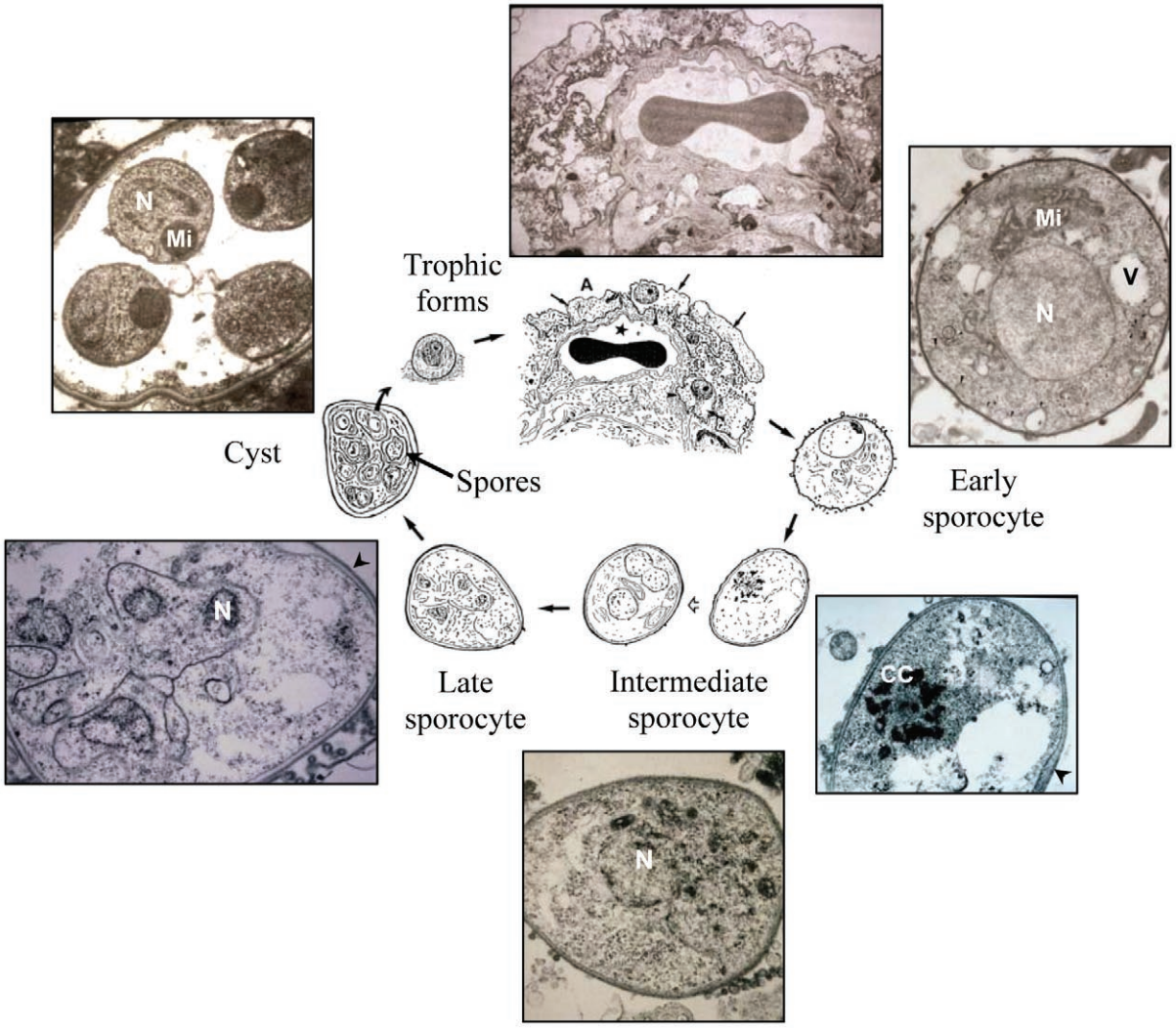


Fig. 1: a hypothetical *Pneumocystis* life cycle illustrated by transmission electron micrographs and corresponding interpretation drawings of organisms developing in mammalian lungs. Mononuclear thin-walled trophic forms (small arrows) are attached to type 1 epithelial alveolar cell that is close to a capillary vessel (star). Following conjugation (Itatani 1996), trophic forms would evolve into thin-walled round early sporocyte in which synaptonemal complex has been reported (Matsumoto & Yoshida 1984, Peter et al. 2001). While electron-lucent layer (arrowhead) develops in intermediate sporocytes, meiotic nuclear division proceeds. An additional mitotic replication leads to a thick-walled late sporocyte (arrowhead) containing eight nuclei. In the mature cyst, the eight spores are fully delineated. These forms are able to leave the cyst and subsequently attach to type I alveolar cells. A: alveolar space; CC: condensed chromosomes and spindle microtubules; N: nucleus; Mi: mitochondrion; V: vacuole. Arrowheads indicate thick cell wall.

