Properties of the Major Antigens of Rat and Human *Pneumocystis carinii*

MICHAEL J. LINKE, MELANIE T. CUSHION, AND PETER D. WALZER*

Cincinnati Veterans Administration Medical Center and Division of Infectious Diseases, Department of Internal Medicine, University of Cincinnati College of Medicine, Cincinnati, Ohio 45220

The major rat and human *Pneumocystis carinii* antigens were analyzed for their susceptibility to treatment with enzymes and other procedures by immunoblotting, immunofluorescence, and light microscopy. Carbohydrate residues were further analyzed by lectin-binding experiments. The 116-kilodalton (kDa) band of rat *P. carinii* was susceptible to proteolytic (e.g., trypsin) and glycolytic (e.g., Zymolyase) treatments but not to a variety of other procedures (e.g., lipase). This moiety reacted strongly with concanavalin A and wheat germ agglutinin, indicating the presence of mannosyl or glucosyl and *N*-acetylglucosamine residues. Immunofluorescence staining and surface labeling suggested that the 116-kDa antigen was located on the *P. carinii* cell wall. The 45- and 50-kDa bands were as sensitive as the 116-kDa band to degradative treatments when studied after immobilization onto nitrocellulose but were more resistant to proteolytic enzymes when studied in situ on whole organisms. These moieties exhibited poor binding to lectins and reactivity by surface-labeling procedures. The 116-kDa band of human *P. carinii* appeared to be a glycoprotein with characteristics similar to those of its counterpart in rats, whereas the human *P. carinii* 40-kDa band exhibited protein and carbohydrate properties more closely related to those of the 45- and 50-kDa rat-derived antigens. We conclude that *P. carinii* antigens are complex glycoproteins and that this information will be helpful in developing strategies for their isolation and purification and study of their function.

Pneumocystis carinii, an organism of uncertain taxonomy, is an important opportunistic pulmonary pathogen. The frequent occurrence of *P. carinii* pneumonia in patients with the acquired immunodeficiency syndrome (27) has stimulated investigative interest in the organism. Much of the recent work in our laboratory and by other investigators has involved analyzing the antigenic characteristics of *P. carinii* obtained from humans or rats (the primary animal model) by the immunoblotting technique (8–12, 18, 21, 22, 35, 40). The different methods of antigen preparation and sources of antibody used in these studies have identified a broad range of immunoreactive moieties and have thus made the results difficult to compare. Nevertheless, some common rat *P. carinii* antigens (e.g., bands of 110 to 120 kilodaltons [kDa] or 45 to 65 kDa) and human *P. carinii* antigens (e.g., bands of 35 to 45 kDa) seem to have been recognized. The data suggest that rat and human *P. carinii* have both shared and species-specific antigenic determinants, but the composition and location of these moieties on the organism are not well understood.

The surface of *P. carinii* has long been known to stain brightly by immunofluorescence, suggesting the presence of highly antigenic structures. The indirect fluorescent-antibody (IFA) technique has been helpful in studying the serologic responses to *P. carinii*, in developing monoclonal antibodies, and in comparing the antigenic characteristics of rat- and human-derived organisms (10, 17, 19, 23, 25, 28, 37–39). Our laboratory has also used this methodology to analyze the effects of selected proteolytic enzymes on the antigenic and morphologic characteristics of *P. carinii* (31).

The relationship between the reactive bands of *P. carinii* identified by immunoblotting and the surface structures detected by immunofluorescence remains poorly defined. We undertook the present study of the major rat and human

P. carinii antigens with the following goals: (i) to analyze their protein and carbohydrate properties, (ii) to assign them a tentative location on the organism, and (iii) to assess their role in maintaining the morphological integrity of the organism.

MATERIALS AND METHODS

Sources of *P. carinii*. *P. carinii* pneumonia was induced by corticosteroid treatment of adult Sprague-Dawley rats, and the organism was recovered from infected lungs and quantitated as previously described (3–5, 16, 36). Briefly, infected lungs were removed en bloc, minced, and homogenized in a Stomacher lab blender (Tekmar, Inc., Cincinnati, Ohio). Erythrocytes were lysed, and the homogenate was washed three times (1,000 × *g*, 5 min) in phosphate-buffered saline (PBS). The fresh material was either used immediately for analysis of *P. carinii* antigens in situ or was lyophilized, stored at −70°C, and later used for analysis of immobilized antigens. Quantitation of *P. carinii* nuclei in fresh lung homogenates was performed with the Diff-Quik stain (American Scientific Products, McGaw Park, Ill.), but this reagent could not be used on frozen material because it did not stain the organism intensely enough to permit accurate enumeration (35). Quantitation of *P. carinii* cysts was performed on all fresh or frozen lung material by the cresyl echt violet stain.

Human *P. carinii* was obtained from lungs from a patient with the acquired immunodeficiency syndrome with histologically proven pneumocystosis and stored at −70°C as previously reported (35). Organisms were prepared for analysis in a manner similar to that for rat *P. carinii*.

Reagents. Protease, trypsin, pepsin, neuraminidase, collagenase, lysozyme, lipase, and sodium periodate were obtained from Sigma Chemical Co., St. Louis, Mo. Pronase, chitinase, and nuclease were from Calbiochem-Behring, La Jolla, Calif. Novozym was a gift from Judith Rhodes, Uni-

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versity of Cincinnati, Cincinnati, Ohio. Zymolyase was obtained from Seikagaku Kogyo Co. Ltd. Tokyo, Japan.

