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Fractionation of *Pneumocystis carinii* Antigens Used in an Enzyme-Linked Immunosorbent Assay for Antibodies and in the Production of Antiserum for Detecting *Pneumocystis carinii* Antigenemia

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Cyst-rich suspensions of *Pneumocystis carinii* were obtained by differential and gradient centrifugation from heavily infected rat lungs. After preparation of an aqueous-soluble extract of the cyst-rich material, the insoluble residue was extracted with 8 M urea. Small amounts of infected human lung tissue and uninfected rat and human lung were processed similarly. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed that both human and rat infected lung extracts contained a large protein (>200,000 daltons). This component was not present in extracts of uninfected lung. In addition, an HCl-soluble extract was prepared from the cyst-rich suspension from infected rat lung. The urea-extracted antigen was most reactive in an enzyme-linked immunosorbent assay. Rabbit antiserum against the HCl-soluble antigen detected circulating antigen in patients' sera in a counterimmunoelectrophoresis assay.

Infections with *Pneumocystis carinii* have resulted in epidemic disease in malnourished young children living in crowded conditions after World War II in Europe and in institutions in Iran. In North America in the early 1970's, pneumocystosis was recognized as a disease of growing importance in both children and adults with malignancies of the types that necessitated aggressive immunosuppressive chemotherapy and in patients with primary immunodeficiencies. With the availability of trimethoprim-sulfamethoxazole as a safe, effective drug for prophylaxis, the prevalence of the disease has decreased. However, the epidemiology of infection with *P. carinii* remains poorly understood.

The immunofluorescence test (IIF) has been the most widely used assay for the detection of circulating antibody in pneumocystosis. Because most patients have impaired immunocompetence, the IIF has not proved to be of great assistance in diagnosis. In the absence of antibody detection the demonstration of antigenemia might, therefore, be a more appropriate approach to serodiagnosis. Only a few reports have been made on assays for antigenemia in pneumocystosis. This approach appeared helpful in young infants (15), but 50% of adult patients with pneumonitis due to causes other than infection with *P. carinii* were shown to have circulating pneumocystis antigen (11).

The enzyme-linked immunosorbent assay (ELISA) has proved to be a sensitive tool for detecting specific antibodies and is applicable to the diagnosis of a wide variety of infectious diseases, including protozoal infections. Because of the efficacy of trimethoprim-sulfamethoxazole, death due to *P. carinii* infection occurs rarely, and infected human lung was not available to us. We, therefore, report our studies on preparing antigens from the lungs of rats infected with *P. carinii* for use in ELISA and for the preparation of hyperimmune rabbit serum for detecting antigenemia in infected humans.

MATERIALS AND METHODS

Source of *P. carinii* organisms. Female Sprague-Dawley rats (Holtzman Co., Madison, Wis.) weighing 150 to 200 g were immunosuppressed by treatment with corticosteroids (4). Cortone acetate (Merck, Sharp & Dohme, West Point, Pa.) was injected subcutaneously at a dose of 25 mg three times weekly for approximately 12 weeks. Tetracycline hydrochloride (Rachelle Laboratories, Inc., Long Beach, Calif.) was added to the drinking water at a concentration of 0.9 mg/ml. At week 5, injections of Bicillin (Wyeth Laboratories, Inc., Philadelphia, Pa.) were started at a dose of 0.05 ml (75,000 U of penicillin G benzathine plus 75,000 U of penicillin procaine) twice weekly and continued until the lungs were harvested.

The rats were killed by intraperitoneal injection of Nembutal (Abbott Laboratories, North Chicago, Ill.)

approximately 12 weeks after immunosuppressive therapy was started. Impression smears from cut surfaces of each lung were stained with toluidine blue O (2) and Giemsa. Occasional lungs showing only scanty cysts of *P. carinii* or contaminated with yeasts or bacteria were discarded. Lungs with numerous *P. carinii* cysts per high-power field were stored at −20°C in RPMI 1640 (GIBCO Laboratories, Grand Island, N.Y.) until processed.

