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Recognition of *Pneumocystis carinii* Antigen on its Surface by Immunohistochemistry and Immunoelectron Microscopy

The aim of this study was to detect the surface antigens in different stages of experimental induced *Pneumocystis carinii* in Sprague-Dawley rats. Immunohistochemical staining with monoclonal (900, 902 and 904) and polyclonal (SP-D) antibodies demonstrated that the *P. carinii* organisms were mostly in the alveolar lumina. The binding sites of the monoclonal (900, 902 and 904) and polyclonal (SP-D) antibodies developed against *P. carinii* were examined at the ultrastructural level by using a post-embedding immunogold labeling. The gold particles were observed evenly on the surface of precyst and cyst stages of the *P. carinii*. In the trophozoite stage, scattered gold particles were seen on the pellicles and tubular expansions. The monoclonal antibodies reacted mainly with pellicles of *P. carinii*, whereas SP-D labeled pellicles, intracystic bodies, cytoplasms of alveolar macrophages, free floating surfactant material in the alveolar spaces, and adjacent type II epithelial cells. In the immunogold labeling, basically no significant differences were found in the precyst, cyst, and ruptured cyst stages. These results indicate that the gold particles were observed adhering to every stage of *P. carinii*, mostly concentrated on the pellicles, and more concentrated in the precyst or cyst stage than trophozoite stage which may be due to an increase in antigen accumulation during development from the trophozoite to the cyst.

Key Words : *Pneumocystis carinii; Immunohistochemistry; Tissue embedding, Post-embedding immunogold labeling*

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INTRODUCTION

P. carinii pneumonia is encountered exclusively in immune-compromised hosts, and recently one of the most common infections and a frequent cause of death in human AIDS patients. Surveys have shown that the prevalence of *P. carinii* pneumonia is up to 85 percent (1, 2). Despite increasing attention to the pneumonia, the causal agent, *P. carinii*, remains an enigmatic organism from a biological point of view because of problems in isolating and culturing the organism (3, 4).

Maddison et a1. (5) suggested that glycoproteins were included in the antigen of *P. carinii*. Until now, studies on *P. carinii* antigens have been dealing mainly with the identification of specific proteins or glycoproteins localized on the electron-dense outer layer of trophozoites, and the electron-dense outer or electron-lucent middle layer of cysts (6-9), but the electron-dense layer could be immunologically variable (10).

Isolation and identification of *P. carinii* antigens are necessary for the biological characterization and for taxonomic studies of *P. carinii*. For these reasons, any researchers have attempted to produce high specific antibodies against *P. carinii*. A recent advance in hybridoma technology has developed several monoclonal antibodies to identify *P. carinii* (11-13). Antigens apparently specific for rat and human *P. carinii* have been identified through the use of monoclonal antibodies (11, 14) as well as polyclonal antibodies (9, 15). Many of these established monoclonal antibodies react with the surface of the organism, but there is little information concerning the ultrastructural localization of antigens identified by these monoclonal antibodies (16).

The aim of this study was to identify the immunohistochemical findings and ultrastructural morphology of the *P. carinii* and to detect the localizing sites of antigenic molecules on *P. carinii* at the ultrastructural level using a post-embedding immunogold labeling method.

MATERIALS AND METHODS

P. carinii Isolation

P. carinii pneumonia was induced in rats by immunosuppression with dexamethasone followed by intratracheal instillation of *P. carinii* (17). Sprague-Dawley rats (250 g) were housed in open cages and provided with drinking water containing dexamethasone (2 mg/liter), tetracycline hydrochloride (500 mg/liter), and nystatin (200,000 U/liter). The rats were maintained on a diet of 8% protein in order to intensify the severity of *P. carinii* infection (18). After five days of immunosuppression, rats were inoculated with *P. carinii* extracts which was prepared by grinding rat lungs with *P. carinii* pneumonia through a 150 mesh sieve. Rats were anesthetized with ether and approximately 500,000 *P. carinii* were injected intratracheally.

After 6-8 weeks of immunosuppression, rats exhibiting respiratory distress were sacrificed by intraperitoneal injection of 0.5 ml of 26% pentobarbital. Using a modified Wright-Giemsa stain (Diff-Quik, Harleco, Dade Diagnostics) we selected good sample of *P. carinii* rat by identification of *P. carinii* organisms (19). Control tissue was obtained from eight uninfected normal Sprague-Dawley rats which were fed a commercial pellet diet.