P. carinii specific antisera were prepared and characterized as previously described (26, 35). Rabbits were hyperimmunized with tissue culture-derived rat *P. carinii* or human *P. carinii* and adsorbed with uninfected tissue culture matrix or normal human lungs, respectively. These antisera did not react with normal rat or human lung, uninfected tissue culture cells, or a series of bacteria and fungi on immunoblots.

Affinity-purified peroxidase and fluorescein-labeled goat anti-rabbit immunoglobulin G (IgG) (heavy and light chains) antibodies were obtained from Kirkegaard & Perry Laboratories, Gaithersburg, Md. These conjugated antibodies displayed no reactivity with *P. carinii* by immunofluorescence or immunoblotting when PBS was substituted for the rabbit antisera.

Biotinylated concanavalin A (ConA) and wheat germ agglutinin (WGA) were obtained from EY Labs Inc., San Mateo, Calif. Horseradish peroxidase-conjugated avidin was obtained from Bio-Rad Laboratories, Richmond, Calif.

Electrophoresis and immunoblotting. Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and immunoblotting were performed by using procedures used in our earlier studies (35, 40). Briefly, samples were solubilized in SDS lysis buffer (2% SDS, 0.06 M Tris [pH 6.8], 1% glycerol, 5% 2-mercaptoethanol). Electrophoresis was performed in 0.1% SDS-discontinuous 12.5% polyacrylamide gel (0.75 mm thick) by a modification of the method of Laemmli (20).

Separated proteins were transferred to nitrocellulose by a method similar to that described by Towbin et al. (32); transfer was performed overnight at 30 V followed by 1 h at 60 V in a Trans-blot apparatus (Bio-Rad). Lanes containing molecular weight standards were stained with fast green, and the remainder of the gel was blocked in 3.0% gelatin. The blots were probed with rabbit antiserum to rat or human *P. carinii*, and the immunoreactive bands were detected with goat anti-rabbit IgG conjugated with horseradish peroxidase followed by development in 4-chloro-1-naphthol substrate solution (Bio-Rad).

Experimental protocol. Two experimental approaches were used.

(i) **Analysis of immobilized *P. carinii* antigens.** For analysis of *P. carinii* antigens, lyophilized rat or human *P. carinii* was used. The preparation was solubilized in sample buffer at a concentration of 5.0 mg/ml, boiled for 3 min, and centrifuged at $17,400 \times g$ for 5 min. Five microliters of each supernatant was run per well of a SDS-polyacrylamide gel and transferred to nitrocellulose as described above. The blots were incubated with 100 μg of the enzyme to be tested in PBS with $MgCl_2$ for 2 h at 37°C; control blots were incubated in PBS without enzyme. In studies where sodium periodate was used as the degradative material in place of the enzyme, *P. carinii* was incubated with 0.5 M sodium periodate in 0.1 M sodium acetate overnight at 4°C; control blots were incubated with sodium acetate alone. The blots were then rinsed with water, washed extensively with PBS, and analyzed by immunoblotting.

(ii) **Analysis of in situ *P. carinii* antigens.** Freshly recovered rat *P. carinii* organisms (10^8 nuclei per ml) were incubated with 100 μg of the enzyme to be tested in PBS with $MgCl_2$ for 2 h at 37°C or overnight at 4°C. In the control preparation, organisms were incubated in PBS without enzyme. As a further test of specificity, addition of the protease inhibitor phenylmethylsulfonyl fluoride to the buffer did not block the

effects of selected glycolytic enzymes (Zymolyase) on *P. carinii*. In experiments where sodium periodate replaced the enzyme, *P. carinii* was incubated with 0.5 M sodium periodate in 0.1 M sodium acetate for 2 h at 37°C or overnight at 4°C; control organisms were incubated in sodium acetate alone. In other studies, *P. carinii* was incubated for 2 h in a boiling water bath rather than being treated with enzymes or sodium periodate. The organisms were then washed and reconstituted in PBS at the initial volume. Samples were removed for IFA and microscopic studies described below, and the remainder was lyophilized. For immunoblotting analysis, the lyophilized *P. carinii* was solubilized in sample buffer at a concentration of 5.0 mg/ml. The samples were boiled, and 10 μl of each sample was run per well of a SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted.

Studies were conducted to determine whether immunoreactive moieties could be liberated from the surface of rat *P. carinii* by treatment with selected proteolytic (e.g., pronase) or glycolytic (e.g., Zymolyase) enzymes. After incubation of the organisms with the enzyme as described above, the preparations were centrifuged at $100,000 \times g$ for 1 h and the supernatants were collected, dialyzed, lyophilized, and then compared with the pellet by immunoblotting. Experiments were also performed to determine whether the antigen detected in the supernatants could be immunoprecipitated by using standard techniques (6). Briefly, each supernatant was divided into two aliquots which were then incubated with normal rabbit serum and protein A-Sepharose (Pharmacia, Uppsala, Sweden) and centrifuged. One supernatant aliquot was incubated with rabbit antiserum to rat *P. carinii*, and the other was incubated with normal pre-bled rabbit (control) serum overnight at 4°C. Immune complexes were precipitated with protein A-Sepharose, and the preparations were washed and analyzed by immunoblotting.

