

Pneumocystis carinii Organisms Obtained from Rats, Ferrets, and Mice Are Antigenically Different

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Pneumocystis carinii infections were developed in animals immunosuppressed by dexamethasone treatment either from activation of latent infection (ferret) or by transtracheal inoculation with *P. carinii*-infected lung tissue from the homologous species (rat or mouse). Convalescent-phase antisera were obtained by stopping dexamethasone treatment after 2 to 4 weeks and allowing animals 5 to 8 weeks for recovery. *P. carinii* harvests from infected lungs were purified by differential filtration, solubilized in buffer containing urea, sodium dodecyl sulfate (SDS), and 2-mercaptoethanol, subjected to SDS-polyacrylamide gel electrophoresis, and blotted to polyvinylidene difluoride sheets for Western immunoblot analysis. These lung preparations are hereafter referred to as *P. carinii* antigens. Convalescent-phase antisera from each animal species were reacted on Western blots of *P. carinii* antigens prepared from organisms isolated from rats, ferrets, or mice. Each combination of *P. carinii* antigens and antisera from the same species of animal reacted with three or more *P. carinii* antigen proteins. Convalescent-phase mouse antisera reacted with *P. carinii* antigens from mice but not rats or ferrets. Convalescent-phase rat antisera reacted with *P. carinii* antigens from rats and mice but not ferrets, and convalescent-phase ferret antisera showed reactions with ferret and mouse *P. carinii* antigens but not rat antigens. These findings indicate antigenic differences among *P. carinii* strains infecting these animals.

Pneumocystis carinii is an opportunistic organism thought to be ubiquitous in the environment. *P. carinii* infects many animal species besides humans, including rats, ferrets, and mice. Several lines of evidence suggest *P. carinii* strains infecting each species are different. Intraspecies *P. carinii* strain variations may also exist (6).

Many investigators have studied the antigenic characteristics of *P. carinii* by using hyperimmune antisera or monoclonal antibodies with various immunologic techniques. Gigliotti et al. (9) determined that *P. carinii* isolates from different host species are not antigenically identical by using monoclonal antibodies that were reactive to rat *P. carinii*. Kovacs et al. (11, 12) also used monoclonal antibodies to rat and human *P. carinii* to demonstrate antigenic differences among *P. carinii* isolates. Linke et al. (14) analyzed *P. carinii* antigens for their susceptibilities to treatment with enzymes by immunoblotting and determined that rat and human *P. carinii* have both shared and species-specific antigenic determinants. Roth and Janitschke (15) used immunofluorescence to demonstrate differences among species with *P. carinii* by using hyperimmune sera.

Convalescent-phase or infection-derived antisera developed in both rats and mice (5, 21, 22) have made it possible to compare naturally occurring antibodies among different species of animals infected with *P. carinii* (3). Walzer and Linke (19) showed that rat and human *P. carinii* isolates have both shared and unique antigenic determinants by examining both hyperimmune and convalescent-phase antisera with immunoblots. Also, rat and human antigens have been compared by immunoblotting with convalescent-phase antisera (4, 10). Walzer and Rutledge (20) also described antigenic differences among rat, mouse, and human *P.*

carinii strains by immunofluorescence assay using convalescent-phase antisera from the same species.

Molecular techniques are also being used to determine strain variation in *P. carinii*. Sinclair, Wakefield, and colleagues (16, 18) compared DNA sequences of the large subunit of the mitochondrial rRNA from rat and human-derived *P. carinii*. These sequences suggest that rat and human *P. carinii* isolates are genetically distinct.

In this study, convalescent-phase antisera from rats, ferrets, and mice that do not react with tissue antigens from the homologous species were used to evaluate strain differences among *P. carinii* antigens from these same species by immunoblotting. The results suggest that *P. carinii* isolates from each species are antigenically different.

MATERIALS AND METHODS

Antigen preparation for blots. Female Sprague-Dawley rats free from *P. carinii* were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. The rats were immune suppressed with dexamethasone (Butler Pharmaceuticals, Indianapolis, Ind.) in drinking water at 1.2 mg/liter. Tetracycline (Butler) was also added to the drinking water at 0.5 g/liter to prevent bacterial infections. After 1 week of immune suppression, the rats were transtracheally inoculated with 0.2 ml of *P. carinii* inoculum containing approximately 10⁶ organisms (1). The infection progressed for 3 to 4 weeks, and the animals were then sacrificed by exsanguination and the lungs were removed. The lungs were minced and then homogenized in a Dounce homogenizer (Blaessig Glass). The *P. carinii* organisms were separated from the homogenate by differential filtration using, in sequence, 10-, 5-, and 3- μ m-pore-size filters. The filtrate was centrifuged at 2,000 \times g for 10 min, the supernatant was discarded, and the pellet was resuspended in sterile phosphate-buffered saline (PBS). A 10- μ l sample of this filtrate was placed on a 1-cm etched

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square on a slide, stained with Giemsa blood stain (Harleco), and evaluated by counting trophozoites to determine the number organisms per ml.