Light microscopy and immunohistochemistry

The lung were taken from each lobe and fixed in a solution of 10% buffered neutral formalin. After fixation, the tissue was processed by standard methods and embedded in paraffin. Sections were cut at 4 μm and stained by hematoxylin and eosin, and Gomori's methenamine silver technique. For immunohistochemical staining, 5 μm thick sections were cut and deparaffinized in xylene and rehydrated sequentially in ethanol, followed by a 30 min incubation in methanol mixed with 0.3% H_2O_2. Then slides were incubated for 1 hour in trypsin solution, and incubated with the primary antibodies, each 1:100 dilution of anti-*P. carinii* monoclonal (900, 902 and 904; Chemicon Inc., USA) and polyclonal (SP-D; Thoracic Research Lab., Mayo Clinic, USA) antibodies for 4 hours. After washing with phosphate buffered saline (PBS), slides were incubated with biotinylated horse anti-mouse antibody (Vector Laboratories, Burlingame, CA) for 30 min, then incubated with an avidin-biotin complex for 1 hour. After washing sections in PBS, peroxidase activity was developed with a diaminobenzidine (DAB) as indicator.

Transmission electron microscopy

The lungs were cut into small pieces, each about 1 mm^3 dimension, and were immediately fixed in phosphate-buffered glutaraldehyde solution (pH 7.2) for 2 hours and later post fixed in 1% buffered osmium tetroxide for 2 hours at 4℃. After processing through a graded series of ethanol and propylene oxide, the tissue was embedded in Epon. Semithin (1 μm) section in thickness was cut and stained with toluidine blue for location of suitable areas for ultrastructural examination. Ultrathin (60-90 nm) sections were obtained by a Porter-Blum MT-1 ultramicrotome, and stained with uranyl acetate and lead citrate, and examined under a Hitachi H-600 electron microscope.

Post-embedding immunogold labeling procedure

Three monoclonal (900, 902 and 904) and one polyclonal (SP-D) antibodies, raised against *P. carinii* pneumonia rats, were employed for immunostaining. *P. carinii* infected rat lungs were fixed in periodate-lysine-paraformaldehyde (PLP) fixative using a standard procedure (20). The tissues were washed with Tris buffer saline (TBS), dehydrated with graded series of ethanol, and embedded in Lowicryl K4M at -20℃ (16). Embedding and polymerization were done in a freezer fitted with a UV light because polymerization of this resin is exothermic and dependent on UV. The polymerized blocks were exposed under tungsten light for 1 week for more consolidation and polymerization. For representative block selection, semithin (1 μm) sections were mounted on slides and stained with toluidine blue. For immunoelectron microscopy, ultrathin (60-90 nm) sections were collected on a membrane-coated slides, labeled, and were blocked with 1% BSA in TBS with 0.1% Tween-20 (TBS-T) for 15 Min. After washing with TBS-T, the slides were incubated with drops of primary antibodies diluted 1:50 with TBS-T for 4 hours (16). After incubation in primary antibodies, the sections were rinsed with TBS-T, and incubated in secondary antibody (GAM-Au, 15 nm) conjugated to colloidal gold. Control sections were incubated parallel to the primary antibodies in TBS-T only. Again the sections were rinsed with TBS-T, followed by water rinse, and stained with uranyl acetate and lead citrate. Sections were removed from the slide and mounted on copper grids for examination under a Hitachi H-600 electron microscope.

RESULTS

Histopathological findings

In cases of massive *P. carinii* involvement in the lungs, the majority of alveoli were distributed by foamy eosin-

