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Isolation of A Major Surface Protein of *Pneumocystis carinii* Using Immunoaffinity Chromatography and Determination of Its Ultrastructural Localization

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Abstract

Pneumocystis carinii has a glycoprotein with a molecular weight of 115 kDa (p115) on the surface of trophozoites and cysts, which has turned out to be a family of glycoproteins with hetrogeneous amino acid sequences. As an attempt to investigate these molecules, a new purification method using immunoaffinity chromatography with a specific monoclonal antibody was developed. p115 was successfully solubilized with detergent and ultrasonication and purified by one step to an apparent homogeneity. Five % of total p115 was isolated, which contained at least 23% of reactivities with the monoclonal antibody, indicating that subfraction of p115, which was reactive with the antibody was purified.

Ultrastructural localization of p115 was determined by immunoelectron microscopy. It showed homogeneous distribution on the pellicle of trophozoites, including tubular expansions, and the outer electron dense layer of cysts. Tubular expansion showed reactions in distinguishable from the pellicle of trophozoites, suggesting a similarity between the two membranes. Their abundance and homogeneous distribution indicate that p115 is a major structural component consisting the pellicle of the trophozoites and the electron dense outer layer of the cysts.

Key words: *P.carinii*, major surface protein, purification, immunoaffinity chromatography, ultrastructure, immunoelectron microscopy

Introduction

Despite increasing medical importance, the inability to grow *Pneumocystis carinii* (Pc) *in vitro* has hampered the investigation of this organism. However, there are several lines of evidence which suggest that the major surface protein, p115 with a molecular weight of 115 kDa (reported molecular weights vary 110 to 120 kDa according to the investigators), plays important roles in host-parasite interactions (Ezekowits *et al.*, 1991; Fisher *et al.*, 1991; Gigliotti and Hughes 1988a; Pottratz and Martin 1990; Pottratz *et al.*, 1991; Zimmerman *et al.*, 1992).

Biochemically, this protein consists of more than a half of the total proteins of *P.carinii* and contains mannose-rich carbohydrates (Tanabe *et al.*, 1989). Immunologically, it is a potent immunogen which provokes production of specific immunoglobulins as well as a specific T-cell response (Fisher *et al.*,

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1991; Linke *et al.*, 1989; Walzer and Linke 1987). *In vitro* experiments suggest that the protein mediates attachment of the organism to the alveolar epithelial cells (Pottratz and Martin 1990; Pottratz *et al.*, 1991). On the other hand, the uptake of *P.carinii* by a host macrophage is mediated by its mannose receptor, which presumably recognizes the mannose-rich major surface protein of *P.carinii* (Ezekowits *et al.*, 1991). It is also reported that passive prophylaxis with a monoclonal antibody against this molecule protects rats and ferrets from *P.carinii* pneumonia (Gigliotti and Hughes 1988a). Major surface protein(s) of rat *P.carinii* is different from that of human *P.carinii* in terms of reactivities with monoclonal antibodies and molecular weights (Gigliotti 1991; Graves *et al.*, 1986b; Kovacs *et al.*, 1988; Kovacs *et al.*, 1989; Linke *et al.*, 1989; Walzer and Linke 1987), indicating both organisms belong to different species. It is speculated that these differences may be the bases for host specificities (Gigliotti 1991; Linke *et al.*, 1989; Walzer and Linke 1987).

More recently, cloning of the genes encoding the major surface protein of rat Pc has been reported (Kovacs *et al.*, 1993). Surprisingly, there are multiple genes with related sequences that code for the glycoproteins.

Despite these investigation, there has been no good and reliable methods to purify p115 (Gigliotti *et al.*, 1988b; Lundgren *et al.*, 1991; Maddison *et al.*, 1982; Radding *et al.*, 1989). We produced a monoclonal antibody against p115 and established a one step purification of the molecule using an immunoaffinity chromatography. Then, immunoelectron microscopy with the antibody revealed ultrastructural localization of the protein, of which only a preliminary report had been published (Gigliotti *et al.*, 1986).