Since fresh human *P. carinii* lung specimens were not available, lyophilized material was used instead. Preliminary studies with rat *P. carinii* indicated that lyophilization had no discernible effect on immunoreactivity. Lyophilized human *P. carinii* was reconstituted at a concentration of 1.0 mg/ml, containing 2×10^6 cysts per ml in PBS with $MgCl_2$. Enzyme, sodium periodate, and boiling treatments were performed as described above. Following washing, the treated *P. carinii* was immediately prepared for immunoblot analysis. *P. carinii* was pelleted ($17,400 \times g$, 5 min) and reconstituted in 100 μl of sample buffer. Samples were boiled, and 10 μl was run per well of a SDS-polyacrylamide gel, transferred to nitrocellulose, and analyzed by immunoblotting.

Analysis of rat *P. carinii* in situ antigens by IFA. Immunofluorescence activity of treated rat in situ antigens was determined by techniques described in earlier studies with slight modifications (26, 38–40). Teflon-coated microslides (10 wells each; Carlson Scientific, Peotone, Ill.) were coated with treated and control *P. carinii* (approximately 10^6 nuclei per well). Slides were fixed in ice-cold acetone. Twenty-five microliters of rabbit antiserum to rat *P. carinii* was loaded into each well and incubated for 30 min at 37°C. The slides were washed in PBS with 0.05% Tween 20 and rinsed with H_2O. Next, 25 μl of fluorescein-conjugated goat anti-rabbit IgG was incubated in each well for 30 min at room temperature. The slides were washed as described above and examined for intensity and pattern of fluorescence staining.

Microscopic studies. Fresh preparations of rat *P. carinii* organisms used in the studies of in situ antigens were analyzed by phase-contrast microscopy. Aliquots of these specimens were also stained with Diff-Quik and examined by
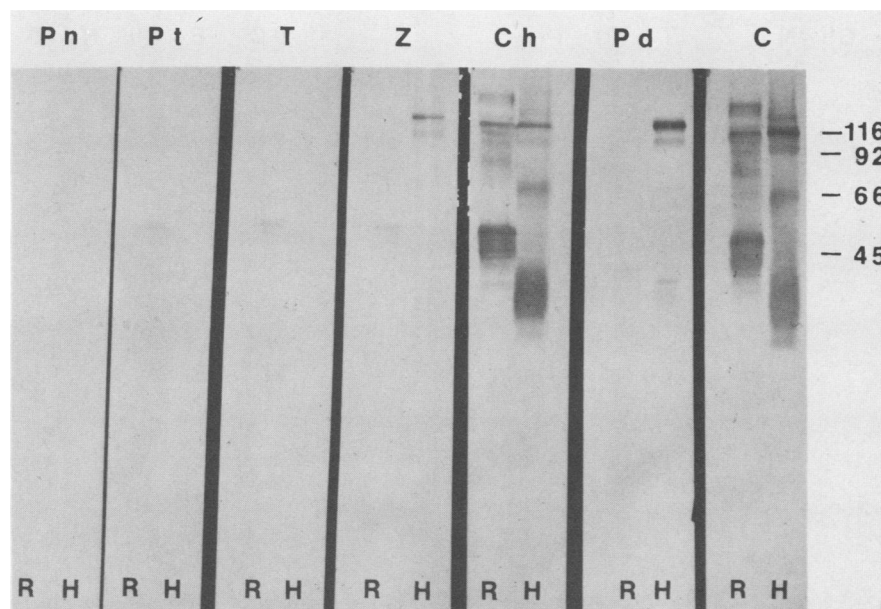
FIG. 1. Immunoblotting analysis of rat (R) and human (H) *P. carinii* following treatment of immobilized antigens with enzymes and sodium periodate. Rabbit antiserum to human *P. carinii* diluted 1/200 was used to detect alterations in the antigenic profiles. Pn, Pronase; Pt, protease; T, trypsin; Z, Zymolyase; Ch, chitinase; Pd, periodate; C, control (untreated). Molecular weight markers (10^3) are on the right.

light microscopy. The effects of enzyme treatment on *P. carinii* morphology were compared with effects on control (untreated) preparations by using procedures and criteria detailed in earlier reports (4, 29).

Lectin-binding studies. We have recently conducted detailed studies of the interaction of rat *P. carinii* with fluorescein-conjugated lectin probes (2). In the assay, *P. carinii* and the lectin were added to a test tube and the percentage of organisms exhibiting fluorescence staining was calculated; the reaction could be inhibited completely in a dose-dependent manner by addition of the specific sugars. Two of the strongest-reacting lectins, ConA and WGA, were chosen for evaluation in the present study. Blots of rat and human *P. carinii* antigens were probed with biotinylated ConA and WGA diluted 1/40 in Tris-buffered saline with 1% Tween for 30 min at room temperature (by the technique of E. Pesanti; personal communication). The blots were washed three times, incubated with avidin horseradish peroxidase (Bio-Rad) diluted 1/1,000 for 30 min, and developed with 4-chloro-1-naphthol substrate solution (Bio-Rad). The *P. carinii* antigen preparations were also studied by immunoblotting to compare regions of immunologic reactivity with lectin staining.

Specificity of the lectin staining was analyzed in the following manner. Blots of human and rat *P. carinii* were incubated with ConA and WGA and washed as described above. The preparations were then incubated overnight with various concentrations (0.1 or 1 M) of the following sugars: mannose, mannan, or glucose, which specifically inhibit ConA; N-acetylglucosamine, which inhibits WGA; and fucose, which does not inhibit either lectin. The blots were washed, probed, and developed as described above. The specific inhibitory sugars markedly reduced the binding of each lectin, whereas the irrelevant sugar, fucose, had no discernible effect.