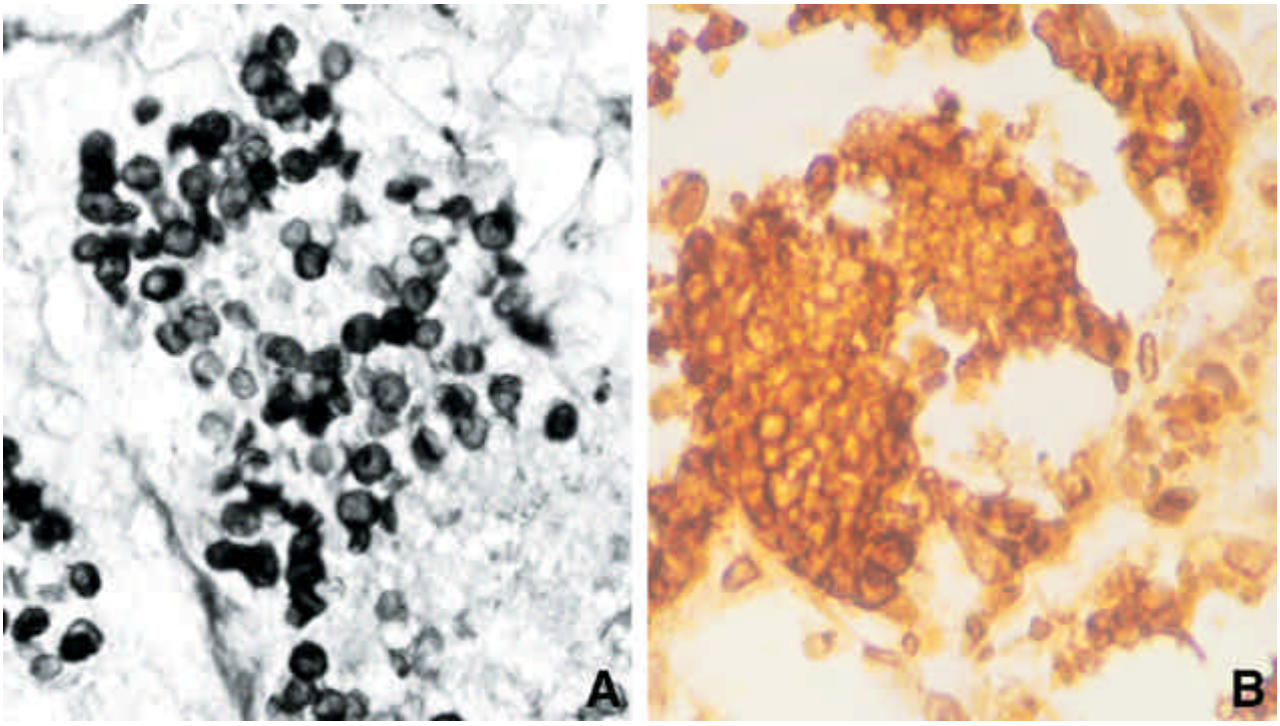

Fig. 1. A: Numerous *P. carinii* cysts showing oval, cup-shaped, and collapsed structures (Gomori's methenamine silver stain). B: Immunohistochemical staining (Pri. Ab=902) of *P. carinii*. The *P. carinii* are well identified mostly in the alveolar lumina (DAB).

ophilic exudate. Within the alveolar space, there was a mixture of *P. carinii* organisms, fibrin, a few alveolar lining cells, and macrophages. In the alveolar septa, there were variable degrees of mononuclear cell infiltrate including lymphocytes and some plasma cells. On Gomori's methenamine silver stain, the organism appeared as almost round, cup or crescent-shaped cysts, occasionally with a thick dark capsular dot (Fig. 1A). In the established cases, the local cellular alveolar response was minimal, though alveolar walls were thickened by proliferated fibroblasts, macrophages, and lymphocytes.

Immunohistochemical findings

Immunohistochemical staining with monoclonal antibodies (900, 902 and 904) demonstrated the foamy eosinophilic material to be densely packed by *P. carinii* cysts and trophozoites. Immunostaining with the monoclonal antibody 902 was more sensitive and intense than others (900 and 904). The polyclonal antibody, SP-D stained more intensely and diffusely than monoclonal antibodies. Immunostained *P. carinii* organisms were mostly detected in the alveolar lumina, but some were present on the epithelial surface of the alveolar walls, in the lumina of bronchioles, and in all levels of the small airways (Fig. 1B).