(Dei-Cas et al. 2004). Morphologically, spores can be spherical, banana-shaped or irregular. Once fully matured, they leave the cyst, probably through a foramen-like structure, to give rise to eight free haploid trophic forms (Yoshida 1989, Itatani 1994).

Transmission has been shown to occur from individual to individual by the airborne route (Walzer et al. 1977, Hughes 1982), although the infective form has not been elucidated. Although *Pneumocystis* DNA has been detected in ambient air, no environmental form has thus far been isolated (Wakefield 1996). The thick-walled mature cyst is certainly best equipped to retain infectivity during transient host-to-host air travel. Furthermore, detection of mature cysts in the bronchial lumen by TEM suggests that they may reach the external environment and be transmitted to other hosts (Dei-Cas 2000).

Exploring *Pneumocystis* growth

The pathogenicity of *Pneumocystis* appears clearly linked to both the proliferation capacity of these fungi and the detrimental inflammatory response elicited by the host (Lebron et al. 2003, Thomas & Limper 2007). However, data about *Pneumocystis* multiplication mechanisms are scarce or controversial. As indicated above, in the infected lungs of patients or immunosuppressed laboratory animals, trophic forms represent 90-95% of the total parasite population. Mainly for this reason, it is currently thought that *Pneumocystis* proliferation results from active binary fission of trophic forms (Cushion 2004, De Souza & Benchimol 2005). Although few ultrastructural micrographs of binary fission have been reported (Richardson et al. 1989), 15 years of studying *Pneumocystis* ultrastructure led us to hypothesise that nuclear division would only occur within the cystic forms (Aliouat et al. 1999, Dei-Cas et al. 2004). In order to test this hypothesis, Aliouat et al. (1999) explored *Pneumocystis* growth in cultures of epithelial alveolar cells (Aliouat 1995, European Concerted Action 1996). Trophic forms, cystic forms containing developing or developed spores ("filled" cysts) and empty cysts were carefully quantified on dry smears stained with either Toluidine Blue O or RAL555, a Giemsa-like stain. The staining features of each parasite stage are detailed in Table. *Pneumocystis* growth kinetics was determined by plotting the parasite population' on a semilogarithmic curve, and doubling time (DT) was calculated at the exponential phase as follows: $DT = \ln 2/\mu$, where μ represents the specific growth rate (i.e., slope of the curve). An increase in the number of filled or empty cysts was observed, showing that cysts contributed to fungal growth. Indeed, the increase in the frequency of empty cysts indicated that the trophic forms were produced from filled cysts; reciprocally, the increase in the number of filled cysts indicated that the trophic forms produced filled cysts. Now, did the trophic forms develop further binary fission? If the trophic forms did not divide, each time one filled cyst produced an empty cyst, eight trophic forms would be released**.** Therefore, the empty cyst versus trophic form curve should be a straight line with a slope equal to eight. Alternatively, if the trophic forms did divide, the slope should obviously be greater than eight. Interestingly, in cultures of *P. carinii* with epithelial alveolar cells, the slope was actually 8.08, suggesting that one trophic form gives rise to only eight trophic forms via cyst production and that no other replication mechanism occurred (Aliouat et al. 1999). This result strengthens the hypothesis proposed by Schmatz et al. (1990, 1991) that the cyst stage is required for proliferation of trophic forms.

