

***Pneumocystis carinii*: History, Classification, Clinical Disease, Pathology, Diagnosis and Control in Laboratory Animals**

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Pneumocystis carinii is a poorly understood organism capable of infecting many mammalian species including man and several laboratory animals. Clinical disease is generally restricted to immunocompromised hosts, in which a potentially lethal pneumonia may occur.

History

P. carinii was first described in 1909 by Carlos Chagas, who mistook it for a cystic form of *Trypanosoma cruzi* in a guinea pig experimentally infected with *T. cruzi*. In 1910, Antonio Carini observed similar cysts in rats with experimental trypanosomiasis, but suspected the cysts were from an unknown organism. He sent samples to his colleague, Laveran, for further examination. In 1912, Laveran's students, Delanoe and Delanoe, found similar cysts restricted to the lungs of *Trypanosoma*-free sewer rats, and named the new organism *Pneumocystis carinii*. *P. carinii* was first associated with clinical pneumonia in humans just after World War II, in European orphanages plagued with malnutrition and overcrowding. From that time until the 1980s, *P. carinii* pneumonia was uncommon, occurring primarily in patients immunocompromised by cancer therapy or congenital immune deficiencies.⁵ Its incidence then rose markedly, coincident with the spread of acquired immunodeficiency syndrome (AIDS).^{49,54,55} In fact, it is the leading cause of opportunistic infection and death in AIDS patients.¹⁰³ *P. carinii* was once thought ubiquitous in laboratory animal colonies, but the increasing use of barrier-raised animals has led to a significant decrease in the prevalence of *P. carinii* infection in immunocompetent laboratory animals. Ironically, its incidence has actually increased due to the increased use of immunodeficient animals such as athymic T-cell deficient nude mice and severe combined immunodeficient (SCID) mice.

Taxonomy

The taxonomic classification of *P. carinii* has been the object of considerable debate, with the majority of investigators favoring a protozoa over a fungal classification. Elucidation of its taxonomic status has been hindered by the inability to grow the organism in non-animal systems. It can be grown on several tissue culture cell lines but is limited to a 10 - to 15 - fold increase, and continuous passage has not been achieved.¹⁰³ Recently, *P. carinii* has been propagated on an axenic culture medium, but only to levels similar to those attained in tissue culture.¹⁰ Further development of cell-free culture systems may eventually aid in the classification of this organism.

Historically, proponents of a protozoal classification for *P. carinii* based their arguments on its susceptibility to antiprotozoal drugs and on ultrastructural morphology.^{3, 84, 109} Investigators favoring a fungal classification countered that the organism stains with common fungal stains, and that its ultrastructure resembles that of certain fungi, not protozoa.^{4, 28, 83} Elaborate molecular biology techniques developed in the past decade have yielded new evidence supporting fungal classification. Analysis of 16S-like^{14, 77} and mitochondrial DNA⁵⁹ suggests *P. carinii* is closely related to the ascomycetes, such as *Saccharomyces cerevisiae*. However, the picture is by no means clear. Watanabe et al.¹⁰⁵ examined 5S rRNA sequences and found *P. carinii* to be closely related to another family of fungi, which they called Protista fungi (Rhizopoda/Myxomycota/Zygomycota group). Perhaps the best classification is that offered by Frenkel *et al.*,¹⁷ who suggest that *P. carinii* is a phylogenetically old parasite without close relatives.

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Host Range

P. carinii has been reported to infect a broad range of mammalian species including mice, rats, guinea pigs, rabbits, cats, dogs, ferrets, horses, foxes, chimpanzees, macaques, marmosets, humans, cows, sheep, goats, pigs and several wild rodents 4, 33, 52, 65, 67, 68, 70 Numerous studies have shown that *P. carinii* isolated from rats and humans have both shared 26, 40, 41, 81, 99 and distinct antigens. 24, 40, 41, 81, 99 In addition, antigenic variation has been demonstrated among isolates from different individuals in both rat and human cases of *P. carinii* pneumonia. 40, 41 Species-specific antigens have also been identified on mouse isolates of *P. carinii*, although mouse- and rat-derived *P. carinii* appear to be closely related when compared to human isolates. 17, 21, 51, 91, 102 Interspecies transmission has been reported in studies using nude and SCID mice to which human and rat isolates of *P. carinii* have been transmitted. 21, 66, 88 In one report, passage of a rat isolate through nude mice eventually resulted in loss of infectivity to rats, suggesting some host adaptation. 21 Analysis of rRNA sequences have revealed a close relationship between rat and human isolates of *P. carinii*, although limited differences exist. 14, 73, 86 Taken together, these results suggest that *P. carinii* isolates from various hosts are closely related, but bear distinct differences.