Transmission electron microscopy

Large number of cysts and trophozoites of *P. carinii* filled many alveoli and attached to alveolar type I epithelium (Fig. 2). Close examination of the cysts or trophozoites' attachment sites to the alveolar wall showed intimate contact between the plasma membrane of the type I epithelial cells and the membrane of the *P. carinii* organisms, which appeared to adapt its shape to the contours of the cell. The trophozoites varied considerably in shape as a result of compression and interlocking with adjacent organisms. The pellicle of the trophozoite was composed of an electron-dense outer layer and an inner plasma membrane, whereas in the precyst and cyst stages, an electron-lucent middle layer appeared between the outer dense layer and inner plasma membrane (Fig. 3). The internal structure of precysts consisted of finely granular cytoplasm, with irregularly scattered layers, electron-dense granules and nuclear regions with ill-defined membrane, and occasionally a prominent nucleolus. Many cysts appeared empty or collapsed, and some of which contained well identified intracystic bodies. Short tubular expansions were occasionally present on the external surface of the cyst pellicles. Collapsed cysts were also observed, appearing as crescent. Fibrin clumps, cell debris, and surfactant membranous material were present

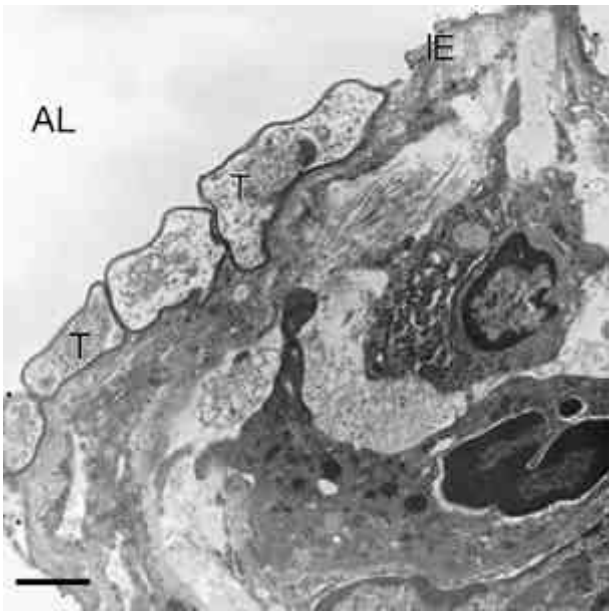

Fig. 2. Electron micrograph illustrating the relationship of *P. carinii* with the alveolar epithelium. The *P. carinii* are closely attached to the alveolar type I epithelium. AL: alveolar lumen, T: trophozoite, IE: type I epithelial cell (Uranyl acetate and lead citrate, ×13,600).

in a few alveoli. Macrophages had phagocytic trophozoites or cysts, which were identifiable within phagosomes, and many were undergoing lysis. There was also an evidence of proliferated fibroblasts and collagen fiber production in the interalveolar septa.

Post-embedding immunogold labeling

Immunogold labeling using the monoclonal antibodies produced consistent specific labeling with negligible background. Three monoclonal (900, 902 and 904) antibodies analysed in this study reacted mostly with the pellicle of *P. carinii*. A positive reaction was observed on both electron-dense outer and electron-lucent middle layers of the pellicles of *P. carinii* cysts using colloidal gold as a marker (Fig. 4). Occasionally positive reactions were observed in scattered cytoplasmic areas or on the intracystic bodies within the cysts. In the trophozoites, most immunogold particles adhered to the surface of the organisms, and some adhered to the tubular expansions (Fig. 5). One polyclonal (SP-D) antibody gave positive labeling of the trophozoites, pellicles and intracystic bodies of the cysts, free floating surfactant material in

