INFECTION AND IMMUNITY, Oct. 1986, p. 96–103
0019-9567/86/100096-08$02.00/0

Vol. 54, No. 1

Analyses of Rat *Pneumocystis carinii* Antigens Recognized by Human and Rat Antibodies by Using Western Immunoblotting

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Received 17 March 1986/Accepted 11 July 1986

The major *Pneumocystis carinii* antigens inducing antibody responses in infected hosts were identified by Western immunoblotting techniques. The biochemical nature of these antigens was also elucidated. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by protein staining revealed a major component with a molecular weight (MW) of greater than 205,000. This major component disappeared and a new major protein staining component of approximately 110,00 to 116,000 MW appeared when electrophoresis was done in the presence of β-mercaptoethanol. Periodic acid-Schiff staining revealed that this major component contains carbohydrate moieties. A major component in the 55,000- to 60,000-MW region was visible with periodic acid-Schiff stain, but not with a protein stain, after electrophoresis in the presence of β-mercaptoethanol. The majority of sera tested from humans with diagnosed pneumocystosis and from rats allowed to recover from steroid-induced pneumocystosis reacted strongly with 110,000- to 116,000-, and 55,000- to 60,000-MW components. These sera often, but not always, detected antigens with MWs of approximately 170,000, 125,000, and 30,000 to 32,000. The data suggest that the antigenic composition of *P. carinii* is relatively complex and that rat and human *P. carinii* probably share antigenic determinants. Competitive studies between infection-derived human and rat antisera for the major rat *P. carinii* components revealed competition; rat antisera appeared to recognize a greater range of antigenic epitopes than did human antisera. Protease treatment of the antigenic components that had been immobilized on nitrocellulose paper destroyed their antigenic reactivity with rat antibody. Treatment with sodium periodate decreased reactivity of this 110,000- to 116,000-MW component and completely destroyed the reactivity of the 55,000- to 60,000-MW component with rat antibody.

Pneumocystis carinii is a ubiquitous organism infecting many mammalian species, including man (14). Serological surveys indicate that detectable antibodies to *P. carinii* occur in 40 to 75% of the healthy adult population (9, 12). These data suggest that *P. carinii* often occurs as a subclinical infection (14). Severe *P. carinii* pneumonitis (PCP) similar to that seen in humans can be induced in rats by giving corticosteroids and a low-protein diet (17). In rats and humans, antibody titers against *P. carinii* usually rise upon recovery.

P. carinii has been long recognized as an important opportunistic pulmonary pathogen in immunocompromised hosts (9, 12). Recently, increased interest in pneumocystosis has developed because of the high incidence of clinical pneumocystosis in patients with acquired immune deficiency syndrome (AIDS) (18). At present, definitive diagnosis requires the use of potentially hazardous invasive procedures to obtain lung tissue or aspirates that can be stained for microscopic demonstration of *P. carinii*. Noninvasive tests include assays for detection of *P. carinii* antigenemia or antibodies in the sera of patients (9). Little information is available on the nature of the *P. carinii* antigens involved in antigenemia or in the immune response. The lack of information is in part due to: (i) difficulty in growing *P. carinii* in vitro; (ii) difficulty in obtaining purified preparations of the organism for antigenic analyses; and (iii) lack of knowledge about the life cycle of *P. carinii*, which further complicates the isolation of antigens associated with different stages of parasite development.

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In the present study, the Western immunoblotting technique was used to identify *P. carinii* antigens that were reactive with antibodies in sera obtained from humans and rats. We demonstrated that antibodies from both sources recognize common antigenic molecules. In addition, preliminary studies describe several biochemical characteristics of the major parasitic antigens.

MATERIALS AND METHODS

***P. carinii* isolation.** *P. carinii* was obtained from the lungs of adult male Sprague-Dawley rats (weighing 200 to 250 g) given a cortisone acetate and low-protein diet regimen as described by Walzer et al. (17). Diseased animals were sacrificed, and the organisms were isolated from the lungs by one of two methods. The first method was similar to that of Walzer et al. (16), which uses collagenase and hyaluronidase for digestion of lung tissue and discontinuous Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.) gradients to separate organisms from lung debris. Modification of this method, using Percoll gradients, have been previously described (5). The cyst- and trophozoite-containing fraction of the gradient was removed, diluted 5:1 in Hanks balanced salt solution without Ca and Mg, and washed several times at 17,000 × *g* for 10 min. Samples of the fraction were stained with toluidine blue 0, and cyst counts were made by the method of Ikai et al. (6). This preparation is called *P. carinii* whole digest (PCDW). Lungs from germfree and normal Sprague-Dawley rats were processed in a similar fashion except that the preparations were not subjected to Percoll gradients, as this procedure did not allow recovery of enough protein for use as antigen controls. These prepara-

TABLE 1. Characteristics of rat sera used in Western immunoblotting analysis

Rat group and no.[a]	Age (wk)	Wk between stopping injection of cortisone and serum sample taken	Serological analysis	
			ELISA	Western immunoblotting
I				
1	6	NA[b]	<1:50[c]	–[d]
2	6	NA	<1:50	–
3	10	NA	<1:50	–
4	21	NA	<1:50	–
II				
5	36–64 (adult)	NA	1:800	+
6	36–64 (adult)	NA	1:1,600	+
7	36–64 (adult)	NA	1:800	+
8	36–64 (adult)	NA	<1:50	–
9	36–64 (adult)	NA	1:400	+
III				
10	36–48 (adult)	10	1:6,400	+
11	36–48 (adult)	2	<1:50	–
12	36–48 (adult)	2	1:6,400	+
13	36–48 (adult)	2	1:800	+
14	36–48 (adult)	2	1:100	±
15	36–48 (adult)	2	1:1,600	+
16	36–48 (adult)	2	1:1,600	+
17	36–48 (adult)	2	1:800	+

[a] Groups: I, germ-free Sprague-Dawley rats (6 to 21 weeks old); II, normal adult (36 to 54 weeks old) Sprague-Dawley rats; III, adult (36 to 48 weeks old) Sprague-Dawley rats immunosuppressed with cortisone (25 mg twice a week for 4 weeks followed by 12.5 mg once a week for 8 weeks for a total of 12 weeks).