Biotin labeling of rat *P. carinii* surface proteins. Two 1.0-ml aliquots of rat *P. carinii* containing 10^8 nuclei were prepared. Ten micrograms of sulfo-NHS-biotin (Pierce Chemical Co.,

Rockford, Ill.) were added to one aliquot, while the other served as the control (24). Both samples were incubated for 10 min at room temperature. Organisms were examined for surface labeling by reaction with fluorescein-labeled avidin (ICN Biomedicals, Inc., Cleveland, Ohio). Teflon-coated microslides were prepared as described above with control and experimental organisms. Twenty microliters of the fluorescein-avidin conjugate diluted 1/200 was incubated in each well for 30 min at room temperature. The slides were washed in PBS with 0.1% Tween 20, rinsed with water, and examined for intensity and pattern of fluorescence activity. Specifically labeled proteins were identified by SDS-PAGE followed by blotting. The blots were probed with horseradish peroxidase-conjugated avidin diluted 1/1,000 in PBS with 0.1% Tween 20 for 10 min, washed, and developed by using a 4-chloro-1-naphthol substrate system.

RESULTS

Analysis of immobilized *P. carinii* antigens. The typical pattern of reactivity of the control (untreated) preparations of rat and human *P. carinii* can be seen in Fig. 1 to 3. As in our earlier studies (35, 40), the principal antigens of rat *P. carinii* were bands of about 116, 50, and 45 kDa. Several other moieties of higher and lower molecular weights were identified, but their presence and staining intensity varied among the different blots. As reported previously (35), the most prominent human *P. carinii* antigens were at 116 kDa and a wide band of about 40 kDa. A variety of other moieties were detected with this antiserum, the most consistent of which were about 66 and 92 kDa.

Treatment of the immobilized rat and human *P. carinii* antigens with pronase, protease, and trypsin ablated almost all reactivity; a faint signal of the 50-kDa moiety of rat *P. carinii* remained (Fig. 1). Zymolyase and sodium periodate destroyed the reactivity of the rat *P. carinii* antigens and lower-molecular-weight human *P. carinii* entities but left the 116- and 92-kDa antigens of human *P. carinii* at least
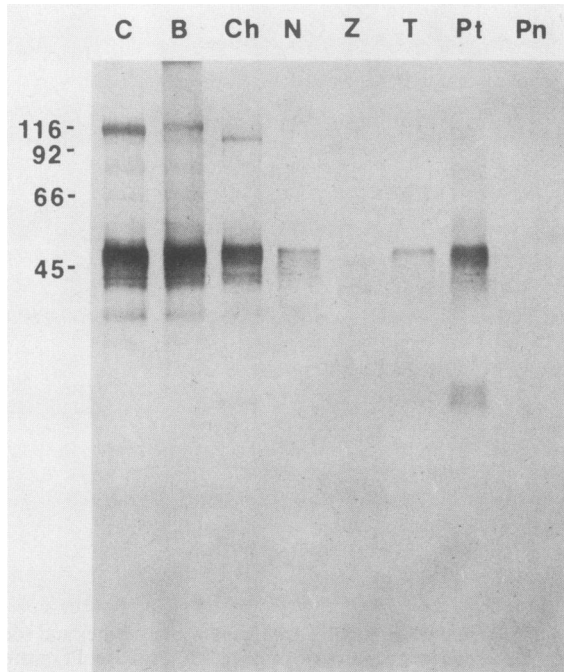
FIG. 2. Immunoblotting analysis of rat *P. carinii* following treatment of in situ antigens with enzymes and other procedures. Rabbit antiserum to rat *P. carinii* diluted 1/200 was used to detect alterations in the antigenic profile caused by these treatments. C, Control; B, boiling; Ch, chitinase; N, Novozym; Z, Zymolyase; T, trypsin; Pt, protease; Pn, pronase. Molecular weight markers (10³) are on the left.
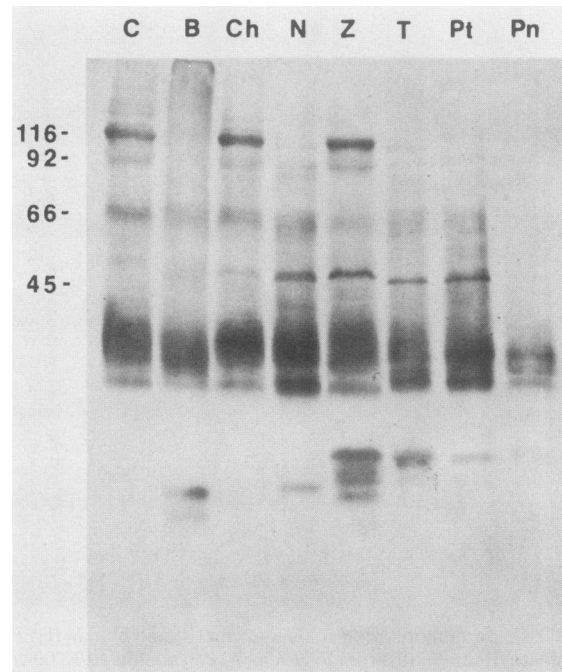

FIG. 3. Immunoblotting analysis of human *P. carinii* following treatment of in situ antigens with enzymes and other procedures. Rabbit antiserum to human *P. carinii* diluted 1/200 was used to detect alterations in the antigenic profile caused by these treatments. C, Control; B, boiling; Ch, chitinase; N, Novozym; Z, Zymolyase; T, trypsin; Pt, protease; Pn, Pronase. Molecular weight markers (10³) are on the left.

partially intact. Pepsin, collagenase, lipase, nuclease, neuraminidase, lysozyme (data not shown), and chitinase had little or no effect on rat or human *P. carinii* antigens.