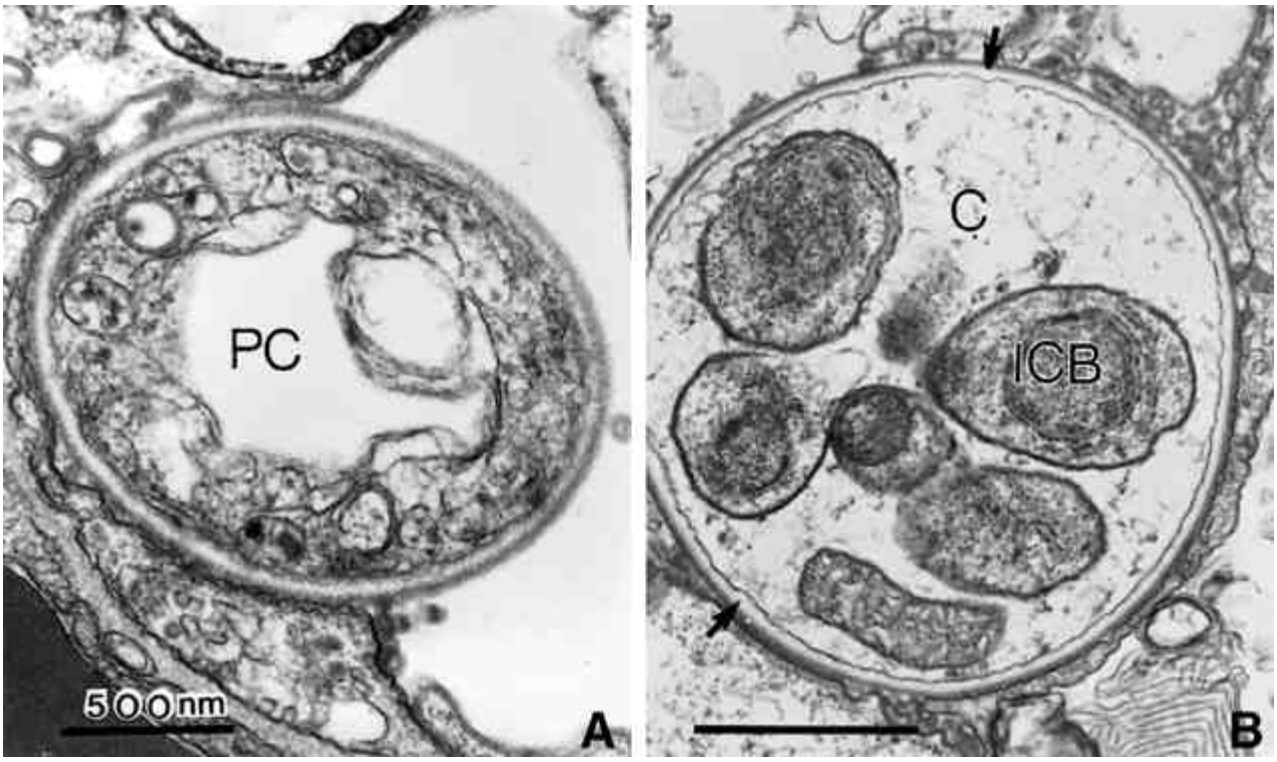

Fig. 3. A: A precyst shows some intracytoplasmic organelles and surface pellicle with outer electron-dense layer, middle electron-lucent layer and inner single plasma membrane. B: A mature cyst containing five intracystic bodies shows a fully developed trilayered pellicle with an electron-lucent middle layer (arrows) between the outer electron-dense layer and inner plasma membrane. The intracystic bodies show a nucleus, a few rough endoplasmic reticulum, amorphous granular particles, and one layered pellicle. PC: precyst, C: cyst, ICB: intracystic body (Uranyl acetate and lead citrate, A: ×39,000, B: ×26,000).

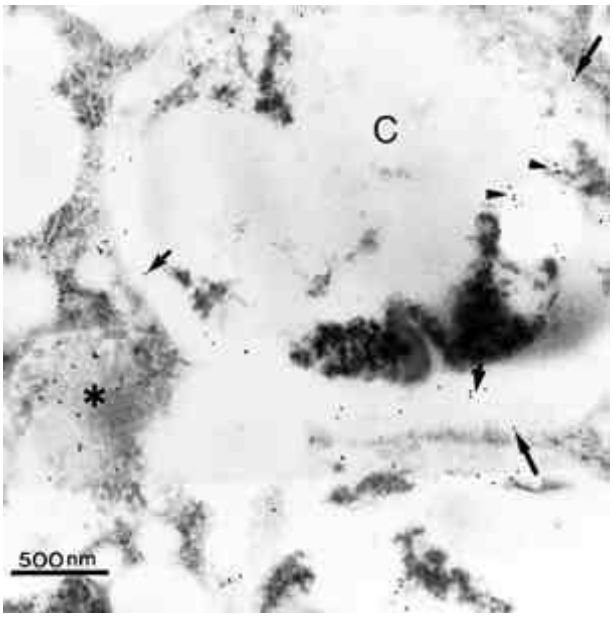

Fig. 4. Ultrastructural localization of antigenic molecules in *P. carinii* using post-embedding immunogold labeling technique with a monoclonal (902) antibody. Surface pellicle of a cyst shows immunogold particles on its outer layer (long arrows) and middle layer (short arrows). There are some scattered gold particles in the cytoplasm of the cyst (arrow heads) and tubular expansions of trophozoite (asterisk). C: cyst, ×36,000.