P. carinii organisms were isolated from lung tissue by the method of Walzer et al. (18) with minor modifications. Semifrozen lung tissue was finely diced with a scalpel and stirred in Hanks balanced salt solution without calcium or magnesium (HBSS) with a magnetic stirrer for 2 h at 4°C. The smallest particles were filtered through a 20-mesh stainless steel screen and held at 4°C. The lung fragments retained on the screen were again stirred in HBSS. The second filtrate was combined with the first and centrifuged at 12,000 × *g* for 15 min. The sediment was washed twice with HBSS and the same centrifugation conditions.

The final sediment was digested with 0.2% collagenase (Sigma Chemical Co., St. Louis, Mo.) and 0.2% hyaluronidase (ICN Pharmaceuticals, Inc., Cleveland, Ohio) in HBSS for 1 h at 37°C in a rotating water bath (100 oscillations per min). The digest was centrifuged at 12,000 × *g* for 15 min, and the sediment was digested a second time for 30 min. After the second digestion the sediment was washed three times, suspended in 10 ml of HBSS, and applied to a Ficoll-Hypaque gradient (Ficoll was obtained from Sigma and Hypaque from Winthrop Laboratories, New York, N.Y.). Two solutions were each prepared in HBSS: (i) 16% Hypaque and (ii) 4% Ficoll plus 16% Hypaque. Gradients of four layers of 5 ml each were prepared by diluting solution (ii) in solution (i). The top layer was 25%, followed by 50%, 75%, and 100% Ficoll-Hypaque, respectively. Each gradient was overlaid with 5 ml of the *P. carinii* suspension and centrifuged at 650 × *g* for 30 min at 22°C. The corresponding layers of each gradient tube were then pooled and washed three times in HBSS, with centrifugation at 12,000 × *g* for 30 min. The final sediment from each layer was stained with Giemsa and toluidine blue O, and counts were made (6). The washed sediment from the 100% Ficoll-Hypaque layer consistently contained the highest concentration of cysts and the least amount of contaminating rat lung material. This cyst-rich material was therefore used as the source of parasites for soluble antigens. The other layers were discarded. Six batches of cyst-rich material were the sources of the six lots of antigens characterized in Table 1.

HBSS-extracted antigen. The final suspension of *P. carinii* cyst-rich material was sonicated with a standard probe, heat system sonicator (model 375) pulsed at a 25% duty cycle with 100% power for 2 periods of 3 min each. By using phase microscopy, we established that these sonication conditions were the mildest capable of rupturing the cysts. The extract was clarified by centrifuging at 12,0000 × *g* for 30 min. The supernatant fluid was removed, stored at −20°C, and designated the HBSS antigen.

Urea-soluble antigen. The residual pellet from the HBSS antigen was suspended in 5.0 ml of 8 M urea (8 M urea in 0.05 M Tris-hydrochloride–0.3 M KCl–2.0 mM EDTA [pH 8.0]) (16) and sonicated at 25% duty cycle with 25% power for 5 min. The extraction was continued without stirring for 1 h at 4°C and then clarified by centrifuging at 12,000 × *g* for 2 h. The supernatant fluid was removed, dialyzed against 0.05 M phosphate-buffered saline (pH 7.2) (PBS), stored at −20°C, and designated urea antigen.

TABLE 1. Comparison of activities of HBSS, urea, and HCl antigens directly bound to ELISA plates

Antigen lot	No. of cysts per ml	Protein in HBSS antigen (μg/ml)	OD_{490} of 1:64 serum dilution		
			HBSS	Urea	HCl
1	3.4×10^6	120	0.49[a]	1.07	0.08
			0.30[b]	0.39	0.05
2	2.1×10^7	30	1.20	1.56	0.42
			0.21	0.27	0.09
3	3.8×10^6	60	0.58	1.02	0.08
			0.16	0.11	0.01
4	1.4×10^8	100	0.95	1.07	ND[c]
			0.30	0.14	
5	1.6×10^9	370	0.85	1.04	ND
			0.18	0.12	
6	7.6×10^8	590	0.86	1.15	ND
			0.33	0.19	

[a] Parasitologically proved positive case.
[b] Healthy Serum Bank donor.
[c] ND, Not determined.