Materials and Methods

Preparation of P. carinii (Pc)

Pc was propagated in nude rats and prepared as described previously (Furuta *et al.*, 1984; Watanabe *et al.*, 1987). Briefly, nude rats (rnu/rnu) were nasally inoculated with Pc (10^5 cysts), which was first isolated from an immunosuppressed conventional rat. Rats were maintained in laminar air flow racks on commercial pellets sterilized by radiation and autoclaved water including tetracycline (1 g/L). Rats were injected with cortisone acetate (25 mg/rat) twice a week, until they were emaciated and cyanotic, 4 to 8 weeks after inoculation. Then the rats were sacrificed by intraperitoneal administration of Pentobarbital sodium (Abbot Laboratories, North Chicago, IL), and lungs were excised and washed with saline (5 ml × 4) through a vinyl tubing inserted into bronchus (bronchioalveolar lavage). Collected fluid was centrifuged (30 g × 5 min) to remove contaminating host cells, and supernatant was centrifuged (400 g × 10 min) to collect Pc as a pellet. Microscopic observation showed the samples contained 97% trophozoites, about 1% of Pc cysts and less than 2% rat cells (Watanabe *et al.*, 1987b) on an average. These were used as a partially purified Pc.

Production of monoclonal antibodies

BALB/c mice were immunized with subcutaneous injection of partially purified Pc (4×10^5) mixed with complete Freund's adjuvant, followed by the same amount of Pc with incomplete Freund's adjuvant 30 days later. After 14 days the same number of Pc suspended in saline was injected intraperitoneally. After three days the spleens were removed and hybridomas were produced (Nogami *et al.*, 1988). High titer antibodies reactive with *P.carinii* were screened by ELISA using sonicated Pc as a target (see below). Positive clones were further screened for specific antibodies by indirect immunofluorescent antibody staining using crude unfixed Pc as a target. Rat cells were used as a negative control. After cloning repeated three times by limiting dilution, a hybridoma line Pc-1 was established.

ELISA

Partially purified Pc was sonicated in 5 volume of phosphate buffered saline (PBS) and diluted 100 fold in PBS. Fifty μl was added to each well in a 96 well polystyrene microplate (Nunc-Immuno Plate 96-F, Nunc Inter Med, Roskilde, Denmark) and incubated at room temperature for 1 hr. The plate was washed 3 times with 0.05% Tween 20 in PBS, and 100 μl of hybridoma supernatant was added to each well. After incubation at room temperature for 1 hr, the plate was washed 3 times as before. Subse-

quently 50 μl of biotinylated anti-mouse Ig antibody (Vector Lab., Burlingame, CA) (1:1,000 in PBS and 1% bovine serum albumin (BSA)) was added to each well. After incubation at room temperature for 1 hr, the plate was washed 3 times and 50 μl of Avidin D-peroxidase conjugate (Vector Lab) (1:1,000 in PBS and 1% BSA) was added for incubation for 1 hr at room temperature. After 3 washes, 200 μl of 10 mg 4-amino antipyrin, 10 ml phenol (1 mg/ml) and 50 μl H_2O_2 in 30 ml of PBS (pH 6.4) was added as substrate and incubated for 30 min at room temperature. OD at 550 nm was determined for each well. OD of the wells to which PBS was added instead of a primary antibody was designated as a control. OD higher than twice of control was considered to be significantly high.

Indirect immunofluorescent antibody staining (IIFA)

Partially purified Pc, consisting mostly of Pc trophozoites, were used as an antigen. The samples were incubated with hybridoma supernatant at room temperature for 1 hr. After 3 washes with PBS, they were incubated with a biotinylated anti-mouse IgG antibody (Vector Lab) (1:100 in PBS and 1% BSA) at room temperature for 1 hr. The secondary antibody was preincubated with rat spleen cells to remove the crossreaction with rat IgG. Following 3 washes in PBS, they were incubated in fluorescein isothiocyanate labeled avidin D (Vector Lab) (1:100 in PBS and 1% BSA). After 3 washes in PBS, samples were observed with a Nikon Fluorescence microscope.

Dot blotting

Two μl of sample was blotted onto a nitrocellulose filter (Schleicher and Schuell, Keene, NH). After air dry the membrane was incubated in 5% skim milk in PBS for 30 min, and reacted with culture supernatant of hybridoma Pc-1 or control monoclonal antibody TFS-4, mouse IgG1 antibody against human small-cell lung cancer (Watanabe *et al.*, 1987a), at room temperature for 1 hr. Following three washes with PBS, the membrane was incubated with an anti-mouse IgG antibody conjugated with alkaline phosphatase (1:1,000 in 5% skim milk in PBS) (Tago, Copenhagen, Denmark) at room temperature for 30 min. After three washes membranes were detected with nitrobluetetrazolium (30 mg) and bromochloroindolyl phosphate (15 mg) in 100 ml of 0.1 M $NaHCO_3$ (pH9.8) and 1mM $MgCl_2$. To estimate the reactivities, developed filters were scanned with Epson GT-6000 scanner and Color Magician III. Relative densities of the dots were analyzed with a software NIH image 1.44.