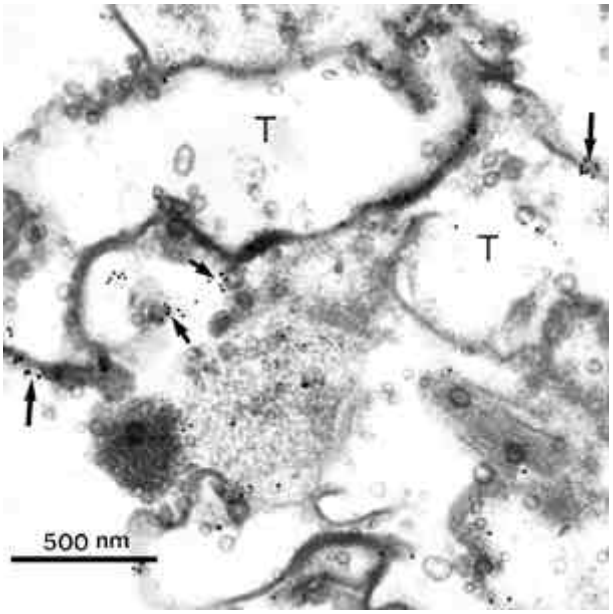

Fig. 5. In the trophozoites, immunogold particles (Pri. Ab= 902) adhered to the surface pellicle (long arrows), and some adhered to the tubular expansions (short arrows). T: trophozoite. ×55,000.

the alveolar space, type II epithelial cells, and macrophages (Fig. 6). Generally, the distribution of the gold particles on the surface of cysts was more homogenous

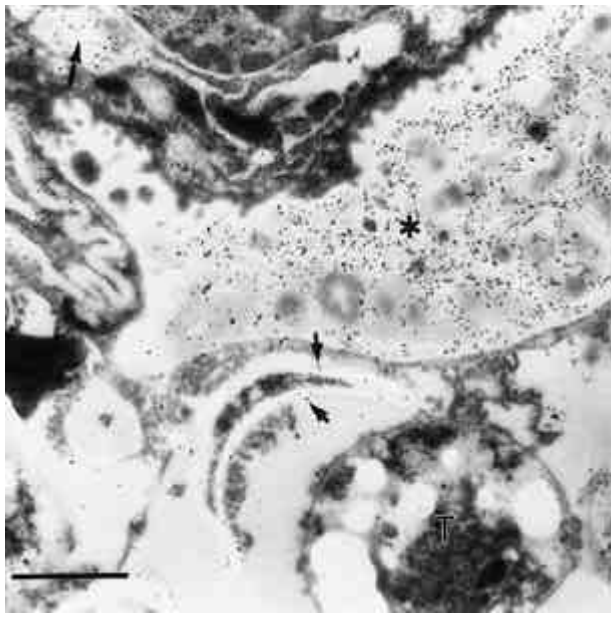

Fig. 6. Ultrastructural localization of antigenic molecules in *P. carinii* using polyclonal (SP-D) antibody. Immunogold particles are labeled on the surfactant material (asterisk), type II pneumocyte cytoplasm (long arrow) as well as the pellicles of the collapsed cyst (short arrows) and trophozoites (T). ×21,500.

than that of trophozoites. The density of immunogold labeling in trophozoites was lower than in the cysts.

DISCUSSION

Immunohistochemical techniques for *P. carinii* have been advocated to facilitate identification of the organism. Early efforts with polyclonal antibodies proved disappointing and did not enhance morphologic or serologic identification of *P. carinii* (21, 22). Recently several monoclonal antibodies have proven useful in the detection of *P. carinii* following formalin fixation and paraffin embedded tissues (11, 13, 23, 32). In this study, we found that all of monoclonal (900, 902 and 904) and polyclonal (SP-D) antibodies demonstrated the *P. carinii* organisms mostly in the alveolar lumina, occasionally on the alveolar epithelial surfaces, and in the lumina of all level of the small airways.