Mating

Pneumocystis species as well as biotrophic fungi of plants (O'Connell & Panstruga 2006) usually develop sexual reproduction within their host (Cushion et al. 2007). In contrast, most fungi developing in animals do not appear to complete a sexual cycle (Sexton & Howlett 2006). Some exceptions to this rule may nevertheless be noted. *Aspergillus nidulans* is sometimes able to sexually reproduce in vivo in human as well as in animal tissues, thus producing cleistothecia and Hülle cells (Doby & Kombila-Favry 1978, Dei-Cas & Vernes 1986, Mitchell et al. 1987). More recently, the existence of an anamorph state of *Candida lusitaniae* was questioned since 100% of 76 clinical isolates were able to mate and undergo meiosis once put in contact with a sexually compatible strain (François et al. 2001). This result indicates that *C. lusitaniae* still uses meiosis as a source of genetic variability; this could explain the higher propensity of *C. lusitaniae* to acquire antifungal resistance (François et al. 2001).

Pneumocystis species also present exceptions to the rule. TEM micrographs as well as recently identified mating genes located in the *Pnemocystis* genome (The *Pneumocystis* Genome Project available from http://pgp.cchmc.org/) provide evidence for the existence of conjugation between trophic forms. Itatani (1996) reported the occurrence of binucleated trophic forms in the lungs of infected rats. The existence of a cytoplasmic isthmus and the asymmetrical position of both nuclei on one side indicate that both trophic forms probably fused at one definitive site and that one nucleus migrated toward the other. Moreover, the close apposition and orientation of spindle pole bodies associated with each nucleus are strongly suggestive of conjugation (Itatani 1996).

More recently, fungal homologues involved in pheromone/mating signalling cascades have been identified in the *Pneumocystis* genome, strengthening the hypothesis of the existence of a sexual cycle in *Pneumocystis*. Generally, the mating process is initiated after mutual secretion of pheromones by fungal cells of opposite mating types. Pheromone secretion is also stimulated by environmental stress like nutrition deprivation (Li et al. 2007). Pheromones recognise a heterotrimeric G-coupled transmembrane receptor located at the cell surface of the opposite mating type. In turn, the mitogen-activated protein kinase (MAPK) signal transduction cascade is activated (Li et al. 2007). Once activated, MAPK controls many cell effectors that halt the mitotic cell cycle, initiate transcription of genes involved in mating and eventually allow the fusion of both cells (Harigaya & Yamamoto 2007).

Using degenerate PCR and library screening, Thomas et al. (1998) identified a gene encoding for a MAPK in *P. carinii* that is homologous to other fungal MAPKs involved in differentiation and proliferation. A few years later, heterologous expression of *P. carinii* MAPK (PCM) was shown to restore pheromone signalling in *Saccharomyces cerevisiae* Fus3/Kss1 double mutants (Vohra et al. 2003b). PCM was mainly expressed in *P. carinii* trophic forms (Vohra et al. 2003b). PCM was then reported to phosphorylate the *P. carinii* homologue of *Schizosaccharomyces pombe Ste11* (*Ste12* in *S. cerevisiae*), which encodes a transcriptional factor necessary for the pheromone-induced expression of genes required for mating (Vohra et al. 2003a). Once conjugation has occurred, activation of *Ste11* indirectly turns on *Mei2*, which plays pivotal roles in both the induction and the progression of meiosis (Harigaya & Yamamoto 2007). PC*Mei2*, a homologue of *S. pombe Mei2*, has recently been identified using the *Pneumocystis* Genome Project database (Burgess et al. 2008). The same research group also identified another kinase, PC*Ran1*, as an *S. pombe Ran1* (*Pat1*) homologue. *Ran1* is known to directly phosphorylate and inhibit the activity of *Mei2*. Indeed, Burgess et al. (2008) have shown phosphorylation of Pc*Mei2* by PC*Ran1* in vitro as well as higher expression of PC*Ran1* in cystic forms, thus suggesting that meiosis is inhibited in this life cycle stage. Moreover, both genes exhibited functional activity in meiotic control when expressed in *S. pombe*.

P. carinii Ste3, an a-factor pheromone receptor homologue, was identified from an expressed sequence tag (EST) database that was created as part of the *Pneumocystis* Genome Project (Smulian et al. 2001). It further confirms the existence of a sexual replication cycle in vivo (Smulian et al. 2001). This G-protein-coupled receptor was later reported to be exclusively expressed in a sub-population of trophic forms (Vohra et al. 2004). This finding is consistent with the expression pattern of pheromone receptors in other fungi. So far, no ligand has been identified for this receptor. Genes encoding functional elements of the pheromone response signal transduction cascade, like *Ste12* and *Ste20* orthologues, are clustered around the *Pneumocystis Ste3* gene (Smulian et al. 2001). *P. carinii Ste20* (*PCSTE20*) was later shown to be expressed following adherence of the fungus to extracellular matrix components and lung epithelial cells (Kottom et al. 2003). Heterologous expression of *PCSTE20* conferred pseudohyphal growth and also revealed the gene to be functional in mating signalling pathways in *S. cerevisae* mutant strains (Kottom et al. 2003). Additional orthologues of fungal genes associated with either the mating/sexual mode of replication or stress/nutritional deprivation were identified in the *P. carinii* EST database, thus pointing to these conditions as triggers of *Pneumocystis* mating (Cushion et al. 2007).