HCl-soluble antigen. Cyst-rich material from the 100% Ficoll-Hypaque layer was treated with HCl (5, 14). Briefly, pelleted cyst-rich material was suspended in 0.2 M HCl, homogenized on a Vortex mixer, and heated in a boiling water bath for 10 min. After cooling to room temperature, the pH was adjusted to 7.2. The antigenic extract was then clarified by centrifuging at 12,000 × *g* for 30 min and dialyzed against PBS.

Three lots of HCl-solubilized extracts were treated with equal volumes of 10% trichloroacetic acid for 15 min and centrifuged at 12,000 × *g* for 1 h. The supernatant fluids were dialyzed against PBS and stored at −20°C.

Protein determinations. The method of Lowry et al. (8) was used to measure protein.

SDS-PAGE. All sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) chemicals, including molecular weight markers, were obtained from Bio-Rad Laboratories, Richmond, Calif. SDS-PAGE was performed in vertical slabs (100 by 140 by 0.7 mm). The gel used was a Tris-sulfate-borate discontinuous system (12, 16). The upper reservoir buffer was 0.04 M boric acid–0.041 M Tris–0.1% SDS, pH 8.64; upper stacking gel was 30 mg/ml (3.0%) acrylamide with 2 mg/ml (0.2%) *N,N'*-methylene bisacrylamide in 0.0267 M H_2SO_4–0.541 M Tris, pH 6.10; lower running gel was 8% acrylamide with 0.1% *N,N'*-methylene bisacrylamide in 0.0308 M HCl–0.4244 M Tris, pH 9.18; lower reservoir buffer was the same as the running gel buffer. All samples, each containing 5 μg of total protein, were treated with 1.0 mg of SDS in the presence of 8 M urea at 60°C for 15 min without disulfide-reducing agents. Samples were stacked at 7 mA per slab and run at 15 mA per slab. Protein bands were stained with silver stain (10).

Human sera. Sera were obtained from 20 patients with parasitologically proved pneumocystis pneumo-

nitis. These sera were chosen because they reacted in the IIF with cysts of *P. carinii* (13). A control serum nonreactive in IIF was obtained from a healthy Centers for Disease Control Serum Bank donor.

Immunization of rabbits. New Zealand rabbits weighing approximately 2.5 kg were immunized with (i) a suspension of 2×10^7 whole *P. carinii* cysts from rat lung, (ii) HBSS antigen extracted from 2×10^7 cysts, or (iii) HCl antigen extracted from 2×10^7 cysts. In addition, a saline extract of normal rat lung was used as an immunogen (9). Antigens were emulsified in Freund complete adjuvant and injected intramuscularly. The animals were boosted twice at monthly intervals with similar doses of antigen in incomplete adjuvant. Blood was collected 10 days after the last injection. The sera were stored at −20°C until use.

ELISA. ELISA (3) was performed in Cooke microtitration plates (1-223-29) obtained from Dynatech Laboratories, Inc., Alexandria, Va. (17). Antigens diluted in sodium carbonate buffer (pH 9.6) were allowed to adsorb onto the plates for 3 h at 37°C, then stored overnight at 4°C before use. Sensitized plates were stable for at least 1 month. Plates were washed with PBS containing 0.05 M Tween 20. Serum dilutions were made in PBS-Tween containing 1% bovine serum albumin. Volumes in each step of the assay were 0.2 ml. The substrate was H_2O_2 with *o*-phenylenediamine as the chromogen (H_2O_2-OPD). Each reaction was allowed to take place over a period of 30 min. The enzyme substrate reaction was stopped by the addition of 0.025 ml of 8 N H_2SO_4. The optical densities at 490 nm (OD_{490}) of the reactions were measured in a Flow Multiskan reader (Flow Laboratories, Inc., Hamden, Conn.).

Block titrations of the HBSS and urea antigens and peroxidase-labeled goat anti-human immunoglobulin G (IgG) (heavy and light chains) and anti-rabbit IgG (heavy and light chains) (Cappel Laboratories, Cochranville, Pa.) were made to determine optimum dilutions.