Analysis of in situ *P. carinii* antigens. The immunoreactivity of the 116-kDa band of rat *P. carinii* was completely removed by treatment with pronase, protease, trypsin, Zymolyase, and Novozym when compared with the control preparation (Fig. 2). Boiling and chitinase partially reduced the intensity of this moiety and slightly altered its migration pattern. The 45- and 50-kDa bands were very sensitive to treatment with pronase and Zymolyase, but some reactivity remained after treatment with protease, trypsin, and Novozym. Chitinase and boiling had little effect on these moieties. Lower-molecular-weight bands found after treatment with some of these enzymes (e.g., protease) presumably represented degradation products.

The 116- and 92-kDa bands of human *P. carinii* were very sensitive to treatment with pronase, protease, trypsin, Novozym, and boiling, whereas Zymolyase and chitinase had little or no effect (Fig. 3). With the exception of pronase, all enzymes had little effect on the immunoreactivity of the 66- and 40-kDa moieties. Multiple low-molecular-weight bands representing breakdown products were found. The results of overnight treatment with enzymes on the reactivity pattern of rat and human *P. carinii* antigens were similar to those achieved with 2-h treatment (data not shown). However, digestion overnight with Zymolyase greatly reduced the signal of the human *P. carinii* antigens and further resolved the lower-molecular-weight bands.

IFA studies. All treatments except chitinase and boiling reduced the reactivity of rat *P. carinii* by the IFA technique

(Table 1). Control organisms displayed typical immunofluorescent reactivity with a sharp, defined rim pattern of fluorescence encircling the cyst forms. Treated organisms displayed either no fluorescence staining (Novozym, Zymolyase, and pronase) or a weak, diffuse pattern (trypsin and protease).

Microscopic studies. Treatment of *P. carinii* with enzymes and other procedures resulted in a 5- to 10-fold reduction in organism count, but the magnitude of this effect did not correlate well with alterations in organism morphology or immunoreactivity (Table 1). Proteolytic enzymes caused the most pronounced changes (Fig. 4). When examined by phase-contrast microscopy, the organisms displayed distortion of their cell walls and generalized swelling. On preparations stained with Diff-Quik, the nuclei of the intracystic

TABLE 1. Effects of enzyme treatment of rat *P. carinii* organism number, morphology, and IFA reactivity

Treatment	Phase-contrast microscopy result	Quantitation (nuclei/ml)	IFA result[a]
Control	Slight	1.3×10^7	+++
Boiling	Slight	ND[b]	+++
Chitinase	Slight	1.4×10^6	+++
Novozym	Slight	4.6×10^6	−
Zymolyase	Slight	3.2×10^6	−
Trypsin	Moderate	1.9×10^6	+
Protease	Moderate	3.3×10^6	+
Pronase	Heavy	1.8×10^6	−

[a] +++, Bright; +, weak; −, no staining.
[b] ND, Not done because of increased clumping of the organisms.
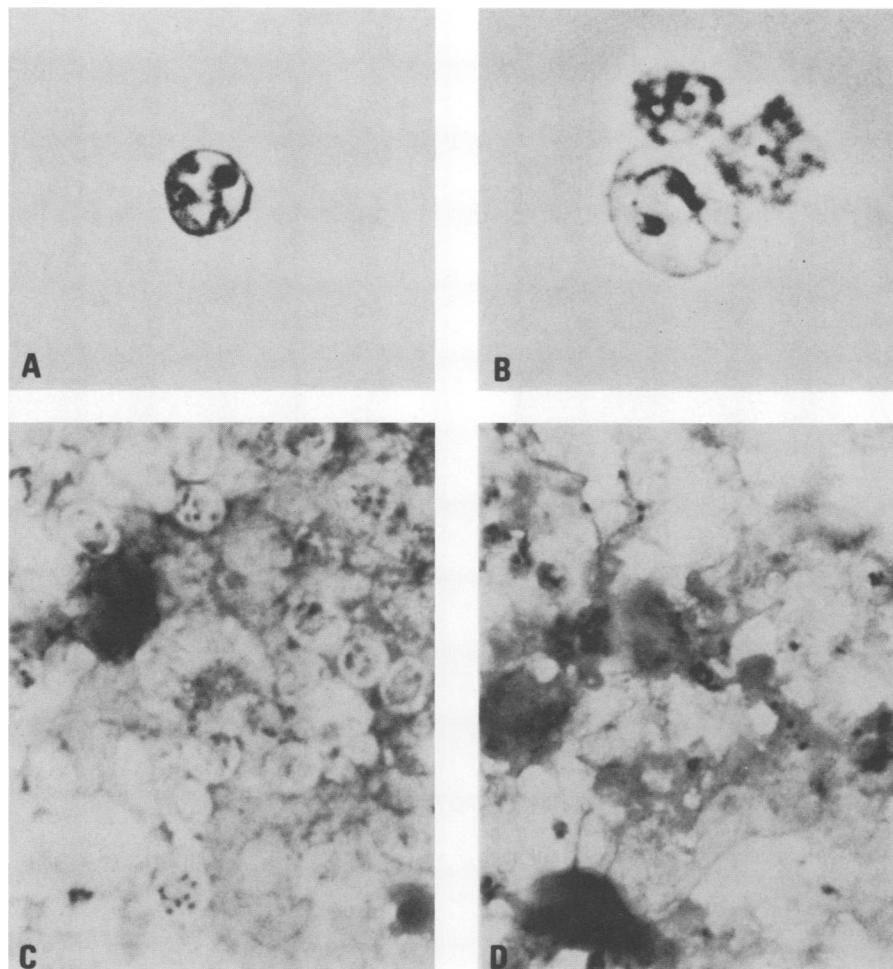
FIG. 4. Effects of enzyme treatments on the morphologic characteristics of *P. carinii* as assessed by phase-contrast microscopy (A, B) and in preparations stained with Diff-Quik (C, D) (magnification, ×1,000). Note the control (untreated) *P. carinii* cyst with typical morphology (A) and the swollen, distorted cyst with attached clumping of trophic or intermediate forms following protease treatment (B). Control cysts have up to 8 nuclei of the intracystic bodies with a nonstaining cell wall (C), whereas organisms treated with pronase have more prominent nuclei and apparent loss of the cell wall (D).