[b] NA, Not applicable.

[c] Dilution of serum which produced an OD_{405} that was 0.2 above the mean OD_{405} of the negative control wells (negative rat serum diluted 1:100).

[d] Reactivity of serum antibodies to major *P. carinii* antigen (MW, 110,000): +, positive; ±, weakly positive; –, negative.

tions were designated as germfree lung digest (GFLD) and normal rat lung digest (NRLD), respectively.

In the second method, infected rat lungs were lavaged extensively to remove *P. carinii* organisms, as described by Masur and Jones (10). Hanks balanced salt solution (5 to 10 ml) was allowed to remain in the lung for 2 to 3 min with gentle massage of the lungs with the fingers and then withdrawn by syringe. The irrigant was placed in a sterile polyethylene centrifuge tube on ice. The procedure was repeated five times, bringing the total volume of Hanks balanced salt solution injected to 25 to 50 ml. The irrigant was centrifuged at 40 × *g* in a centrifuge (Ivan Sorvall, Ink., Norwalk, Conn.) for five min at 4°C. This low-speed centrifugation removed crude debris and blood cells, but most of the *P. carinii* cysts remained in the supernatant. The supernatant was centrifuged at 1,800 × *g* for 10 min to sediment the *P. carinii* organisms. The pellet was suspended in Hanks balanced salt solution and washed several times, and cyst counts were made as previously described (6). This preparation, called *P. carinii* lavage (PCL), was stored at −20°C for future use.

Sera. The following human sera were used: (i) seven samples obtained from AIDS patients (ages, 18 to 32 years) that were confirmed positive for PCP by lung biopsies and had antibody titers ranging from <1:50 to 1:3,200; (ii) six random samples from normal individuals (ages, 21 to 40 years) with no record of PCP but having some antibody to *P. carinii* (titers ranging from 1:50 to 1:400); and (iii) five serum samples from infants (ages, 6 to 18 months) having antibody titers ranging from 1:50 to 1:200 (Table 1). Sera from the following Sprague-Dawley rats were used. (i) Eight samples were from adult rats that had been treated with cortisone acetate and a low-protein diet for 8 to 12 weeks and then allowed to recover from PCP for 2 to 10 weeks. Cortisone acetate was administered twice a week for 4 weeks at a concentration of 25 mg per dose and then tapered off to 12.5 mg per dose once a week for 8 weeks. The antibody titers of these rats ranged from <1:50 to 1:6,400; (ii) Five samples were from normal adult rats that had not been immunosuppressed and had antibody titers ranging from <1:50 to 1:1,600. (iii) Four samples were from Sprague-Dawley germfree rats (6 to 21 weeks old) that had no detectable antibody titers (<1:50) as measured by indirect enzyme-linked immunosorbent assay (ELISA). Characteristics of the sera are described in Tables 1 and 2.

Protein determinations. Protein determinations of the antigen preparations were performed by the method of Bradford (1) with bovine serum albumin as the standard. The assay reagents were obtained from Bio-Rad Laboratories, Richmond, Calif. In addition, the number of cysts per milliliter of suspension was determined as described by Ikai et al. (6).

ELISA for detection of *P. carinii*-specific antibodies. A soluble *P. carinii* antigen preparation prepared from PCDW was used in the indirect ELISA for antibody determinations. The soluble *P. carinii* antigen preparation was prepared and the ELISA was performed as described by Graves et al. (5). A positive antibody titer of any given serum sample was defined as the reciprocal of the dilution which had a mean optical density at 450 nm (OD_{450}) that was 0.2 above the mean OD_{450} of the negative control serum at a 1:100 dilution. The negative control serum was a pool of sera from normal Sprague-Dawley rats that showed negligible antibody activity in the ELISA under the conditions used.

Gel electrophoresis. Whole *P. carinii* (either PCDW or PCL), GFLD, or NRLD preparations and molecular weight (MW) standards were solubilized with a sample buffer (0.5% sodium dodecyl sulfate [SDS], 0.002% bromophenol blue, 0.02 M Tris hydrochloride, 7% glycerol [pH 6.8]) for 3 min at 100°C with or without a disulfide-reducing agent (1.25% 2-β-mercaptoethanol; BME). Samples containing 4 to 30 μg of protein were electrophoresed in a 5 to 15% polyacrylamide gradient slab gel (0.75 by 155 by 135 mm) with the use of the discontinuous buffer system containing SDS as described by Laemmli (7). The numbers of *Pneumocystis* cysts per microgram of protein applied to the gel were approximately 1×10^5 to 2×10^5 in the PCDW and PCL preparations, 2×10^1 to 5×10^1 in the NRLD preparation, and 0 in the GFLD preparation. Electrophoresis was performed at 15 mA per gel until the ion front reached the bottom of the gel, which was approximately 4.0 h. MW standards used throughout the studies included myosin (205,000), β-galactosidase (116,000), phophorylase *b* (97,400), bovine albumin (66,000), egg albumin (45,000), glyceraldehyde-3-phosphate dehydrogenase (34,700), carbonic anhydrase (29,000) trypsinogen (24,000), β-lactoglobulin (18,400), and lysozyme (14,300). The MW standards were purchased from Sigma Chemical Co., St. Louis, Mo. The separated proteins in the gels were then either transferred to nitrocellulose paper (13) or stained. One set of parallel gels was stained with either 0.2% Coomassie blue in 50% methanol and 7% acetic acid or periodic acid-Schiff (PAS) stain (see below). The Coomassie

blue-stained gel was destained in 7% acetic acid–5% methanol.