Characterization of the monoclonal antibody

The subclass of the antibody was determined by ELISA using Pc as a target, the monoclonal antibody as a primary antibody, and subclass specific biotinylated anti-mouse immunoglobulins as secondary antibodies (Miles Laboratories, Naperville, IL).

Characterization of the epitope

Partially purified Pc were incubated in PBS either at 80°C, or at 37C for 15 min (Watanabe *et al.*, 1987a). Pc cells were incubated with or without Pronase E (100mg/ml) (Sigma, St Louis, MO) in PBS (pH 7.4). Pc cells were also incubated in sodium metaperiodate (50mM) (Sigma) in 10mM acetate buffer (pH 4.5), at room temperature for 1 hr or in acetate buffer. After treatments, the cells were washed in PBS and stained with IIFA using the monoclonal antibody Pc-1. As a positive control, NCI-69 cells (human small cell lung cancer cell line) were incubated in Pronase E and stained with monoclonal antibody TFS-4 (Watanabe *et al.*, 1987a). Kato III cells (human gastric cancer cell line) were incubated in metaperiodate and stained with monoclonal antibody ST-4-39 (Watanabe *et al.*, 1985).

Solubilization of antigen

100 μl of partially purified Pc were sonicated in 0.9 ml PBS containing 2 mM phenylmethylsulfonylfluoride (PMSF) and 1 mM EDTA, using a Model 5202 Sonicater (Ootake Seisakusho, Tokyo) at 100 W for 30 sec. Samples were kept on ice and sonication was repeated ten times. One ml PBS with 2% Nonidet P-40 and 0.002% 2-mercaptoethanol was added and the sonicate was kept on ice overnight. Supernatant was collected after centrifugation at 20,000×g for 30 min.

Preparation of immunoaffinity column

The monoclonal antibody was produced as ascites in mice, precipitated with 50% ammonium sulfate, dialyzed against distilled water, and lyophilized. Forty mg of antibody was reacted with 2 ml of Affigel-10 (Bio-Rad Lab. Richmond, CA) according to the manufacturer's instruction and used as an immunoaffinity column.

Immunoaffinity purification

Solubilized antigen was dialyzed three times with 2 ml of 10 mM PBS with 0.5% Nonidet P-40 (NP-40), 0.001% 2-mercaptoethanol, 2 mM phenylmethylsulfonylfluoride and 1 mM EDTA using Centricon 30 (W.R. Grace and Co., Beverly, MA), and applied to the immunoaffinity column (1 cm × 3 cm) at a flow rate of 15 ml/hr at 4C. Perfusion was repeated three times. After washing with 20 ml PBS, proteins were eluted with 0.2M glycine-HCl buffer (pH 2.5) containing 0.5% NP-40, and a one ml fraction was collected in microfuge tubes containing 50 μl of 1M Tris-HCl (pH8.0). Five μl of each fraction was analyzed on a 7.5% SDS polyacrylamide gel electrophoresis under reducing conditions and stained using Coomassie brilliant blue or Daiichi Ag Stain (Daiichi-Kagaku, Tokyo). To confirm the specificity of affinity purification, control experiment was performed using a control column which contained an irrelevant monoclonal antibody TFS-4 (Watanabe *et al.*, 1987a).

Protein assay

Protein concentration was assayed with the method of Lowry *et al.* (Lowry *et al.*, 1951) using bovine serum albumin as a standard. Concentration of p115 was estimated by scanning SDS-PAGE gel stained with Coomassie brilliant blue as described in dot blotting.