bodies appeared somewhat enlarged and there was no evidence of the cyst wall. Glycolytic enzymes caused few consistent alterations in *P. carinii* morphology or tinctorial properties, while boiling resulted in increased organism clumping.

Analysis of solubilized immunoreactive moieties of rat *P. carinii*. These experiments compared the supernatants and pellets of in situ rat *P. carinii* antigen preparations for the presence of immunoreactive bands after treatment with selected enzymes (Fig. 5). The pellet of the control preparation displayed the typical antigen recognition pattern (Fig. 1 to 3), whereas no bands were found in the supernatant. Pronase digestion removed the reactivity of the 116-kDa band and left a faint, diffuse band of about 45 kDa in the pellet. The supernatant contained a thin, distinct band of approximately 116 kDa, a doublet in the region of 92 kDa, a diffuse band around 45 kDa, and several other moieties. Harsher pronase treatment digested these bands (data not shown). Treatment with Zymolyase reduced the intensity and distorted the recognition of bands in the pellet. A prominent 116-kDa band was detected in the supernatant along with a 92-kDa band and several others. These addi-

tional bands may have resulted from partial digestion of the major immunoreactive bands. Note that pronase and Zymolyase seemed to be less destructive to *P. carinii* antigens than in the experiment displayed in Fig. 2. The reasons for this are unclear but may be related to the fact that the rabbit antiserum was used at a lower dilution (1/100 here versus 1/200 in Fig. 2) and hence was more sensitive in detecting *P. carinii* antigens. In data not shown, the 116-kDa band in the supernatant of the Zymolyase-treated preparation was immunoprecipitated with the rabbit antiserum but not with normal (control) rabbit serum.

Lectin-blotting studies. Probing rat *P. carinii* with ConA revealed an intensely staining band of about 116 kDa and a weakly reactive broad band of 45 to 50 kDa (Fig. 6). These bands correlated very well with the major entities detected by immunoblotting. WGA displayed a 116-kDa band similar to that found with ConA but showed no reactivity with 45- to 50-kDa moieties. Rather, this lectin reacted with several moieties of about 32 to 40 kDa which correlated to some degree with entities detected by immunoblotting. We have previously detected these lower-molecular-weight bands as well as moieties of >116 kDa on occasion with some sources
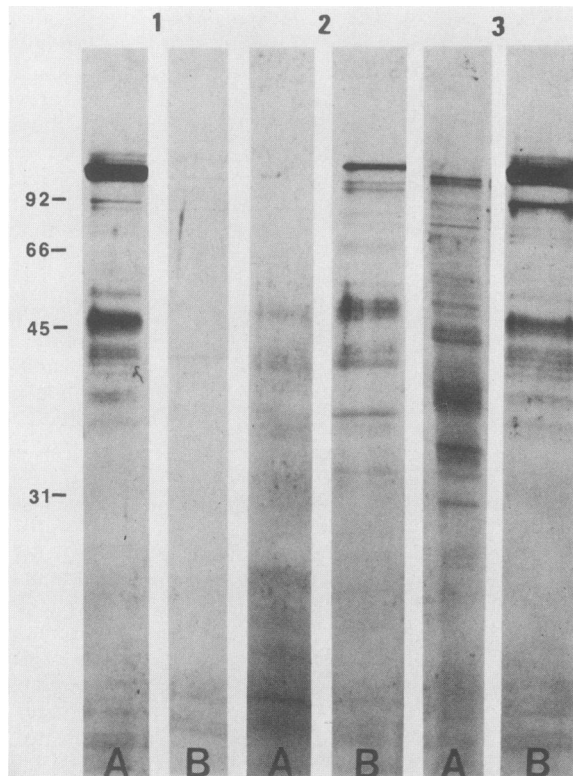
FIG. 5. Immunoblotting analysis of cleaved rat *P. carinii* antigens following in situ treatment with pronase and Zymolyase. The pellet (lanes A) is compared with the supernatant (lanes B) of each preparation after enzyme treatment. Rabbit antiserum to rat *P. carinii* diluted 1/100 was used to detect the antigens. 1, Control; 2, pronase; 3, Zymolyase. Molecular weight markers (10^3) are on the left.
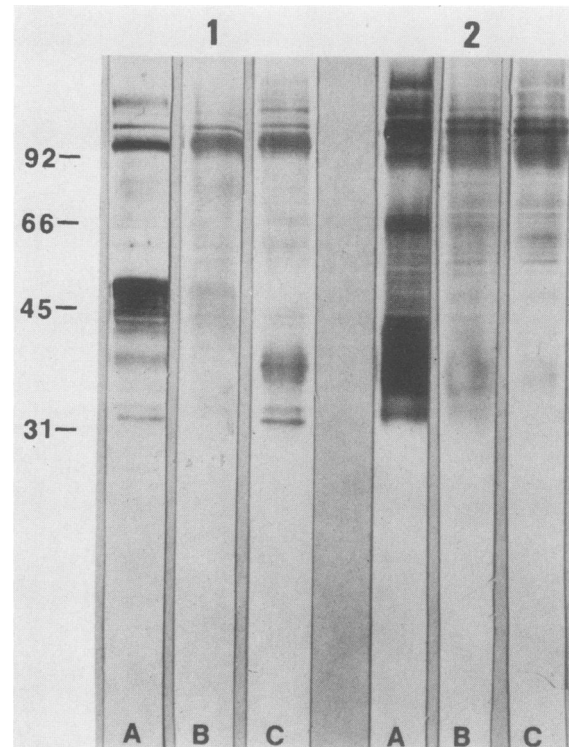