Electrophoretic transfer and immunoblotting. Proteins separated by SDS slab gel electrophoresis were transferred to nitrocellulose paper by a method similar to that of Towbin et al. (13). Briefly, the proteins were electrophoretically transferred to nitrocellulose paper (0.45-μm pore size) at 60 V for 6 h. In most cases, a portion of the membrane containing the MW standards, one lane each of *P. carinii* and GFLD, was stained with amido black (0.2 g of amido black in a mixture of 90 ml of methanol, 20 ml of acetic acid, and 80 ml of distilled water). The other portion of the membrane was incubated with 1% Tween 20 in phosphate-buffered saline (PBS) for 1 h at 37°C or overnight at 4°C to saturate unoccupied protein-binding sites. The sheets were cut into 0.5-cm-wide strips (designated as electroblots) and subsequently incubated overnight at 4°C with either test sera or positive *P. carinii* sera diluted 1:100 in PBS containing 0.05% Tween 20 (PBS-Tween 20 buffer). Positive *P. carinii* sera (titer of 1:6,400) were a pool of high-titered sera obtained from rats that had recovered from clinical pneumocystosis and is designated as infection-derived rat sera. After incubation with various sera, the strips were washed four times in PBS-Tween 20 buffer over a period of 30 min. The membrane strips were then incubated for 2 h with alkaline phosphate-conjugated goat anti-human immunoglobulin G (IgG) (γ chain specific) or alkaline phosphate-conjugated rabbit anti-rat IgG (both obtained from Sigma). Both conjugates were diluted 1:1,000 in PBS-Tween 20 buffer. This was followed by five washes in PBS-Tween 20 buffer. Finally, the antigenic bands were developed by addition of substrate consisting of 25 mg of β-naphthyl acid phosphate and *O*-dianisidine tetrazotized sodium salt in 50 ml of 0.06 M sodium tetraborate buffer (pH 9.7) containing 29.3 mg of $Mg(SO_4)_2$. The substrate solution was filtered and used immediately. The antigenic reactive bands were allowed to develop for a minimum of 3 min and a maximum of 5 min. The reaction was stopped by washing in a solution of methanol-acetic acid-water (5:1:5, (vol/vol/vol) followed by a final rinse in water. All incubation steps of the immunoblotting technique were carried out with a rocking platform.

Competitive studies with human and rat sera. Rat *P. carinii* antigens that had been separated by SDS-polyacrylamide gel electrophoresis (PAGE) with slab gels and then transferred to nitrocellulose membrane were used in these studies. In one part of this study, three individual electroblots containing *P. carinii* (PCDW) antigens were reacted with positive rat serum (titer, 1:3,200). Following incubation overnight at 4°C and extensive washing with PBS-Tween 20 buffer, electroblot no. 1 was reacted with positive human serum (obtained from an individual who had recovered from PCP and had an antibody titer of 1:3,200); electroblot no. 2 was reacted with normal human serum (antibody titer, 1:50); and electroblot no. 3 was reacted with PBS-Tween 20 buffer. Following a second incubation at 4°C overnight, the electroblots were washed and electroblots no. 1 and 2 were incubated with goat anti-human IgG conjugated with alkaline phosphatase, and electroblot no. 3 was incubated with rabbit anti-rat IgG conjugated with alkaline phosphatase. Electroblot no. 3 served as a positive control. The immunoblotting procedure was then completed as described above. In part two of this competitive study, three electroblots were initially reacted with positive human serum (the same as that used in part one), incubated, and washed as described above. The electroblots were then reacted with positive rat sera (antibody titer, 1:3,200; electroblot no. 1), germfree rat serum (antibody titer, <1:50; electroblot no. 2), or PBS Tween-20 buffer only (electroblot no. 3) and incubated overnight at 4°C. Following this incubation, all of the electroblots were washed extensively, electroblots no. 1 and 2 were reacted with rabbit anti-rat IgG conjugated to alkaline phosphatase, and electroblot no. 3 was reacted with goat anti-human IgG conjugated to alkaline phosphatase. Electroblot no. 3 served as a positive control. The electroblots were washed and developed with a substrate as described above under immunoblotting. As negative conjugate controls, electroblots were reacted with anti-rat or anti-human IgG conjugate only.

TABLE 2. Characteristics of sera from patients and controls used in Western immunoblotting analysis

Patient group and no.[a]	Age (yr)	Time between diagnosis of PCP and serum sample taken	Serological analysis	
			ELISA	Western immunoblotting
I				
2	1.5	NA[b]	1:200[c]	–[d]
3	<1	NA	1:100	–
4	<1	NA	1:100	–
5	<1	NA	1:200	–
6	<1	NA	<1:50	–
II				
7	27	NA	1:50	+
8	29	NA	1:400	+
9	40	NA	1:100	±
10	31	NA	1:100	+
11	21	NA	1:100	+
12	26	NA	1:200	+
III				
13	27	1 day	1:200	+
14	25	1 day	1:3,200	+
15	28	<1 wk	1:3,200	+
16	32	<1 wk	1:800	+
17	Unknown	5 days	<1:50	±
18	18	3 days	1:800	+
19	24	3 mo	1:400	+

[a] Groups: I, infants (6 to 18 months of age) hospitalized for injury or bacterial infections other than PCP; II, healthy adults with no evidence of PCP; III, confirmed AIDS patients (adults) who had PCP that was confirmed by bronchial biopsy.
[b] NA, Not applicable.
[c] Dilution of serum which produced OD_{405} that was 0.2 above the mean OD_{405} of the negative control wells (negative human serum diluted 1:100).
[d] Reactivity of serum antibodies to major *P. carinii* antigen (MW, 110,000): +, positive; ±, weakly positive; –, negative.

Staining for carbohydrate. Polyacrylamide gels through which PCDW were electrophoresed as described above were stained for carbohydrates with the PAS reagent stain as described by Fairbanks et al. (4).