Life Cycle

The life cycle of *P. carinii* is unknown but is generally accepted to include four basic stages: trophozoites, precysts, cysts, and intracystic bodies. Trophozoites (trophic forms) are 1-5µm long, uninucleate, ameboid structures with a double-layered wall. This form adheres to alveolar walls and is the most active metabolically. Precysts are approximately 5µm long, oval, smooth, and have a thick cell wall. 28 Cysts are 4-6µm long, with a three-layered cell wall. Cysts are thought to be the major immunogenic stage, although shared epitopes with trophozoites have been demonstrated. 40, 51, 72 Intracystic bodies, usually eight in number, are found within precysts and cysts.

Based on these forms, Cushion et al. 8 have proposed a complex life cycle with both sexual and asexual phases. In the former, cysts (the putative infectious stage) are inhaled by a susceptible host, then rupture and release intracystic bodies. At least some of these intracystic bodies are believed to be haploid, based on demonstration of structures indicative of meiotic division in early precysts. 50 These haploid forms theoretically represent isogametes capable of copulation. Diploid trophozoites resulting from this copulation then develop into cysts, completing the sexual phase. The asexual phase, which involves binary fission of trophozoites, is thought to account for the majority of *P. carinii* multiplication within the lung. Evidence for this mode of replication is based on the ultrastructural examination of lungs, where events resembling binary fission have been observed. 8, 16, 27, 28 The fate of *P. carinii* outside of the host is unknown, and the possibility of an environmental phase must also be considered. 12 Like taxonomic classification of *P. carinii*, definitive elucidation of the life cycle has been hindered by the inability to grow the organization in cell-free systems, and growth on axenic cultures may bring clarification.

Pathogenesis

P. carinii pneumonia is thought to result from reactivation of a latent infection, and occurs primarily in the immunocompromised host. 92 Latent infection appears to be highly prevalent in man as well as in many conventional mice and rat colonies. 41, 49, 58 The actual mode of transmission is unknown, but is probably limited to the aerosol route, 35 with cysts expelled during exhalation and coughing. Cysts are then inhaled by another individual. In the pulmonary alveoli, intracystic bodies are released and develop into trophozoites. These trophozoites tenaciously attach to and interdigitate with type I 32, 38 and possibly type II pneumocytes. 42, 53 The alveolar surface lining layer, composed of phospholipids, apoproteins, mucopolysaccharides and surfactant, may nourish and even protect the organism.

The adherence mechanism is poorly understood. *P. carinii* has been found tightly interdigitating with alveolar and adjacent trophozoite cell membranes. 42 Numerous glycoproteins, which function as adhesins in other organisms, have been identified on its surface. 9, 44, 57, 63 One glycoprotein, gp120, has been shown to bind fibronectin and may function in the formation of a fibronectin bridge between *P. carinii* and host epithelium. 62 *P. carinii* has been shown to attach to fibronectin receptors on alveolar epithelium 60 and macrophages, 61 but the significance of this attachment in pathogenesis is unknown.

In the immunocompetent host, immune mechanisms are able to control growth or spread of the organism by a probable combination of cell-mediated and humoral immunity. In the immunocompromised host, these mechanisms fail, and *P. carinii* multiplies and eventually causes necrosis of type I pneumocytes with subsequent damage to alveolar basement membranes. Type II pneumocytes may proliferate in an attempt to repair the defect. Sufficient damage to the alveolar-capillary barrier leads to pulmonary edema and less successful gas exchange. 98 The nutritional status of the host may also play a role in susceptibility. It has been proposed that decreased intake or increased loss of protein and decreased absorption in certain diarrheic conditions may impair the immune system and allow unrestrained growth of *P. carinii* 36, 68