FIG. 6. Analysis of immobilized rat (1) and human (2) *P. carinii* antigens by lectin blotting and immunoblotting. Rabbit antisera to rat and human *P. carinii* diluted 1/200 were used in the immunoblots, and biotinylated lectins were used in lectin blotting. Lanes: A, immunoblot; B, blot with ConA; C, blot with WGA. Molecular weight markers (10^3) are on the left.

of antibody, but their nature and significance are unclear (35).

Probing human *P. carinii* with ConA demonstrated an intensely staining band in the region between 116 and 92 kDa and a weakly reactive band in the 40-kDa region. Note that the 116-kDa band detected by this lectin is a doublet, whereas the 116-kDa moiety detected by antibody is a single thick entity. WGA reacted in a manner similar to that of ConA with the 116- and 92-kDa regions but showed little or no reactivity in the 40-kDa region.

Surface-labeling studies. Reaction of fluorescein-conjugated avidin with biotinylated surface proteins of rat *P. carinii* revealed heavy labeling of the cyst cell wall. The reaction pattern resembled that detected by immunofluorescence. Several biotinylated protein-containing moieties were detected following SDS-PAGE, blotting, and reaction with horseradish peroxidase-conjugated avidin (Fig. 7). The most prominently labeled band occurred in the region of 116 kDa.

DISCUSSION

Immunoblotting studies of *P. carinii* have been hampered by the lack of a reliable in vitro cultivation system and, hence, a standardized antigen preparation. Infected lung homogenates and bronchoalveolar lavage fluid have served as the main sources of organisms, and specimens (particularly those obtained from humans) differ considerably in quality, quantity, and storage conditions. A variety of pro-

cedures have been used to purify and solubilize *P. carinii* and to control for the presence of host tissues or other microbes which frequently contaminate these preparations. Since methodologic differences can alter the antigen profile of cloned preparations of organisms (e.g., *Trypanosoma cruzi*) on Western blots (immunoblots) (30), it is likely that these factors have contributed significantly to the different immunoreactivity patterns found in studies of *P. carinii*.

Our previous studies identified major antigens of rat *P. carinii* at 116, 50, and 45 kDa (35, 40). The 116-kDa band appears to correspond to a 110- to 120-kDa antigen which has been found in human-, rabbit-, ferret-, and mouse-derived *P. carinii* (10–12, 18, 34). This moiety is frequently recognized by serum antibodies of animals exposed to the organism, and administration of a monoclonal antibody directed against the 116-kDa band provides partial protection against experimental corticosteroid-induced pneumocystosis (9). The 45- and 50-kDa antigens of rat *P. carinii* can appear either as distinct moieties or as a broad-based band on the immunoblot. It is unknown whether these antigens are derived from the 116-kDa band or represent separate entities. The 45- and 50-kDa bands are frequently recognized by sera of animals exposed to *P. carinii* and seem to correspond to bands of 45 to 65 kDa found by other workers (12, 18).

Major antigens of human *P. carinii* have been found at 116, 92, 66, and 40 kDa (35). The heterogeneous, broad-based 40-kDa band is similar to a 35- to 45-kDa band found by others (18). This moiety is not only the most common *P. carinii* antigen identified by lung specimens but is also the most common band recognized by serum antibodies of
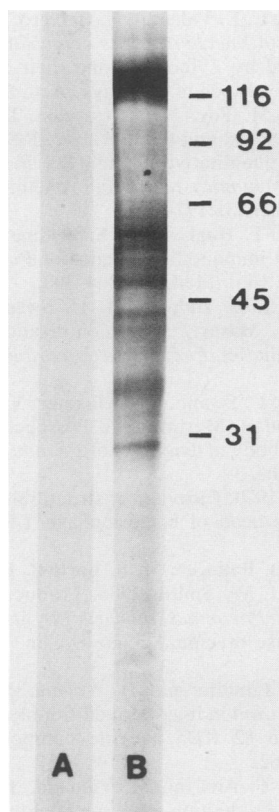
FIG. 7. Analysis of biotinylated rat *P. carinii* surface proteins following separation by SDS-PAGE and blotting to nitrocellulose. Biotin-labeled proteins were detected with horseradish peroxidase-labeled avidin. Lanes: A, control (unlabeled); B, biotinylated *P. carinii*. Molecular weight markers (10^3) are on the right.

different patient populations (P. D. Walzer, unpublished data).