Treatment of *P. carinii* antigens with protease, trypsin, and sodium metaperiodate. Rat *P. carinii* antigens separated by SDS-PAGE with slab gels and transferred to nitrocellulose membrane were then treated with enzymes or sodium metaperiodate following incubation for 1 h at 37°C in PBS containing 1% Tween 20. Following 5 washes in PBS, individual nitrocellulose strips containing *P. carinii* antigens were treated for 2 h at 37°C with either 100 μg of protease (type X) per ml or 100 μg of acetylated trypsin (type V-S) per ml made up in PBS (pH 7.4) and supplemented with 0.02 M $MgCl_2$. Both trypsin and protease were from Sigma. In addition, antigen-containing strips were exposed to 0.05 M

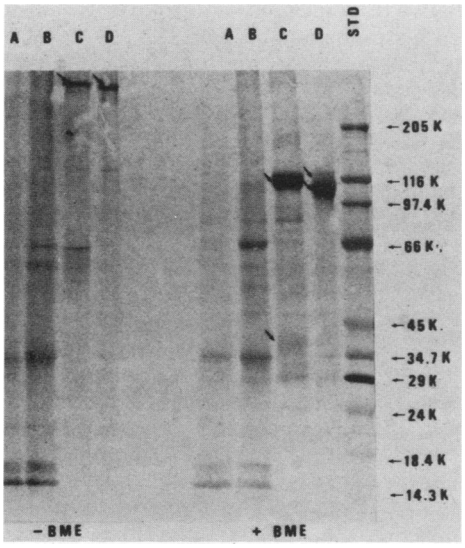

FIG. 1. SDS-PAGE of *P. carinii* obtained from infected rat lungs. SDS-treated samples (15 μg) were electrophoresed on 5 to 15% gradient gels with or without BME (1.25%). Lanes A, GFLD; B, NRLD; C, PCL antigen; D, PCDW antigen; STD, MW standards (numbers on the right, in thousands). The gel was transferred to nitrocellulose paper and stained with amido black. The arrows in lanes C and D indicate the major protein staining bands.

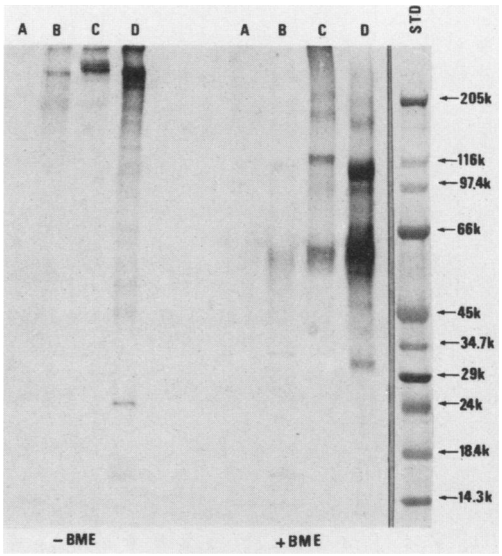

FIG. 2. Western immunoblot analysis of *P. carinii* with infection-derived rat sera. The antigen extracts were identical to those described in the legend to Fig. 1 except that 4 instead of 15 μg of protein was loaded per lane, electrophoresed, and transferred to nitrocellulose paper. However, the lanes were immunoblotted with infection-derived rat sera instead of being stained with amido black. Lanes: A, GFRD; B, NRLD; C, PCL; D, PCDW; STD, MW standards (numbers on the left, in thousands).

(final concentration) sodium metaperiodate (Sigma) in 0.01 M sodium acetate buffer (pH 4.5) for 18 h at 4°C. As controls, antigen-containing strips were treated with either PBS or acetate buffer and run in parallel. After incubation, the strips were washed in PBS-Tween 20 buffer three times and then immunoblotted, as described above, with normal and infection-derived rat sera. Controls to show that enzymes were active against known proteins and CHO cells indicated that the enzyme systems were functional (data not shown).

RESULTS

Analyses of *P. carinii* proteins by SDS-PAGE. Studies were conducted in attempts to identify proteins unique to *P. carinii*. Extracts from PCDW, NRLD, GFLD, and PCL were separated by SDS-PAGE and stained with amido black. In the absence of the BME, a relatively dense band was seen in the MW region of greater than 205,000 for PCL and PCDW (Fig. 1, lanes C and D). The PCDW band appeared to be slightly lower than the PCL band. A faint band of >205,000 MW occurred in NRLD (lane B) but no band of >205,000 MW was detected in GFLD (lane A). In addition, a band in the MW region of about 125,000 was visible for both PCDW and PCL but not NRLD or GFLD. The other bands seen were also present in NRLD or GFLD and were considered to be host protein contaminants.

When the same antigen extracts were treated with BME, a different pattern occurred. PCDW had a dense band at 110,000 MW and a lighter band at 125,000 (Fig. 1, lane D). PCL (Fig. 1, lane C) had a dense band at 116,000 MW and a light, more diffuse band at 37,000 MW. NRLD, but not GFRD, had a very faint band in the 116,000-MW region (lanes B and A, respectively). Both PCL and PCDW had a faint, diffuse band in the MW region of about 170,000 that was not seen in NRLD or GFLD. The other bands seen were also present in NRLD or GFLD and were considered to be host protein contaminants as was the case with the gels without BME.