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Immunity to *P. carinii*

The exact mechanism of protection in the immunocompetent host is unknown but probably involves cell-mediated and humoral immunity. The importance of cell-mediated immunity was first demonstrated by Furuta et al.,¹⁸ who transferred immunized spleen cells to infected nude mice and observed a significant reduction in lung cyst numbers. In another study, Furuta et al.²⁰ demonstrated that peak delayed-type hypersensitivity reactions in experimentally infected mice correlated well with decline in lung cyst numbers, confirming the involvement of cell-mediated immunity. Recently, specific components of the cell-mediated immune system have been examined with respect to *P. carinii* infections. SCID mice reconstituted with spleen cells from immunocompetent mice were able to resolve *P. carinii* pneumonia, but lost this ability when depleted of CD4+ (T helper) cells. In contrast, depletion of CD8+ (T suppressor) cells had no effect.²⁹ Conventional mice experimentally depleted of CD4+ T cells developed progressive *P. carinii* pneumonia.^{29,69} Similarly, in AIDS patients the onset of *P. carinii* pneumonia is usually associated with marked depletion of CD4+ cells (<200 cells/mm³ of blood).⁴⁸ Taken together, these findings indicate cell-mediated immunity is required, though the exact mechanism is still unknown.

Several lines of evidence suggest that humoral immunity may also play a role. Furuta et al.¹⁸ demonstrated that transfer of rabbit antisera to infected nude mice increased survival time and decreased mortality, but did not prevent *P. carinii* multiplication. In other studies, passive immunoprophylaxis with a monoclonal antibody to a surface antigen on *P. carinii* resulted in markedly reduced *P. carinii* in the lungs of immunosuppressed ferrets and rats.²³ However, the presence or absence of serum antibody in cases of natural infection has not been shown to correlate with severity of disease.^{11, 45} It is thought that antibody may function as an opsonin¹⁸ and may aid in complement-mediated lysis or inhibit attachment of *P. carinii*.²³

Macrophages also play a critical role in *P. carinii* immunity. In vitro, in the presence of anti-pneumocystis serum, they ingest and rapidly degrade *P. carinii*.⁴⁷ Further evidence for their importance is found in studies utilizing C3H/HeJ mice. These mice, which have inherent macrophage defects, were found to be more susceptible to steroid-induced *P. carinii* pneumonia.⁹⁴ However, the inherent susceptibility of nude mice to *P. carinii* pneumonia indicates that macrophages alone are not capable of controlling *P. carinii* growth. The contribution of neutrophils is unknown; alone, they do not appear to play an important role in *P. carinii* clearance.³⁷

Clinical Signs

Clinical signs are similar in most laboratory animal species. These include weight loss, cyanosis, rough hair coat and rapid, labored respiration. A chronic wasting disease has been reported in infected nude mice,^{22, 107} with weight loss, development of dry, scaly skin and rapid, labored respiration. Clinical disease has been more severe in nude mice older than 4-6 months of age^{82,102} and in mice housed in microisolator cages. The latter may be due to increased parasite load in the environment or factors such as overcrowding and elevated humidity. This is somewhat ironic, as housing uninfected mice in microisolators helps to prevent *P. carinii* infection.¹⁰² Disease in SCID mice is characterized by a 30-45 percent reduction in median lifespan, progressive weight loss, debilitation, roughened hair coats, kyphosis, and varying degrees of dyspnea and cyanosis. Cyst burden increases with age, particularly after 60 days of age.⁶⁴ Hematocrit is strikingly elevated in moribund animals due to hypoxia-induced polycythemia. In one report, female mice had more trophozoites, alveolar epithelial proliferation, and chronic inflammation than males, but males had an apparent higher cyst density⁶⁴ and a slightly shorter lifespan than females. Lifespan in both sexes may be further shortened by concurrent infection with other infectious agents. Housing affected mice in individually ventilated (positive pressure) cage racks has been shown to decrease life span further.¹⁰² The clinical course of disease is relatively long, possibly due to the effectiveness of nonlymphoid inflammation. This marked inflammation may, however, eventually contribute to mortality.¹⁰²