We attempted to couple the HCl antigen to polystyrene plates both directly, with the antigen diluted (100 μg/ml) in carbonate buffer as above, and indirectly by precoating the plates with poly(L-lysine) (7). For the latter, plates (76-001-05) obtained from Flow Laboratories, Inc. (McLean, Va.) were treated with 0.1 ml per well of a 0.5-mg/ml solution of poly(L-lysine) (approximately 40,000 daltons; Sigma, type VII-B). Fifteen minutes later, the plates were washed, and antigen at a concentration of 100 μg/ml was added to each well in a volume of 0.05 ml. In a preliminary unpublished study, we had found this concentration of a glycoprotein satisfactory in a similar ELISA for schistosomiasis. The plates were sealed and held overnight at 37°C. Plates were then washed with PBS, the wells were filled with PBS containing 5% fetal calf serum, and the plates were held at 4°C overnight. PBS containing 5% bovine serum albumin and 0.001% Tween 20 was used as a serum diluent and for all washes between reagent steps. Sera were diluted in 0.05-ml volumes, and the plates were incubated at 37°C for 1 h. Conjugate was allowed to react at 37°C for 1 h and OPD-H_2O_2 at room temperature for 30 min.

CIE. The methodology used for counterimmunoelectrophoresis (CIE) was that of Pifer et al. (14). SeaKem (ME) agarose (Marine Colloids Div., FMC Corp., Rockland, Maine) was prepared at a concentration of 0.75% in 0.05 M Veronal buffer (pH 8.2) containing 0.05% thiomerosal. Glass slides (50 by 75 mm) were coated with 10 ml of agarose. One or two rows of wells, 3 mm in diameter and 2 mm apart, were cut in each slide. Undiluted human serum was placed in the cathodic well, and rabbit antiserum to the HCl *P. carinii* antigen was placed in the anodic well. One or two slides were electrophoresed at room temperature in a Shandon electrophoresis bath (Shandon Scientific Co., Inc., Sewickley, Pa.) containing 1 liter of the 0.05 M Veronal buffer. A constant current of 10 mA was used. Initially, the voltage was 35 V for one slide or 18 V for two slides. As a marker, bromcresol purple in Veronal buffer was placed in a cathodic well on each slide. This dye has an electrophoretic mobility slightly slower than that of albumin. When the bromcresol purple had migrated through the anodic well, the electrophoresis was stopped. One slide required electrophoresis for approximately 1 h, and two slides required 2 h. After electrophoresis slides were held in a humid chamber at 22°C for 24 h, after which they were dried. The nonprecipitated protein was removed by soaking in buffer, and the slides were stained with thiazine red.

RESULTS

The reactivities in ELISA of the HBSS, urea, and HCl antigens prepared from cyst-rich material from six batches of infected rat lung are compared in Table 1. Preliminary studies showed that the parasite material before sonication must contain at least 3×10^6 cysts per ml to obtain antigens of adequate activity (data not shown). Because the ratios of cysts to rat lung particles were not identical in the six cyst-rich preparations, the protein concentrations of the HBSS antigens did not correlate with the cyst counts. Total protein of the HBSS antigen derived from 7 to 10 heavily infected rats was 3.7 mg for lot 5 and 5.9 mg for lot 6. Total protein of the urea-extracted antigens was 6.6 mg for lot 5 and 6.2 mg for lot 6. A block titration with a proved positive and a nonreactive serum was made with each lot of antigen to determine the optimal concentration of each preparation for sensitizing ELISA plates. For the urea antigens, this concentration varied from 0.8 μg/ml (lot 6) to 10 μg/ml (Lots 1 to 3).

The urea antigen invariably showed greater activity in ELISA with the positive serum and a greater differential in activity between the positive and negative sera than did the HBSS antigen (Table 1). All of the rabbit anti-*P. carinii* sera reacted with HBSS and urea antigens in ELISA with OD_{490} at a 1:64 dilution of serum varying from 0.36 to 1.16.