***P. carinii* antigens detected by rat and human antibodies.** Experiments were done to determine which rat *P. carinii* proteins were reactive with antibodies found in infection-derived rat sera. Proteins from the four preparations used for analysis in Fig. 1 were separated by SDS-PAGE, electrophoretically transferred to nitrocellulose paper, and immunoblotted as described in Materials and Methods. The reactivity profiles of the *P. carinii* proteins are presented in Fig. 2. Without BME, most prominent antigenic bands were greater than 205,000 MW for both PCL and PCDW (lanes C and D). A distinct band in the region of 24,000 MW was also seen for PCDW. No reactive band was seen for GFLD (lane A), whereas NRLD showed faint bands at >205,000 MW (lane B). Immunoblots of antigens treated with BME revealed that PCL (lane C) had major reactive bands at 116,000 and 55,000 to 60,000 MW. PCDW (lane D) had reactive bands in the regions of 110,000, 55,000 to 60,000, and 32,000 MW. None of these bands occurred with GFLD (lane A), whereas NRLD showed a faintly reactive band at 110,000 MW and a denser band in the 55,000 to 60,000-MW region (lane B) (Fig. 2). Thus, the prominent protein bands of PCDW that were highly reactive with infection-derived rat sera included the 110,000-MW band with BME and the >205,000-MW band without BME (lane D). The prominent PCL bands were 116,000 MW with BME and >205,000 MW without BME (lane C). In addition, reactive antigens occurred in the region of approximately 55,000 to 60,000 MW for both PCDW and PCL preparations with BME (lanes C and D). This reactive band(s) was diffuse and was probably made up of more than one antigenic molecule. This band(s) was not seen in the absence of BME (lanes C and D). With BME, an antigenic reactive band was also seen around 32,000 MW for PCDW (lane D). Antigenic reactive bands

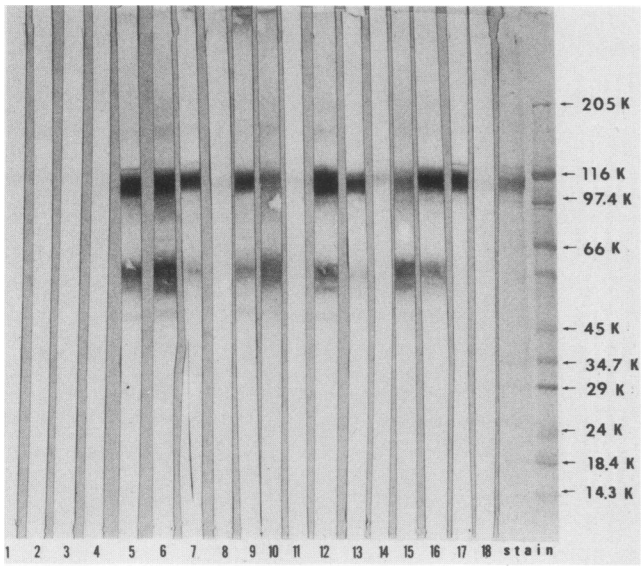

FIG. 3. Western immunoblot analysis demonstrating reactivity of antibodies in rat sera to antigens present in *P. carinii*. *P. carinii* proteins (PCDW) were separated by SDS-PAGE (5 to 15% gels) and transferred to nitrocellulose paper, which was cut into 0.5-cm-wide strips, and each of the strips containing separated antigens (4 μg) was blotted with germfree rat serum (lanes 1 to 4), normal rat serum (lanes 5 to 9), or sera from rats that had recovered from PCP (lanes 10 to 17). PCDW antigens located on the nitrocellulose strip on lane 18, were reacted with rabbit anti-rat IgG conjugated to alkaline phosphatase and served as a conjugate control. The amido black-stained lanes represent electroblots of separated PCDW proteins and MW markers (numbers on the right, in thousands).

were also found in normal rat lung preparations in the MW region of 55,000 in the presence of BME (Fig. 2, lane B). No antigenic reactive bands were found in the GFLD preparations.

The above studies were done with a single pool of infection-derived rat sera. To determine which antigens most often stimulate the immune response, we tested the antigens against individual rat and human sera. General characteristics of the rat and human groups and their antibody titers as determined by indirect ELISA are summarized in Tables 1 and 2, respectively. A preparation of PCDW (200 μg containing 4×10^7 cysts) was treated with electrophoresis sample buffer containing BME, loaded onto two 5 to 15% gradient gels that had been prepared with preparative combs (no individual slots except one for the protein standards), and then electrophoresed. Following transfer of the separated proteins to nitrocellulose paper, individual strips (0.5 cm wide) of the nitrocellulose paper, each containing about 4.0 μg of protein, were immunoblotted with individual test serum. An important observation is that the majority of rat and human serum samples having ELISA titers of 1:100 or greater reacted significantly with similar *P. carinii* antigens in the MW regions of approximately 110,000 and 55,000 to 60,000 (Fig. 3 and 4). To a lesser degree, many of the rat and human sera reacted with a band in the MW region of about 125,000. Sera from germfree rats (Fig. 3, lanes 1 to 4) having antibody titers of less than 1:50 showed no reactivity with *P. carinii* antigens. Little variability in reaction patterns could be seen between antibody-containing sera of normal adult rats and antibody-containing sera from diseased (PCP) animals that had recovered (Fig. 3, lanes 5 to 9 versus lanes 10 to 17). Three rat sera, one from the normal group (Fig. 3, lane 7) and two from the recovery group (Fig. 3, lanes 13 and 17) showed a strong reaction with the 110,000-MW antigen but a fairly weak reaction with the antigen in the MW region of 55,000 to 60,000.

In similar experiments with human sera, serum from only one infant (Fig. 4, lane 2) reacted with *P. carinii* antigens (110,000 MW). Several sera from both normal and PCP-recovered humans reacted strongly with the 110,000-MW antigenic component but to a lesser degree, if at all, with the 55,000- to 60,000-MW component. A few of the normal and PCP human sera (Fig. 4, lanes 7 to 12 and 13 to 19, respectively) reacted with molecules in the MW regions of approximately 30,000 to 32,000, 36,000, and 170,000.

Competitive study of antibodies of human and rat sera for *P. carinii* antigens. If antibodies in human and rat sera recognized similar *P. carinii* antigens as suggested in Fig. 3 and 4, then human antibodies should compete with rat antibodies for major rat *P. carinii* antigens. A competitive study was conducted to verify this assumption.