How to investigate the *Pneumocystis* cell cycle?

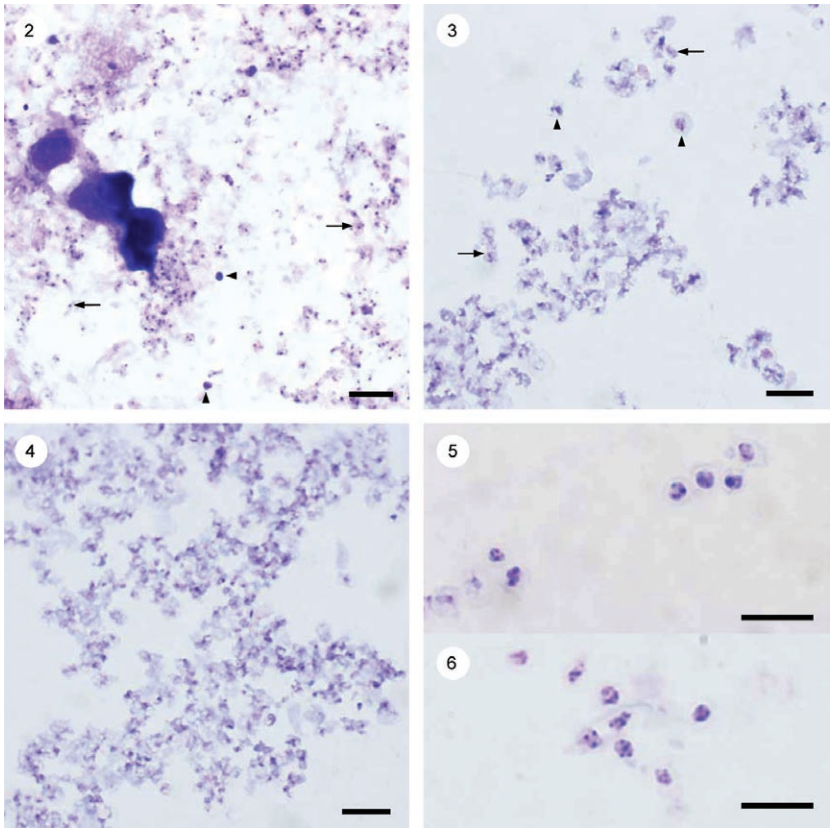
Our aim is to dynamically study, both in vitro and in vivo, the completion of the *Pneumocystis* life cycle. To reach this aim, several obstacles need to be overcome. First of all, no long term culture system of *Pneumocystis* organisms is available, which prevents any attempt to synchronise fungal growth or stage differentiation in vitro. It is thus difficult to pinpoint the outcome of each life cycle stage. Second, *Pneumocystis* organisms are fairly small in size and have a tendency to form aggregates that render purification from host cell debris and stage-to-stage separation difficult. A number of authors have tested several methods such as sucrose density centrifugation (Lim et al. 1973), gravity sedimentation (Taylor & Easmon 1990) or dialysis (Read & Burns 1991) and Percoll gradient (Chin et al. 1996) in order to enrich for fungal organisms against host cell debris (De Stefano et al. 1994).

Several techniques provide partial stage-to-stage purification of *P. carinii*; they include gravitational field-flow fractionation (Bories et al. 1992), immunomagnetic sorting (Aliouat-Denis, unpublished observations), sequential filtrations (Durkin et al. 1991, Vohra et al. 2004), elutriation combined with sequential filtrations (De Stefano et al. 1994) and flow cytometry (De Stefano et al. 1992). The last two procedures only provide good enrichment of cystic forms (95-96%) and their infectivity has not been tested.