Absence of activity with the positive human serum was observed when direct linking of the HCl antigen in carbonate buffer (pH 9.6) to the polystyrene plate was attempted. Five additional sera, all of which had OD_{490} greater than 0.30 at a 1:64 dilution of serum when tested with the

urea antigen, also failed to react with the HCl antigen under the same conditions (Table 2). However, one of the two rabbits immunized with the HCl antigen showed some reactivity (OD_{490} of 0.57). In contrast, when the wells of a polystyrene plate were pretreated for 15 min with poly(L-lysine) and, after washing, the HCl antigen was added, binding of antigen did occur. This was evidenced by strong reactivity (OD_{490} greater than 1.1 at 1:64 dilutions of sera) with sera of rabbits immunized with either HBSS or HCl antigens (Table 2). The human sera again failed to react.

HBSS antigen did not show activity in Ouchterlony with 20 human sera that were positive serologically by IIF. Similarly, urea antigen and HCl antigen failed to react with 6 and 20 sera, respectively, that were positive serologically in ELISA.

All of the rabbit antisera against *P. carinii* reacted with HBSS, urea, and HCl antigens from infected rat lung in Ouchterlony gel diffusion and immunoelectrophoresis. In addition, in immunoelectrophoresis a cathodic arc due to antibody against rat IgG and a weak anodic arc were formed against normal rat serum. These antibodies were absorbed with glutaraldehyde-insolubilized normal rat globulin (1).

Figure 1 shows the reactivities of absorbed rabbit anti-HBSS and anti-HCl sera against HCl and HBSS antigens. The strong band between the HCl antigen and the anti-HCl serum linked with a strong band between the rabbit anti-HCl serum and HBSS antigen. At least two additional bands were formed between this antiserum and HBSS antigen. The reactions of the rabbit anti-HBSS serum with HBSS and HCl antigens were similar but slightly weaker (Fig. 1). The strong band between the anti-HCl serum and HCl antigen linked with one of a number of bands formed with the urea antigen (Fig. 2). The anti-HCl rabbit serum did not react in gel diffusion with extract of normal rat lung. None of the rabbit anti-*P. carinii* sera reacted in Ouchterlony with 20 sera from proved cases of pneumocystosis.

The HCl antigen did not migrate under the conditions used for immunoelectrophoresis but formed a strong band at the point of application (Fig. 3).

TABLE 2. Activity of HCl antigen from *P. carinii*-infected rat lung in ELISA plates prepared with poly(L-lysine)

Serum	OD_{490} of 1:64 serum dilution on:	
	Untreated plate	Plate prepared with poly(L-lysine)
Human[a]		
1	0.08	0.02
2	0.02	0.17
3	0.01	0.27
4	0.01	0.14
5	0.02	0.00
Rabbit		
Anti-*P. carinii* HBSS serum absorbed with NRG[b]	0.05	1.17
Anti-*P. carinii* HCl I absorbed with NRG	0.20	1.31
Anti-*P. carinii* HCl II absorbed with NRG	0.57	1.38
Normal rabbit	0.02	0.00

[a] The OD_{490} of the 1:64 dilutions of these sera in ELISA with the urea-extracted *P. carinii* antigen ranged from 0.79 to 1.25.

[b] NRG, Normal rat globulin.

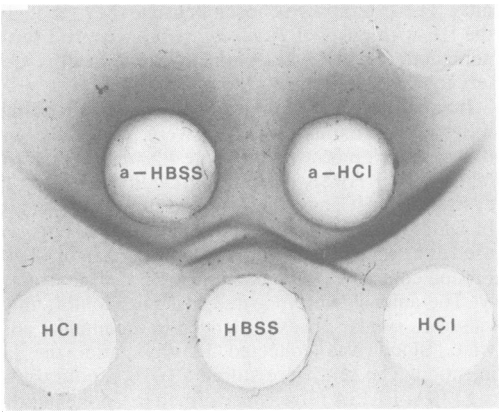

FIG. 1. Activities of *P. carinii* HCl and HBSS antigens with rabbit anti-HBSS and anti-HCl sera after absorption with normal rat globulin.