Immunoelectron microscopy

A nude rat lung with Pc pneumonia was perfused *in situ* with ice cold 4% periodate-lysine-paraformaldehyde (PLP), cut into small pieces (2×5×5 mm), and fixed in the same fixative for five hours (Hayashi *et al.*, 1989). The tissues were washed in PBS, with increasing concentrations of sucrose and finally placed in 25% sucrose-5% glycerol-PBS. The specimens were embedded in OCT Compound^R (Tissue-Tek, Miles Laboratories, Elkhart, IN) and quickly frozen in dry ice-hexane. Then the tissues were sectioned at 10 μm thickness with a cryostat, mounted on poly-L-lysine-coated glass slides and air-dried. The culture supernatant of the hybridoma was reacted with frozen sections at 4C overnight, followed by five washes in PBS each for 1 hr. As a second-layer of antiserum, horseradish-peroxidase-labeled Fab' fragment of anti-mouse IgG was prepared as described previously (Hayashi *et al.*, 1989) and absorbed with rat spleen cells prior to use. The incubation was carried out overnight at 4C, followed by the washing in the same way, and fixation with 1% glutaraldehyde for 15 min. at 4C. After washing with PBS, the tissue specimens were preincubated with 0.02% 3,3'-diaminobenzidine, 10 mM sodium azide and 1% dimethyl sulfoxide in 50 mM Tris buffer (pH 7.6) for 30 min, and then the peroxidase reaction was developed by adding 0.005% H_2O_2 for 5 min. The sections were post-fixed with 1% osmium tetroxide for 1 hr, dehydrated in a graded ethanol series and embedded in Epon 812. As a control, the monoclonal antibody was either omitted or replaced with the irrelevant monoclonal antibody TFS-4 (Watanabe *et al.*, 1987a).

Results

Properties of the monoclonal antibody Pc-1

By screening hybridoma clones with ELISA and IIFA, a monoclonal antibody which specifically reacts with a surface antigen of Pc but not with rat cells, was produced. The subclass was determined to be IgG1 k. The ring-like staining revealed by IIFA (Fig. 1) suggests that the antigen is located on the cell surface, which was confirmed by immunoelectron microscopy (Fig. 4). Pc-1 did not react with *P.carinii* isolated from human (data not shown).

The reactivities of Pc trophozoite and cyst with monoclonal antibody Pc-1 were lost after incubation at 80C for 15 min. The reactivity was also lost by Pronase treatment. However periodide treatment did not affect the reaction. These observations suggested that the epitope is more likely a peptide than a carbohydrate.

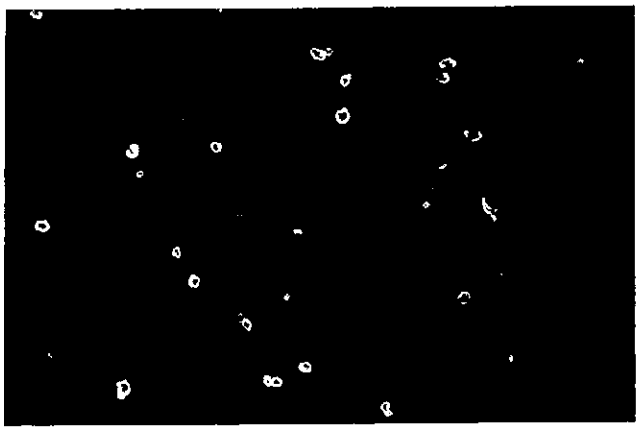
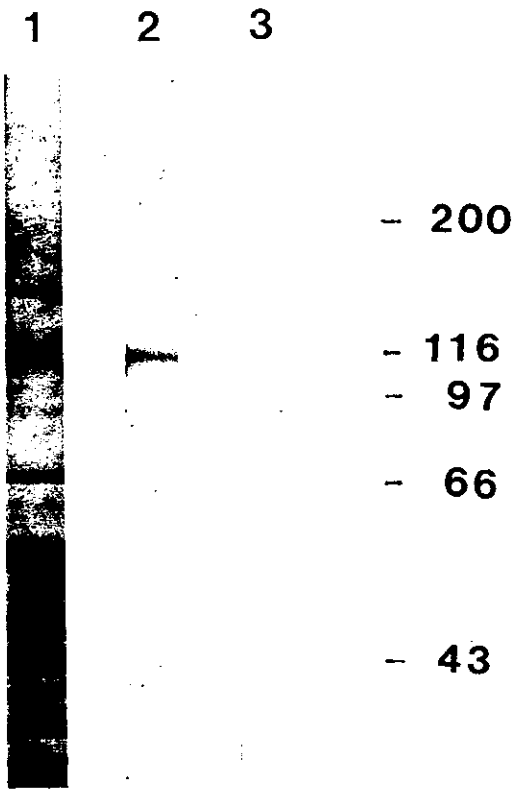


Fig. 1 Indirect immunofluorescence antibody staining.
Partially purified Pc were stained by IIFA using monoclonal antibody Pc-1. Ring-like staining seen in some organisms suggests that the antigen is located on the surface of the organism. Magnification ×1000.