Immunoblot reactions of *P. carinii* with positive rat serum (ELISA titer, 1:3,200) (Fig. 5, lane 3) and positive human serum (ELISA titer, 1:3,200) (lane 6) showed one intensely stained antigenic region of 110,000 MW and a broad, diffuse region of about 55,000 to 60,000 MW. These lanes served as controls for the experiment and were similar to the results seen in Fig. 3 and 4. When an electroblot of PCDW antigens was immunoblotted initially with infection-derived rat serum, followed with positive human serum (AIDS patients with confirmed PCP; ELISA titer, 1:3,200), and finally developed with anti-human (IgG) conjugate, effective competition took place (Fig. 5, lane 4). The 110,000- and the diffuse 55,000- to 60,000-MW regions decreased in intensity.

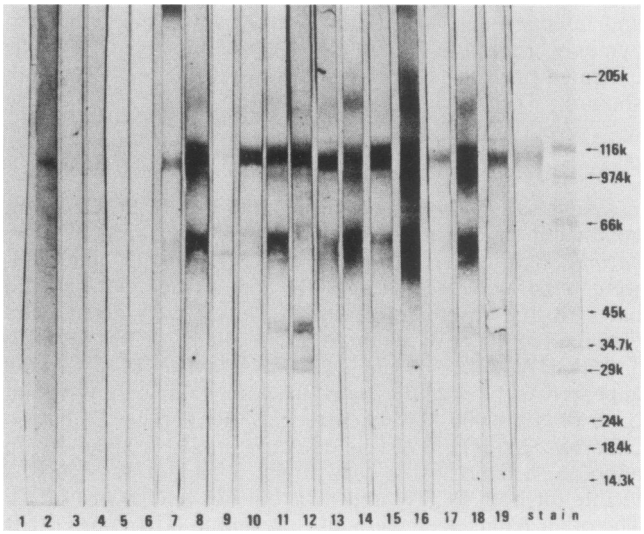

FIG. 4. Western immunoblot analysis demonstrating reactivity of antibodies in human sera to *P. carinii* antigens. Immunoblots were prepared as described in the legend to Fig. 3. Individual strips (0.5 cm wide) of nitrocellulose containing the separated PCDW antigens (4 μg) were immunoblotted with infant (6 to 18 months old) sera (lanes 2 to 6), normal adult sera (lanes 7 to 12), or sera of AIDS patients diagnosed as having PCP (lanes 13 to 19). Lane 1 is an electroblot of PCDW proteins that was reacted with goat anti-human IgG conjugated to alkaline phosphatase and served as a conjugate control. The amido black-stained lanes represent electroblots of separated PCDW proteins and MW markers (numbers on the right, in thousands).

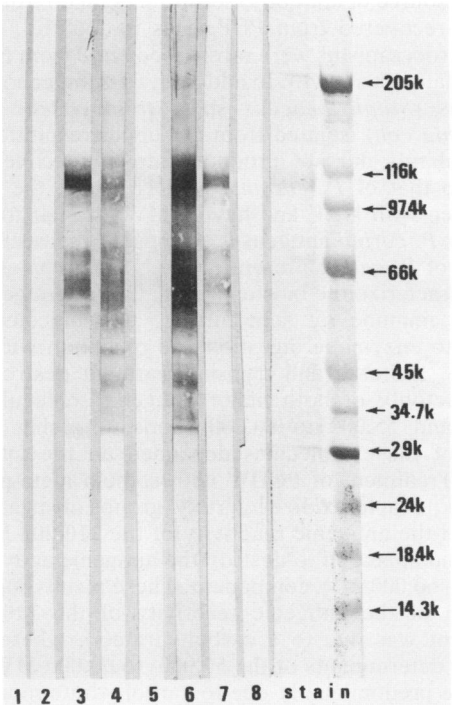

FIG. 5. Competition between rat antibodies and human antibodies for *P. carinii* antigens. Initially, electroblots of PCDW (4.0 μg per strip) were reacted with either positive rat serum (titer, 1:3,200 by ELISA; lanes 3, 4, and 5) or positive human serum (titer, 1:3,200 by ELISA; lanes 6, 7, and 8) or left untreated (lanes 1 and 2). Following appropriate incubation (see Materials and Methods), the electroblots were reacted with the following (lanes): 1, 2, 3, and 6, PBS; 4, positive human serum; 5, negative human serum (less than 1:50 titer by ELISA); 7, positive rat serum; 8, negative germfree rat serum (no titer by ELISA). Rabbit anti-rat IgG conjugate was then added to lanes 2, 3, 7, and 8, and goat anti-human IgG conjugate was added to lanes 1, 4, 5, and 6, and this was followed by incubation and addition of substrate as described in Materials and Methods. The numbers on the right indicate the sizes of MW markers (in thousands).

Under similar conditions except that normal human serum (titer, 1:50 by ELISA) was used as the second antibody instead of positive human serum (lane 5), no distinct antigenic reactive bands were seen. Using positive human serum as the primary antibody reactant followed by positive rat serum as the secondary antibody and anti-rat IgG conjugate (Fig. 5, lane 7), we observed a small amount of competition since the intensity of the 110,000-MW reactant band decreased slightly from that of the control (lane 3). The intensity of the diffuse 55,000- to 60,000-MW region decreased considerably. Using negative rat serum (germfree sera; <1:50 titer) as the second antibody (instead of positive rat serum) in this situation (lane 8), we saw no reactive bands. The conjugate controls, goat anti-human IgG and rabbit anti-rat IgG conjugated to alkaline phosphatase (lanes 1 and 2, respectively), were negative. The above described experiments were conducted with several different infection-derived rat sera and human sera having significant titers (1:800 to 1:3,200), and similar results were obtained in each case.

Effects of enzymatic and chemical treatments on antigenicity of *P. carinii* major antigenic components. Treatment of electroblots of separated *P. carinii* antigens with either acetylated trypsin (type V-S) or protease (type V) had a dramatic effect on the antigenicity of the major 110,000- and 55,000- to 60,000-MW antigens of *P. carinii*. Essentially, the reactivity of these antigens for rat antibody was completely lost when protease was used and drastically reduced when trypsin was used. Sodium metaperiodate treatment decreased the activity of the 110,000-MW antigenic component and appeared to destroy the antigenic activity of the 55,000- to 60,000-MW component completely. Sodium acetate buffer (0.01 M, pH 4.5) alone had no effect on the antigenicity of *P. carinii* antigens.