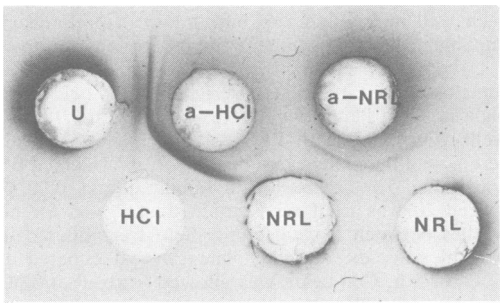

FIG. 2. Activity of *P. carinii* urea (U) and HCl antigens and normal rat lung urea-extracted antigen (NRL) with rabbit anti-*P. carinii* HCl and anti-normal rat lung sera.

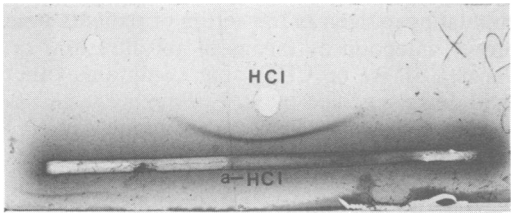

FIG. 3. Immunoelectrophoretic pattern developed with *P. carinii* HCl antigen and rabbit anti-HCl serum.

To further characterize the HCl-soluble antigen, three lots of HCl extracts were treated with trichloroacetic acid. These preparations were no longer active in gel diffusion with the rabbit anti-HCl sera. The HCl antigens did apparently contain some proteins that were measurable by the method of Lowry et al. (8), but almost no bands were detected by silver stain after SDS-PAGE. In Fig. 4 the SDS-PAGE patterns of extracts from infected and uninfected lungs of both humans and rats are compared. Extracts from infected human lung tissues were processed in 8 M urea and isotonic buffer, respectively (Fig. 4, lanes 2 and 5). Both of these fractions contained a significant protein band (>200,000 daltons) (arrow). This band was not found in identically processed extracts from uninfected human lung tissues (Fig. 4, lanes 3 and 4). A similar band of protein of slightly greater molecular weight (dotted-line arrows) was found in extracts from infected rat tissues (Fig. 4, lanes 6 and 7) but not in extracts from healthy rat tissues, even at a much higher protein concentration (Fig. 4, lane 8).

A number of agar-buffer systems for CIE (employing Noble agar or various types of agarose, or Veronal buffers of slightly varied concentrations) resulted in demonstrable reactions between the rabbit anti-*P. carinii* sera and their homologous antigens. However, only the conditions detailed above resulted in the formation of a weak band between a human serum with antigenemia and a rabbit anti-*P. carinii* serum, both of which were kindly supplied as reference reagents by L. L. Pifer. Our rabbit serum prepared against HCl-extracted *P. carinii* and absorbed with normal rat globulin was observed by L. L. Pifer in her laboratory to detect circulating *P. carinii* antigen in human sera. In addition, she identified antigen in one of our sera which we had identified as having antigenemia.

Attempts were made to detect circulating antigens in a reverse ELISA. The gamma-globulin fraction of a rabbit anti-HCl serum was absorbed to microtitration plates. After incubation with patients' sera, enzyme-conjugated anti-human IgG Fc was added. No reactions were observed with any of the sera (data not shown).