Purification of a major surface protein of Pc with an immunoaffinity column

As shown in Fig. 2, eluted fractions contained a single band of protein with a molecular weight of 115 kDa on SDS-PAGE under reducing conditions, which was detected by silver staining. In a single step, the antigen was purified to an apparent homogeneity. None of the elution fractions from the control column contained any detectable proteins.

Purified protein preserved specific reactivity with monoclonal antibody Pc-1 (Fig. 3). None of the elution fractions from the control column revealed positive reactions with Pc-1 or the control monoclonal antibody.

Estimated yield was summarized in the Table 1.

Fig. 2 Analysis of the purified protein on a sodium dodecylsulfate polyacrylamide gel electrophoresis under reducing conditions detected by silver stain.
1) Solubilized Pc proteins contain p115, which shows a broad band.
2) A protein eluted from the immunoaffinity column containing the monoclonal antibody Pc-1 has a molecular weight of 115 kDa.
3) The corresponding elution fraction from the control column containing an irrelevant monoclonal antibody TFS-4 reveals no protein.
Sizes of molecular weight markers are shown on the right.

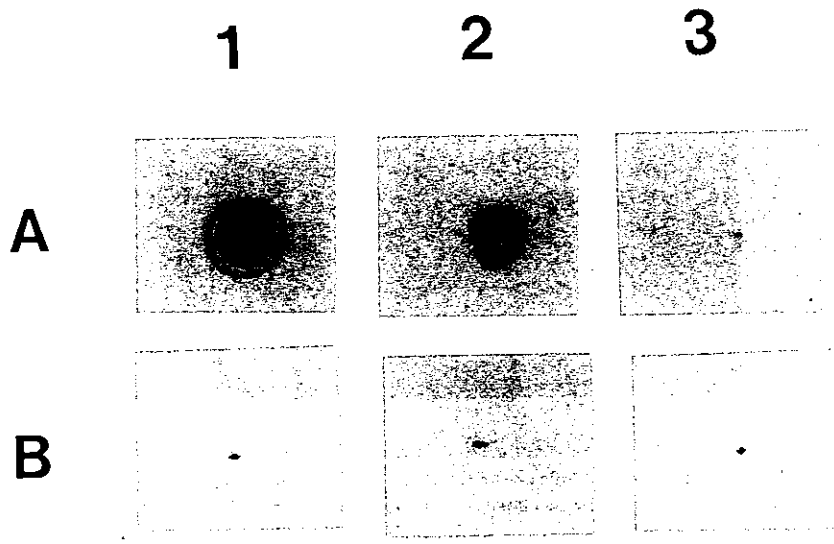


Fig. 3 Dot blotting of a purified protein.
A) Reactivity with a mouse monoclonal antibody Pc-1.
B) Reactivity with a control monoclonal antibody TFS-4.
1) Solubilized fraction of *P.carinii*.
2) Elution fraction from an affinity column containing a mouse monoclonal antibody Pc-1.
3) Corresponding elution fraction from a control column containing an irrelevant monoclonal antibody TFS-4.
An elution fraction from the Pc-1 immunoaffinity column as well as solubilized fraction showed specific reactivity with the monoclonal antibody Pc-1, but not a corresponding fraction from a control column.

Table 1 Purification of p115 with an immunoaffinity column

	Protein	p115		Reactivities with Pc-1			
	μg	μg	Yield %	spec. activity U*/μg	× purification	total activity U	Yield %
Homogenate	840	420					
Solubilization	290	126	100	0.045	1	13	100
Affinity (sup)	240	104	83	0.005	0.12	1.3	10
(eluate)	6.9	6.9	5.5	0.43	9.6	3	23

*Reactivity of the elution fraction from the affinity column was arbitrarily defined as unity.