PAS staining of *P. carinii* proteins separated on SDS-polyacrylamide gels. The above results indicated that *P. carinii* antigenic components (110,000 and 55,000 to 60,000 MW) contained carbohydrate moieties. To confirm this observation, PCDW preparations (30 μg per lane containing 6×10^6 cysts) with or without BME were electrophoresed on SDS-polyacrylamide gels (5 to 15%) and directly stained with PAS stain (Fig. 6). For comparison, identical preparations were stained with Coomassie blue. Four major components which correspond to MWs of approximately 125,000, 110,000, 55,000 to 60,000, and <14,000 were detected in PCDW preparations that were treated with BME and stained with PAS stain (Fig. 6, lane B, PAS stain). Without BME, components in the MW regions of >205,000 and 125,000 and one large component at the top of the gel were observed (Fig. 6, lane A, PAS stain).

DISCUSSION

Analyses of stained electrophoretic patterns of SDS-PAGE gels run in the presence of BME revealed one major protein in PCDW and PCL that was not found in GFLD and in only trace amounts, if at all, in NRLD. The major protein

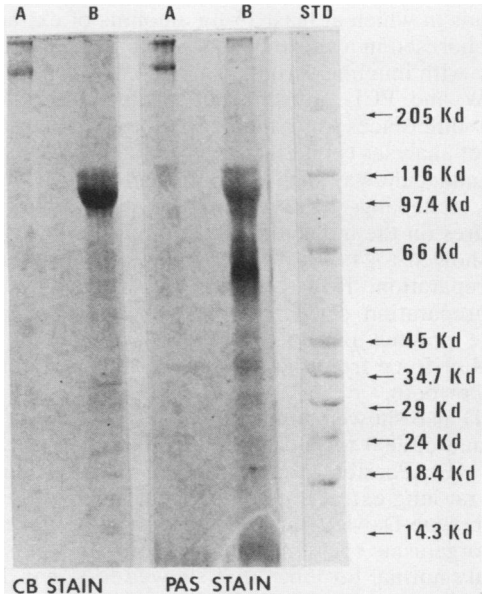

FIG. 6. PAS stain of *P. carinii*. PCDW (30 μg) was dissociated with SDS in the presence or absence of BME and then analyzed on 5 to 15% gradient gels. Individual sections of the gel containing PCDW that had been untreated (lanes A) or treated with BME (lanes B) were stained with either PAS or Coomassie blue (CB). MW standards (indicated by numbers on the right, in thousands) were stained with Coomassie blue.

bands observed for PCL (MW, 116,000) and PCDW (MW, 110,000) were especially broad and dense. Further studies are needed to determine whether they are composed of more than one protein band. When the reducing reagent was omitted from the samples, a major >205,000-MW band was seen with both PCL and PCDW. A 125,000-MW band was also seen for both preparations. These changes in the electrophoretic pattern of these extracts in the absence of BME suggest that the larger protein molecule (MW, >205,000) was composed of two or more subunits held together by disulfide bonds. Since the major antigenic components (116,000 for PCL and 110,000 for PCDW) seen in the presence of BME disappeared in the absence of BME, they are probably part of the larger >205,000-MW bands. Maddison et al. (8) also found a molecule at >200,000 MW in soluble rat *P. carinii*-infected lung preparations treated with 8 M urea but no reducing agent. Possibly the >200,000-MW protein reported by Maddison et al. (8) is the same as the >205,000-MW protein found in our preparations.

When analyzed by Western immunoblotting techniques (Fig. 2), a relatively complex and somewhat variable antigenic pattern was found for *P. carinii* obtained by the digestion and lavage methods. BME-treated PCDW showed prominent antigenic bands at 110,000 and 55,000 to 60,000 MW, whereas BME-treated PCL showed prominent antigenic bands at 116,000 and 55,000 to 60,000. Both preparations showed weakly reactive bands at about 170,000 MW. In addition, a weakly reactive 32,000-MW antigenic band was seen with PCDW. Perhaps the relatively harsh treatment of PCDW with digestive enzymes and the Percoll gradient procedures degraded the 116,000-MW band into smaller (110,000 and 32,000) components. The minor 37,000-MW band seen in PCL after protein staining was not detected by immunoblotting with infection-derived rat sera. Possibly, this band represents host material. It is interesting that the 55,000- to 60,000-MW region did not stain significantly with amido black (Fig. 1), even under experimental conditions in which at least 15-μg amounts of extracts were electrophoresed in a single lane of a slab gel and yet reacted strongly with immune serum.

PCDW and PCL antigens not treated with BME also showed differences in banding patterns in Western immunoblot analyses (Fig. 2). In the >205,000-MW region, the PCDW molecule was slightly lower than the PCL molecule, possibly reflecting the effect of the digestion or Percoll procedures on the molecule. In addition, the PCDW preparation showed a 24,000-MW band that was not seen in the PCL preparation. However, this was not always seen in every preparation of PCDW. The band may represent a cleavage product of the >205,000-MW band, an antigen released or freed from the organism in this particular preparation, or both.

NRLD also showed antigenic bands at >205,000 MW (no BME) and 55,000 to 60,000 and 110,000 (very weak) MW (with BME). Possibly, these were *P. carinii* proteins since normal rat lung extracts obtained from lungs of the healthy male Sprague-Dawley rats do contain low numbers of *P. carinii* organisms (personal observation). In this study we used only normal rat lungs that showed less than 10^5 cysts per g of lung by the method of Ikai et al. (6). This contrasts with the 10^7 to 10^9 cysts per g of lung present in diseased rat lungs used to prepare PCDW. Less than 10^2 *Pneumocystis* cysts were added to each NRL lane as contrasted with approximately 10^6 cysts per PCL or PCDW lane.