Gross and Histologic Lesions

In advanced cases of disease, lungs appear grossly enlarged and solid with a firm, rubbery consistency, and may fail to collapse.¹⁰¹ In addition, gray-brownish areas of consolidation may be present. Histologic lesions of exudative alveolitis are similar in all species with *P. carinii* pneumonia. Mild infections consist of slight interstitial thickening with mild lymphocytic infiltrates.¹⁰² As the disease progresses, alveoli become distended with homogeneous, honeycombed foamy material (Figures 1 and 2, next page). This material contains proliferating and degenerating organisms, cell debris, antibody-antigen complexes, protein and fibrin. Alveolar macrophages become the prominent inflammatory cell type. Interstitial inflammatory response is usually minimal, with occasional thickening of alveolar walls and a lymphoplasmacytic infiltrate. In severe lesions, the majority of alveoli are filled with characteristic foamy material, and the interstitium may become edematous and even fibrotic.^{89, 102} Organisms are not readily identifiable on routine hematoxylin- and eosin- stained sections. Detection requires special stains or immunohistochemical techniques, as discussed below.

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Figure 1. Many alveoli in this section of lung are filled with pale-staining foamy material (hematoxylin and eosin stain).



Figure 2. The amorphous, pale, foamy material that fills the alveoli contains numerous clear spaces and is relatively acellular, except for a few macrophages (hematoxylin and eosin stain).

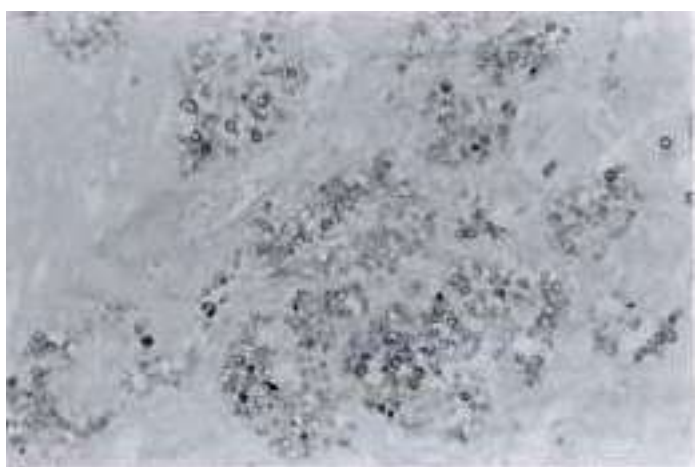


Figure 3. Grocott's methenamine silver stain demonstrates numerous oval cysts embedded in the alveolar material

Diagnosis

Diagnosis of *P. carinii* in rodents has historically been achieved via histologic examination of lung sections treated with various special stains.^{43, 101, 108} The most popular is Grocott's or Gomori's methenamine silver (GMS), which stains polysaccharide moieties on cyst walls¹⁰¹ and intracystic bodies¹⁰⁶ (Figure 3). Periodic acid-Schiff and Papanicolou stains have been used to display the characteristic honeycombed froth associated with *P. carinii*.^{87, 101} All these stains lack specificity, however, and may not detect low numbers of organisms. Added specificity can be obtained with immunohistochemical staining that uses polyclonal antisera against rat and mouse *P. carinii*. This technique also adds sensitivity because, in contrast to such routine histochemical stains as GMS, both trophozoites and cysts are stained and relatively easy to identify. This is especially important in younger animals where trophozoites may be the predominant form. Indirect immunofluorescence techniques have also been utilized on impression smears, but these lack cytoplasmic detail and do not establish a definitive diagnosis.⁷⁹ Immunohistochemical techniques are currently the most common method of *P. carinii* identification in human specimens obtained non-invasively (i.e., by bronchiolar lavage or induced sputum), where differentiation from other organisms is critical.⁴⁹ Immunohistochemical techniques lack sensitivity, however, and may fail to detect antigenic variants of *P. carinii*. These pitfalls have led to the development of molecular diagnostic techniques such as in situ rRNA hybridization, DNA hybridization, and polymerase chain reaction (PCR).^{31, 81} Of these, PCR is the most promising. It has been used to detect *P. carinii* in sputum samples from AIDS patients and tissue samples from infected mice and rats.^{39, 85} It provides specificity and added sensitivity, and may eventually provide a less invasive means of diagnosing *P. carinii* pneumonia.