It is clear that most of p115 did not bind to the column and recovered in the flow through fractions. Only 5% of p115 was isolated in the eluate. However, as for the reactivities with Pc-1, 23% of the original material was recovered in the eluate. Apparent loss of reactivities during affinity chromatography was probably due to acid elution, because the reactivities were relatively unstable and totally de-

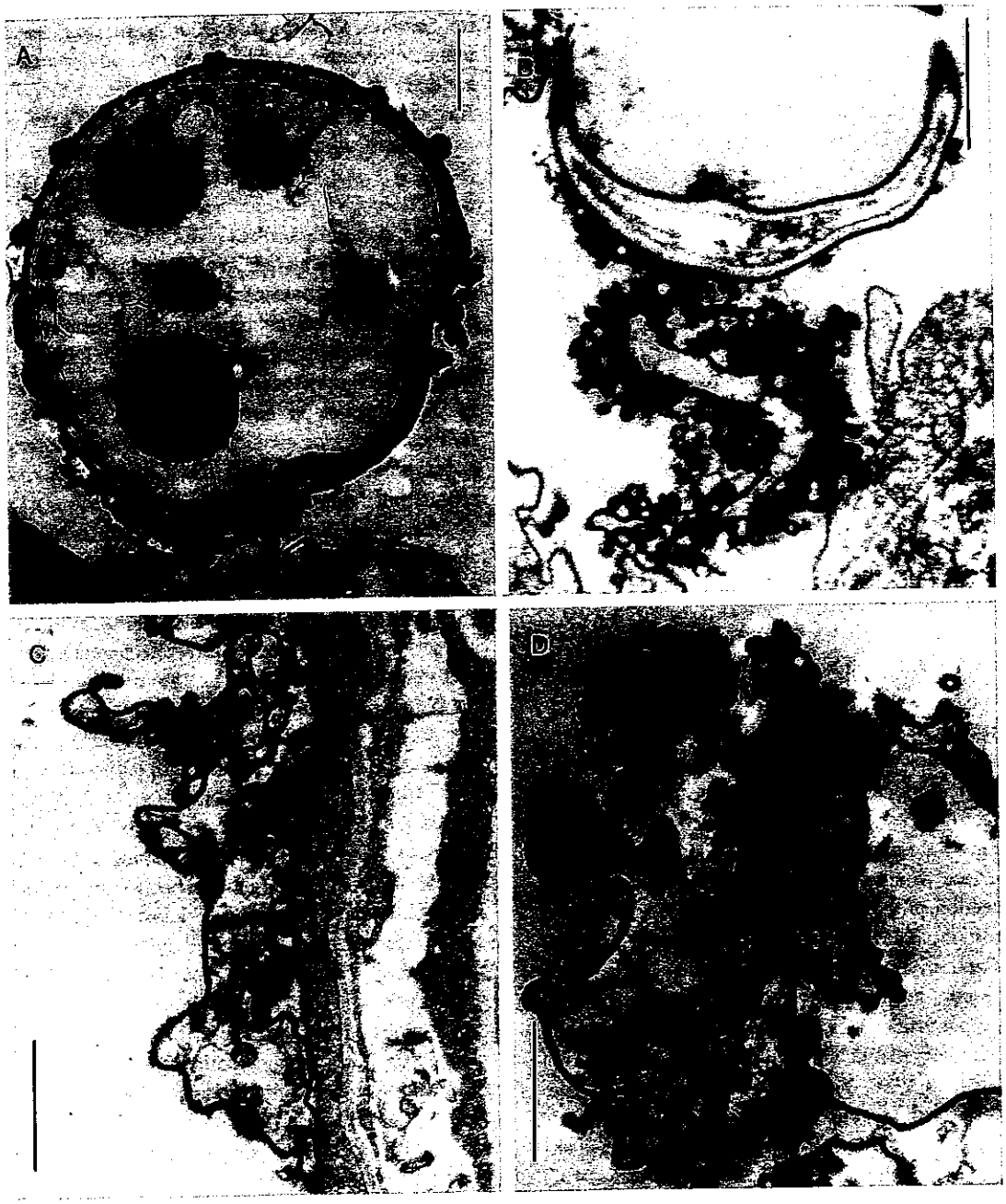


Fig. 4 Immunoelectron microscopy of *P.carinii* using monoclonal antibody Pc-1.
A) A cyst of *P.carinii* reveals positive reaction on the electron-dense outer layer. Several attached tubular expansions are also positive.
B) A crescent shaped cyst shows reactions similar to A. Numerous tubular expansions demonstrate strong reactions.
C) A trophozoite, which attaches to the host epithelial cell on the basement membrane, shows positive reactions. The reactions are essentially identical between the basal and the luminar side.
D) A trophozoite with numerous tubular expansions reveal strong reactions.
E) A control reacted with an irrelevant monoclonal antibody TFS-4.
Bars represent 1 μm.

stroyed after alkali elution (pH11.0) (data not shown).