It is feasible that some of the reactive bands seen with NRL could be due to microorganisms other than *P. carinii* since the source of antibody (serum) was taken from animals that had recovered from PCP. This is doubtful, however, since microorganisms were rarely recovered from NRL after culture (data not shown). In addition, four indigenous microorganisms (*Staphylococcus* sp., *Streptococcus* sp., and *Escherichia coli*) isolated from the upper respiratory tracts of normal rats did not produce antigen reactive patterns similar to that of *P. carinii* or NRL. Thus, the antigenic bands seen with NRL and having MWs similar to those of the major *P. carinii* antigens are probably the results of low numbers of *P. carinii* present in the NRL preparation.

To characterize the biochemical nature of antigens recognized by immune rat sera further, we subjected PCDW extracts to enzymatic digestion and oxidation with sodium periodate. Protease and trypsin treatment destroyed antigenic reactivity of both major antigenic molecules (MW, 110,000 and 55,000 to 60,000), indicating that antigenic activity of these molecules depended on the integrity of protein. Treatment of PCDW with sodium meta-periodate, an agent known to oxidize hydroxyl groups of sugar moieties decreased the antigenic reactivity of the 110,000-MW component and appeared to destroy the antigenic activity of the 55,000- to 60,000-MW component. These results suggest that a portion of the antigenic reactivity of the 110,000-MW component was due to a carbohydrate(s), whereas major antigenic determinants of the 55,000- to 60,000-MW component were predominantly due to carbohydrate moieties.

Our studies, in which gels containing separated proteins from the PCDW were stained with PAS (Fig. 6), confirmed the presence of PAS-staining moieties on the 110,000- and 55,000 to 60,000-MW components. In addition, the 125,000- and >205,000-MW components were stained with PAS. A component in the MW region of about 14,000 was also stained with PAS but not with Coomassie blue. Since these gels were loaded with large amounts (30 μg) of protein, the absence of Coomassie blue-stained bands in the MW regions of 55,000 to 60,000 and 14,000 suggests that proteins were minor components of these molecules. However, protein must be associated with the 55,000- to 60,000-MW molecule to some degree since protease treatment destroyed the antigenic activity (see above).

There has been some evidence presented by other workers suggesting that the major *P. carinii* antigen contains carbohydrates. Maddison et al. (8) reported that their HCl-treated *P. carinii* soluble antigen was possibly a glycoprotein. The stability of their antigen to boiling suggests that the antigen may be, at least in part, carbohydrate in nature. Other indirect evidence of a carbohydrate moiety of *P. carinii* was presented by Opferkuch (11). He showed by cytochemical staining that *P. carinii* contained chiefly neutral glycoprotein. Brzosko and Nowoslawski (2), who used sera from infants with interstitial plasma cell pneumonia, reported that specific immunofluorescence stained the same parasite structures in serial sections of infected lung as were stained by PAS. They showed that the PAS-positive structures and immunofluorescence could be blocked by acetylation (which blocks hydroxyl and amino groups). These results suggested that the hydroxyl, and not amino, groups largely determine the antigenicity of the *P. carinii* glycoprotein moiety.

Our preliminary studies with random rat and human sera showed that the two major rat PCDW antigens (110,000 and 55,000 to 60,000 MW) were detected by the majority of sera from the rats and humans infected with or recovered from *P. carinii* (Fig. 3 and 4). The data demonstrated that human antibodies reacted with rat *P. carinii* antigens, a fact that has long been assumed, based on serological studies (15). The

competition study further confirmed that antibodies in rat and humans infected with *P. carinii* reacted with similar antigenic components (Fig. 5). However, antibodies present in infection-derived rat sera appeared to compete with the human antibodies for the antigenic sites on the major antigenic molecules more effectively than the converse. This suggested that, although common antigenic determinants were recognized by both sources of antibodies, rat sera appeared to recognize a greater range of antigenic determinants than did human sera. This would be expected since we used *P. carinii* antigen derived from rats for this study. A more critical analysis would be possible if we had *P. carinii* antigen from humans available for these kinds of analysis. All rat sera tested that had an antibody titer of ≥1:100 reacted with at least one of the major antigenic components and in most cases with both components. Sera from germfree rats (antibody titer, <1:50) did not react with PCDW molecules. Furthermore, we were unable to demonstrate *P. carinii* cysts in lung digests from these animals. This further supported the conclusion that the immunoblot reactions were specific *P. carinii* antigen-antibody reactions.

The data presented in this paper support the hypothesis that rat and human *P. carinii* share common antigenic determinants. The facts that some of these antigenic determinants were found on the major 110,000-MW component of rat *P. carinii* and that monoclonal antibodies produced in our laboratory (5) reacted with this major molecule further advocate the possible use of these antibodies in serodiagnostic studies of clinical specimens from humans. Competitive studies between our monoclonal antibodies and positive human antisera for the major 110,000-MW component of rat *P. carinii* are in progress and may further support the use of these monoclonal antibodies in diagnostic assays.

Only one infant serum (titer, 1:200) showed antigenic reactivity with one of the two major antigenic components (110,000 MW). The reason(s) why the other infant sera, showing antibody titers of 1:100 or 1:200, did not react in the Western blot study is not known at this time. The fact that many normal adult Sprague-Dawley rats and humans were shown to have antibodies to *P. carinii* antigens is in agreement with other published data (12) and indicates that *P. carinii* is a ubiquitous organism and most animals are probably infected early in life (8). The reason why certain sera contain antibodies that react differently to either the major *P. carinii* antigens (MW, 110,000 and 55,000 to 60,000) or the putative minor *P. carinii* antigens (MW, 170,000, 125,000, and 30,000 to 32,000 [Fig. 3 and 4]) is unknown. Possibly, the stage of the infection when the serum was collected is a factor. Possibly, different antigens are expressed at different stages of the cycle of *P. carinii* and the immune response varies accordingly. Furthermore, some of the variability in reaction by different serum samples may reflect inherent differences in the host responses of individuals. Finally, species or strain differences in *Pneumocystis* may contribute to variability.

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