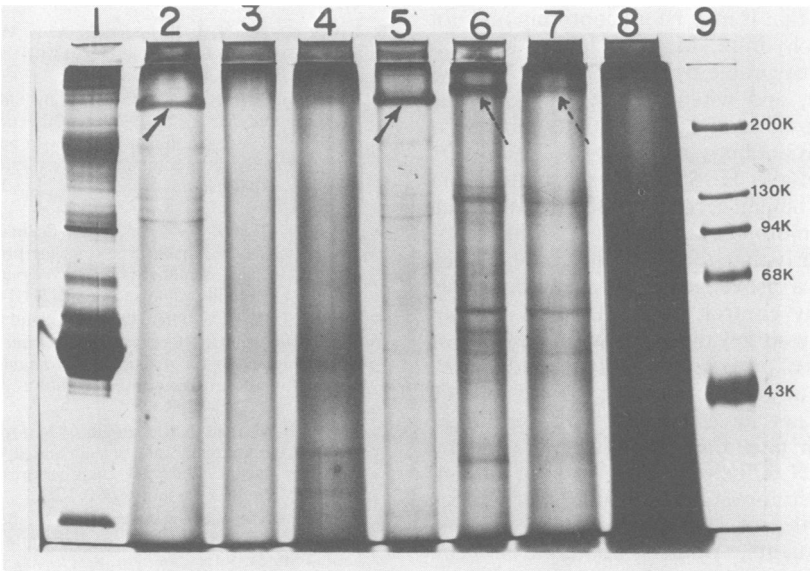

FIG. 4. SDS-PAGE of lung extracts. Electrophoresis of SDS-treated samples was conducted on 8% running gel and 3% stacking gel (see the text). Lane 1, Normal human serum; lane 2, 8 M urea extract of infected human lung; lane 3, 8 M urea extract of uninfected human lung; lane 4, HBSS extract of uninfected human lung; lane 5, HBSS extract of infected human lung; lane 6, HBSS extract of infected rat lung; lane 7, 8 M urea extract of infected rat lung; lane 8, 8 M urea extract of uninfected rat lung; lane 9, molecular weight marker, in descending order: 200K, 130K, 94K, 68K, 43K.

DISCUSSION

The exact yields of *P. carinii* antigenic material were impossible to determine because of the inevitable co-occurrence of proteins of rat lung origin. The various lots of urea antigen had slightly to moderately higher protein content than did the corresponding lots of HBSS antigen.

Urea extraction results in the solubilization of particulate material, possibly including membrane components, which are residual from the HBSS extraction (16). The walls of *P. carinii* cysts are extremely resistant, as shown by their ability to withstand the sulfuric acid-ether treatment in the toluidine blue O stain and resistance to mild sonication (personal observation). The diagnostically important antigens may be associated with the cyst wall, as it is reactive in the IIF assay (13). The higher and more specific reactivity of the urea antigen in ELISA compared with the HBSS antigen is, therefore, not surprising.

Another significant finding in the comparative SDS-PAGE patterns of extracts from both human and rat lung tissue is the presence of a large protein (>200,000 daltons) which is found in infected tissues and in trace amounts, if at all, in uninfected tissues. It is possible that this large-molecular-weight component from infected lung tissues is parasite derived and active in ELISA.

Although no protein band pattern was observed in the SDS-PAGE of the HCl antigen, the data suggest that it may be glycoprotein and not a pure carbohydrate. Material in the HCl extracts was measurable by the technique of Lowry et al. (8), and when the HCl antigen was injected with Freund adjuvant into rabbits, it stimulated an antibody response.

Activity of the HBSS and urea antigens in ELISA with human sera suggested that the urea antigen was more sensitive and could differentiate more clearly than the HBSS antigen between serum from a proved case of pneumocystosis and a healthy control. These antigens did not show activity in gel diffusion with the sera of proved cases of pneumocystosis who had anti-*P. carinii* antibody detectable by IIF. The HCl antigen did not have activity with sera from patients with pneumocystosis when tested by ELISA on gel diffusion. Only the urea antigen was used, therefore, in the evaluation of ELISA for the detection of antibody to *P. carinii* in 461 sera from patients, contacts, and controls. The results of this investigation and of the subsequent study of the specificity of the observed reactions are reported in detail in a companion paper (9).

The only reports of detection of antigenemia in pneumocystosis have been made by L. L. Pifer and her colleagues. Our attempts to detect circulating antigen in the serum of patients with proved infection by means of gel diffusion, reverse ELISA, or CIE using conditions other than those specified by L. L. Pifer were unsuccessful. CIE using our absorbed rabbit anti-HCl sera was therefore evaluated with 430 sera cases of pneumocystosis, contacts, and controls. The data are reported in a companion paper (9).

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