Electron microscopically, *P. carinii* cysts contain two, four, or eight intracystic bodies (sporozoites), presumably arising from mitotic replication (24). After maturation, these intracystic bodies rupture through the cyst wall, yielding free-living *P. carinii* trophozoites. The trophozoites adhere to the alveolar surface and prefer attaching to type I alveolar epithelial cells, although very occasionally some adhere to type II epithelial cells (25). *P. carinii* adhered to alveolar epithelial cells play a central

role in the initiation of the *P. carinii* pneumonia. Potential mechanisms mediating this attachment have been elucidated, and include cell surface glycoproteins, vitronectin, exogenous fibronectin, and components of the parasite cytoskeleton (26-28). Ultrastructurally, the apposition and interdigitation of pellicles of *P. carinii* with the surface membranes of the alveolar type I cells suggest that cytoskeletal components of *P. carinii* may be potentially important in maintaining that the organism shape changes during attachment (29).

Detergent extracts of *P. carinii* contain several glycoproteins that serve as surface antigenic determinants for the organisms, and the major *P. carinii* glycoprotein antigen is roughly 110 to 120 kDa molecular weight, with lesser surface components, 45 and 50 kDa (30). The surface isolated from rodent *P. carinii* are closely related, but immunologically distinct from *P. carinii* derived from humans (26). Gp120, a mannose-rich glycoprotein, is particularly abundant on the pellicle of *P. carinii* organisms (16, 30).

The stabilization of antigens in situ without destroying their antigenicity, while retaining relatively good tissue and cellular morphology, is a common problem in immunohistochemistry (20). The conventional fixatives interact strongly with proteins and thus often denature protein antigens (20). PLP fixative contains sodium-periodate, lysine and paraformaldehyde, and can preserve antigenicity as well as paraformaldehyde, ultrastructural morphology, and glutaraldehyde (20, 31). The antigenic material of *P. carinii* surface area is a carbohydrate in nature, fixation with PLP is best because of its ability to crosslink carbohydrate chains as well as protein. Since carbohydrates (specifically mannose) have been found to be major constituents of the surface of this organism, it is probable that this increase in labeling intensity is due to an increase in carbohydrate (mannose) accumulation (16, 32).

We used several monoclonal antibodies, and basically there were no significant differences among the antibodies. The antigenic activity of *P. carinii* was well preserved, and consistent results were obtained with the monoclonal (900, 902 and 904) and polyclonal (SP-D) antibodies. The localization of antigens on tissues which were applied with monoclonal antibodies is very specific, mostly with the pellicle of *P. carinii*, and occasionally with those situated on the intracystic bodies. Although a small amount of non-specific precipitation of the gold particles was observed in the background, but there did not, appear to be any labeling in the host pneumocytes in the monoclonal antibody group. In order to reduce the amount of background labeling produced by monoclonal and polyclonal antibodies to a minimum, it may be necessary to dilute the primary antisera (33).

The trophozoite, precyst, cyst, and intracystic bodies reacted well with each monoclonal antibody. The intensity of labeling of the electron-dense layer was higher in the precyst, cyst, and ruptured cyst stages than in the trophozoite stage (16). These results suggest that some of the pellicle antigens do not change during the precystic and cystic cycles of *P. carinii*, and also indicate the possibility of antigen accumulation or modification during the development from trophozoite to cyst (16). In addition, the specific antigenic materials are located on the outer layer and middle layer of the pellicles of *P. carinii*, and the labeling of both the electron-dense outer and electron-lucent middle layers indicate that similar antigens are present in both layers (16). In this study, the monoclonal antibody 902 reacts more intensely than others (900 or 904).

SP-D is a collagenous glycoprotein that is synthesized and secreted into the pulmonary air spaces by alveolar type II cells and non-ciliated bronchiolar cells (34). In this study, immunogold labeling using polyclonal (SP-D) antibody revealed a good positive labeling of trophozoites, cysts, free floating surfactant material in the alveolar spaces, and cytoplasm of type II epithelial cells. In the type II cells, SP-D labeled mainly on many vacuoles which contained previously surfactant material. Also SP-D labeled intensely on the cytoplasm of macrophages. In a related report, SP-D may play an important role in facilitating the clearance of *P. carinii* from the alveoli and small airways via enhanced uptake by alveolar macrophages (35).

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