In the present study, two basic experimental approaches were used to analyze the protein and carbohydrate nature of these antigens. As has been done with other microbes (e.g., *Leishmania* sp.) (13), in situ *P. carinii* antigens were evaluated by subjecting whole organisms to specific degradative techniques. This method allowed us to analyze the relationships between individual antigens, cyst surface immunoreactivity, and organism morphology. Experiments were performed in greater detail on rat *P. carinii* than on human *P. carinii* because a good supply of fresh organisms was readily available from these animals.

The other approach involved analysis of immobilized *P. carinii* antigens. Organisms were solubilized, electrophoresed, blotted to nitrocellulose, and then treated with enzymes or sodium periodate. This procedure allowed us to directly analyze the antigens under highly controlled conditions and also enabled us to compare our results with those obtained by other workers who followed a similar protocol (12).

In this study, we found that the 116-kDa band is composed of protein and carbohydrate constituents, on the basis of its susceptibility to proteolytic and glycolytic treatments. The reactivity of this moiety with ConA suggested the presence of glucosyl or mannosyl residues. In fact, the binding is so avid that we could not completely inhibit the reaction with specific sugars, and other workers could not elute the 116-kDa antigen from a ConA affinity column by standard

procedures (8). The 116-kDa band also reacted strongly with WGA, more likely reflecting the presence of *N*-acetylglucosamine residues than sialic acid because neuraminidase treatment had no discernible effect on this antigen. In previous studies using fluorescein-conjugated lectins and whole organisms, ConA and WGA reacted strongly with *P. carinii* but no binding was found with *Limax flavus*, which is specific for sialic acid (2).

Data from the present study as well as from immunoelectron microscopy (10) suggest that the 116-kDa antigen resides in the *P. carinii* cell wall. The 116-kDa band was heavily labeled by surface biotinylation, and enzymatic destruction of this moiety correlated with loss of cyst immunofluorescence. Treatment of *P. carinii* by Zymolyase, a β-1-3 glucanase, cleaved both the cell wall (M. T. Cushion, unpublished data; Y. Matsumoto, S. Matsuda, and T. Tegoshi, J. Protozool., in press) and the 116-kDa band. Since the recovered antigen appeared to be immunologically intact, this procedure might be useful as a purification step.

The carbohydrate components of the *P. carinii* cell surface and 116-kDa antigen bear strong resemblance to certain properties of fungi. Yeast cell walls are composed mainly of polymers of mannose and glucose (1); Zymolyase has been useful in studying yeast cell wall mannoproteins and nucleic acids (14, 15, 33, 41). Moreover, recent studies of rat *P. carinii* rRNA suggest the organism is more closely related to fungi than to protozoa (7; S. L. Stringer, J. R. Stringer, M. Blase, P. Walzer, and M. T. Cushion, Exp. Parasitol., in press; J. I. Watanabe, Y. Nakamura, K. Tanabe, and H. Hori, J. Protozool., in press). Thus, application of the techniques used to investigate yeast cell wall structures and antigens might be very helpful in studying the immunoreactive moieties of *P. carinii*.

The 116-kDa band of human *P. carinii* displayed characteristics similar to those found with the 116-kDa band of rat *P. carinii*, although the human *P. carinii* was more resistant to treatment with Zymolyase, sodium periodate, and chitinase. It seems reasonable to conclude that this moiety is a glycoprotein, but more precise delineation of its properties will require other experimental approaches, evaluation of additional clinical isolates, and careful assessment of such factors as specimen age and storage conditions which might influence interpretation of the results.

Our results indicate the presence of carbohydrate and protein components within the 45- and 50-kDa antigens of rat *P. carinii*. When these antigens were immobilized on nitrocellulose, they were as susceptible to proteolytic and glycolytic treatments as the 116-kDa band. However, treatment of whole organisms by trypsin and protease resulted in only partial degradation of these moieties with a corresponding reduction, but not total loss, of immunofluorescence staining. The location and biochemical properties of the 45- and 50-kDa antigens are poorly understood. These moieties were not labeled by protein-specific surface biotinylation, stained with protein stains, and reacted weakly or not at all with ConA and WGA.

The biochemical characteristics of the 40-kDa antigen of human *P. carinii* more closely resembled those of the 45- and 50-kDa antigens of rat-derived organisms than those of the 116-kDa human *P. carinii* antigen. It is unclear whether the differences between the 40- and 116-kDa bands represent different carbohydrate and protein constituents or different locations on the organism.

Of the other human *P. carinii* antigens, the 92-kDa band exhibited a pattern of reactivity with enzyme digestion and lectin binding which was similar to that found with the

116-kDa band. The 66-kDa moiety paralleled the responses of the 40-kDa entity to enzyme treatment.

The relationship of these antigens to organism morphology is complex. It is interesting that proteolytic enzymes affected both the immunological properties and structural integrity of *P. carinii*, whereas the major influence of glycolytic treatments was on organism immunoreactivity. Thus, carbohydrate residues of these antigens seem to have an important role in eliciting the host immune response.

The present study has analyzed the protein and carbohydrate properties of the major *P. carinii* antigens. This information should be helpful in developing strategies for the isolation and purification of these moieties and in studying their function.

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