Immunoelectron microscopy

As shown in Fig. 4-A, the antigen was detected on the surface of the cysts, corresponding to the electron dense outer layer. Attached tubular expansions were similarly positive. The part of the cell surface where the host tissue attaches showed no reaction, probably because it was inaccessible to the antibody. The absence of the reaction on the surface of intracystic bodies should be due to the same reason. The crescent form organism, which is generally considered to be a ruptured cyst, revealed an essentially identical reaction as the cysts (Fig. 4-B) (Yoshida 1989; Yoshikawa *et al.*, 1988). On the other hand, trophozoites revealed a homogeneous distribution of the antigen on the cell surface, spanning the whole thickness of the pellicles (Fig. 4-C, D). Numerous tubular expansions (Fig. 4-B, D) showed reaction that is indistinguishable from those of the pellicle of the trophozoites (Yoshida 1989; Yoshikawa *et al.*, 1988). Comparison between cysts and trophozoites revealed that the densities of the antigen were similar in both but that reactive band of cysts were wider than that of trophozoites, suggesting outer layer of cysts is thicker than pellicle of trophozoites. Control studies using the irrelevant monoclonal antibody TFS-4, or omitting primary antibody showed absence of the reactions (Fig. 4-E).

Discussion

This paper describes the production of a monoclonal antibody against major surface protein of *P.carinii*, isolation of the native protein using immunoaffinity chromatography, and determination of its ultrastructural localization.

That the monoclonal antibody Pc-1 recognizes the major surface protein is based on its specificity for *P.carinii*, its localization on the cell surface, and its molecular weight of 115 kDa determined on the affinity purified protein.

Characterization of the epitope suggested that it consists of a peptide but not a carbohydrate. However, exact structure of the epitope remains to be elucidated. Though few of the monoclonal antibodies previously reported were well characterized, heat stability of the epitopes and molecular weights on Western blotting suggest that most antibodies recognize carbohydrate moieties of the antigen (Gigliotti *et al.*, 1986; Graves *et al.*, 1986a; Graves *et al.*, 1986b; Kovacs *et al.*, 1989; Lee *et al.*, 1986; Linder *et al.*, 1987; Matsumoto *et al.*, 1987). On the contrary, Western blotting with monoclonal antibody Pc-1 revealed no reaction, because the reactivity with the antibody was lost during SDS-PAGE and Western transfer (data not shown), which indicated liability of the epitope. Thus Pc-1 seems to be different from most of other monoclonal antibodies.

Previous attempts to purify major surface antigen of Pc have utilized combination of several purification steps (Gigliotti *et al.*, 1988b; Lundgren *et al.*, 1991; Maddison *et al.*, 1982; Radding *et al.*, 1989). High specificity of immunoaffinity chromatography using a monoclonal antibody has enabled to purify p115 by one step. Pc-1 column isolated only 5% of p115, which contained at least 23% of reactivities with the antibody, indicating that it selectively isolated subfraction of p115 which is reactive with Pc-1. Peptide nature of the epitope is compatible with the unique characteristics of p115 which is encoded by a multigene family. Thus, this method should be especially useful to study

microheterogeneity of p115.

Immunoelectron microscopy clearly localized the antigen on the pellicle of trophozoites and outer layer of cysts. It is of interest that the tubular expansions showed the reactions indistinguishable from that of the pellicle of the trophozoites (Fig. 4). Tubular expansion is a numerous tube like structure unique to Pc, of which function is unknown. Previously Gigliotti had shown in a preliminary study the existence of the major surface protein on tubular expansions (Gigliotti *et al.*, 1986). Our study clearly demonstrated the similarity of the pellicle membranes of tubular expansions and that of trophozoites in terms of p115. Abundance of the major surface protein on tubular expansions explains why *P.carinii* is so rich in this protein, because the increased surface areas contain much of this molecule.

Depending on its abundance and localization on the cell surface, p115 has been considered to be a major component of the pellicle. Homogeneous distribution on all the pellicle, which was first reported in our study, further supports this hypothesis. It should be noted that p115 is rich in cysteins and that their positions are well conserved, because these cisteins may contribute to the structure of the pellicle, through forming intra- and inter-molecular bonds (Kovacs *et al.*, 1993).

In addition to a constituent of the pellicle, various functions have been attributed to p115. The possibility that different subset of p115 has different functions is of interest. Our method should be useful to investigate this hypothesis.

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