High-speed cell sorting was our chosen strategy to purify *P. carinii* organisms from host cell debris and to achieve stage-to-stage separation with high purity (Martinez et al. 2009). A polyclonal antibody produced in our laboratory was used to label the whole *P. carinii* population. A commercially available monoclonal antibody (Monofluo™ Kit "*Pneumocystis jirovecii (P. carinii)"*, BioRad) that requires trypsin digestion was used to specifically label cystic forms. The epitope recognised by the monoclonal antibody lies in the electron-lucent layer of rodent and human *Pneumocystis* sporocytes and mature cysts (Sukura et al. 1994) and does not cross-react with host cell debris or yeasts (Lautenschlager et al. 1996). The experimental protocol was refined in order to avoid *P. carinii* clumps and to reach the best compromise between efficient cyst labelling and preservation of fungus integrity. The cytological and ultrastructural integrity of trypsin-treated *P. carinii* organisms was assessed. Trypsin treatment affected the outer electron-dense layer that was thinner in trypsin-treated trophic forms than in controls, whereas it completely disappeared in cystic forms, directly exposing the electron-lucent middle layer to the external environment. No other ultrastructural alterations were noted. Separation of cyst forms from trophic forms was achieved by flow cytometry with a purity of 99.6 ± 0.3% in 27 independent cell sorting experiments (Figs 2-6) (Martinez et al. 2009).

Dexamethasone-treated Lou nu/nu rats were used to monitor the impact of the trypsin treatment, co-immunostaining and sorting steps on *P. carinii* infectivity. Following endotracheal inoculation, sorted *P. carinii* had decreased infectivity but remained infectious to the Nude rats (Martinez et al. 2009).

We are currently following stage-to-stage differentiation of *P. carinii* both in vitro and in vivo. Purified life cycle stage populations are endotracheally inoculated to



Figs 2-6: purification of *Pneumocystis carinii* trophic (arrows) and cystic (arrow heads) forms using high speed cell sorting. *P. carinii* organisms are stained with panoptic RAL-555 stain. Scale bar = 10 µm; 2: *P. carinii* organisms recovered from rat lung tissue before immunostaining and cell sorting. Trophic forms (arrows) and cysts (arrowheads) are clearly visible. Numerous lung cell debris (e.g. dark round bodies) are present. 3: whole population of *P. carinii* life cycle stages after cell sorting. No lung cell debris is visible. 4: purified *P. carinii* trophic forms after cell sorting. 5, 6: purified *P. carinii* cystic forms after cell sorting. Purity reached 99.6 ± 0,3% in 27 independent sorting experiments.

Nude rats and the proportions of trophic and cystic forms as well as sporocytes are followed during the course of pneumocystosis. The behaviour of the purified life cycle stages was also studied in vitro both in axenic culture and in co-culture with L2 cells (rat lung epithelial cells, ATCC CCL-149). This approach will allow us to pinpoint the blocking step that prevents the completion of the *P. carinii* life cycle in culture. Ideally, stage-specific genes or proteins as well as genes/proteins involved in stage-to-stage differentiation will be identified by comparing gene expression profiles using microarrays and the 2D gel electrophoresis patterns of purified *P. carinii* trophic or cystic form populations. These transcriptomic and proteomic approaches have already been started as part of an international collaboration network (ANR-ERA-NET "*Pneumocystis*" PathoGenoMics Program, ANR-06-PATHO-009-01).

Estimation of the ploidy level of *P. carinii* has already been reported and haploidy has been predicted for most *Pneumocytis* life cycle forms (Stringer & Cushion 1998). Fifteen gene-specific DNA probes were hybridised to individual bands on seven karyotype forms produced by pulse-field gel electrophoresis (Cushion 1998). Each probe hybridised to a single band in all electrophoretic karyotypes, suggesting that *P. carinii* f.sp. *carinii* is mainly haploid. Similarly, quantitative image analysis of *Pneumocystis* nuclei stained with fluorescent dyes revealed that both trophic forms and spores were haploid (Wyder et al. 1998). Two-dimensional pulse-field gel electrophoresis allowed Cornillot et al. (2002) to identify homologues for at least two chromosomes. These results raised some questions about the ploidy status of the *Pneumocystis* nucleus. Our preliminary experiments of DNA content measurements in *Pneumocystis* cystic forms by flow cytometry confirm that each spore contains one copy of DNA content. In contrast, trophic forms would not only be haploid (unpublished observations).

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