Several serologic assays have been developed in man and rodents. They currently lack standardization and are impractical on a large scale due to the difficulties in obtaining large amounts of the antigen needed for testing.¹⁰⁰ Serologic assays also fail to differentiate previous exposure from active or latent infection.^{41, 101} The potential for crossreactivity with other organisms, such as *Toxoplasma gondii*, has also been reported.³⁰ Tests for antibody include ELISA, ^{19,41} immunoblotting ^{25,41,56,99, 102} and complement fixation.³⁴ Serological assays have been used to evaluate exposure to *P. carinii* in humans, rats and immunocompetent mice. ^{41, 58, 97}

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Control and Prevention

Control of *P. carinii* is of particular concern in colonies of nude and SCID mice, where it is primarily achieved by quarantine and testing, depopulation of infected groups, and repopulation with animals free of the organism.¹⁰² Its elimination from colonies of nude and SCID mice has also been achieved via caesarian rederivation.^{72, 102} Animals may be maintained free of *P. carinii* in isolators or microisolators. Treatment of SCID mice with trimethoprim (100 mg/kg/day) and sulfamethoxazole (500mg/kg/day) in the drinking water reduces mortality but does not eliminate the organism.¹⁰² Furthermore, since these drugs are not microbicidal, continual therapy is necessary, so treatment is not recommended as a control measure on a colony basis.

Colonies of immunocompetent or immunodeficient animals in which *P. carinii* status is important should be periodically monitored, generally by histologic examination. Serologic examination may demonstrate *P. carinii* exposure, but the drawbacks of *P. carinii* serology must be considered. In addition, since immunodeficient mice are unable to mount a meaningful antibody response to *P. carinii*,^{93, 102} a sentinel animal program is necessary if serologic monitoring is employed.¹⁰⁷

Animal Models for *P. carinii* Pneumonia

Laboratory animals are of paramount importance in the study of *P. carinii*. Because standardized tissue culture systems have not been established, cortisone-treated rats are often used as sources of *P. carinii*. In addition, both induced models (i.e., cortisone-treated rats) and spontaneous models (nude mice, SCID mice) have been used to investigate specific aspects of disease such as pathogenesis, host defense mechanisms, and potential treatments. For example, aerosolized pentamidine, currently the most common method of *P. carinii* prophylaxis in AIDS patients, was developed in the rat model.^{13, 25} Several animal models have been utilized in the study of *P. carinii*. The classic model is the corticosteroid-treated rat.¹⁵ Corticosteroids decrease host inflammatory response, inhibit phagocytosis by alveolar macrophages,⁸⁹ and lower serum IgG and IgM levels and antibody response to *P. carinii*.⁹² Corticosteroids also alter alveolar surface carbohydrates¹¹⁰ and decrease T helper cells with a subsequent reversal of the T helper/T suppressor cell ratio.⁹⁶ The model involves treating rats with dexamethasone in the water⁴⁶ or subcutaneous injections of cortisone acetate^{7, 15} or methylprednisolone⁷⁸ for approximately eight weeks, causing apparent activation of latent *P. carinii* pneumonia. Addition of a low-protein diet provides a more uniformly affected animal.⁹⁵ Tetracycline is also routinely added to prevent secondary bacterial infection. Recently this model has been refined by using rats free of *P. carinii* and transtracheally inoculating them with a known quantity of *P. carinii* isolate.^{1, 6}

Athymic mice have also been proposed as potential animal models.^{82, 88} Nudes can be infected by direct contact with affected rats or by intrapulmonary or intranasal inoculation. Intensity of infection in most nude mice is usually light, with minimal clinical signs, so nudes have primarily been recommended for epidemiologic studies.⁹⁰ Other models such as SCID mice, corticosteroid-treated mice,^{2, 94} nude rats,^{18, 22, 104} young rabbits,⁷⁵ cortisone - treated rabbits,⁷⁵ ferrets⁷⁶ and cats⁷¹ have also been utilized. Recent development of a model involving mice selectively and reversibly depleted of T helper cells may aid in studies of immunity to *P. carinii*.⁶⁹

Conclusion

P. carinii remains a poorly understood organism capable of infecting man and several laboratory animal species. It rarely, if ever, causes clinical disease in immunocompetent hosts. However, control and prevention of *P. carinii* pneumonia is extremely important in immunocompromised hosts such as athymic and SCID mice, in which fatal pneumonia can occur.

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