The ferrets (Marshall Farms, North Rose, N.Y.) were not *P. carinii* free. They were immune suppressed with dexamethasone (4.8 mg/liter) in drinking water and were given tetracycline (0.5 g/liter of drinking water) to prevent bacterial infections. A latent *P. carinii* infection was evident in the animals within 2 weeks. At this time, the animals were sacrificed by exsanguination, and the lungs were removed. The *P. carinii* organisms were separated from the lung tissue by the filtration process described above. A 10- μ l sample was also evaluated by Giemsa staining as described above.

BALB/c mice from Harlan Sprague-Dawley were determined to be free of *P. carinii* (2). They were immune suppressed for 10 days with dexamethasone (4.8 mg/liter) and tetracycline (0.5 g/liter) in drinking water and then transtracheally inoculated with 50 μ l of mouse *P. carinii* inoculum, which contained 1.5×10^6 organisms. After the infection had progressed for 3 to 4 weeks, the animals were sacrificed by exsanguination, and the lungs were removed. The filtration and evaluation of the mouse lungs were done as described above.

The rat, ferret, and mouse filtrate samples were prepared as antigens for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The volume of each filtrate sample was adjusted so that 5.8×10^4 to 6.0×10^4 trophozoites would be loaded in each sample well. The filtrate was mixed with SDS-urea loading buffer in a 1:1 (vol/vol) ratio and then heated at 95°C for 5 min. The loading buffer consisted of 1 ml of 0.5 M Tris-HCl (pH 6.8), 800 mg of 10% (wt/vol) urea, 1.6 ml of 10% (wt/vol) SDS, 0.4 ml of 2-mercaptoethanol, 0.2 ml of 0.1% (wt/vol) bromophenol blue, and 4 ml of distilled water.

In addition to the *P. carinii* antigens, control lung antigens from immune-suppressed rats and mice that had not been transtracheally inoculated with *P. carinii* and were *P. carinii* free were prepared as described above for infected lungs. Since the ferrets had latent *P. carinii* infections, an uninfected lung antigen preparation could not be obtained. However, a healthy ferret was sacrificed before immune suppression, and its lungs, which were presumed to have a low burden of organisms on the basis of previous examination by Giemsa staining of a lung from a ferret that was not immune suppressed, were processed for reference antigen. Each of these antigens was mixed in a 1:1 (vol/vol) ratio with the loading buffer described above and then heated at 95°C for 5 min.

Development of convalescent-phase antisera. The convalescent-phase antisera used as the first antibodies in the immunoblots were developed in basically the same manner for the rats, ferrets, and mice. The animals were immune suppressed and infected with *P. carinii* as described previously. The rats and mice were used in groups of 10, and the ferrets were used singly. After the infection had progressed for approximately 3 weeks in the rats and mice, one animal in each group was sacrificed, and the level of *P. carinii* infection was evaluated by Giemsa staining. The counting system used was based on the number of organisms in 1,000 \times fields as follows: greater than 100, 5+; 11 to 100, 4+; 1 to 10, 3+; 2 to 9 in 10 fields, 2+; or 1 in 10 or more fields, 1+. The score for no organisms in 50 fields was 0 (1). If the animal had at least a 3+ infection, immunosuppression was stopped for the rest of the group. To assess the degree of infection of a ferret, a bronchoalveolar lavage was performed, and the number of organisms in the lavage fluid was counted as

described above for rats and mice. Ferrets were found to be moderately (3+) infected at 2 weeks. Immune suppression of the ferrets was stopped after 2 weeks. All animals were allowed to recover for 2 weeks, and then the animals were bled at weekly intervals. Once the serum at a 1:100 dilution demonstrated strong immunoreactivity by Western blotting (immunoblotting), the animals were sacrificed by exsanguination, and the serum was saved. The rats mounted an antibody response in about 4 to 5 weeks, as did the mice. The ferrets needed about 8 weeks to mount a response. The sera collected from rats were pooled, and the sera collected from mice were pooled.

Electrophoresis and immunoblotting. For SDS-PAGE (13), a 4% polyacrylamide stacking gel was used with a 10% polyacrylamide running gel. The stacking gel buffer was 1.0 M Tris-HCl (pH 6.8), the running gel buffer was 1.5 M Tris-HCl (pH 8.8), and the electrode buffer was Tris-glycine (pH 8.3; 0.025 M Tris, 0.192 M glycine, and 0.1% SDS; Boehringer Mannheim, Indianapolis, Ind.). The samples were applied to each well in the same volume and concentration each time. The gels were run at 100 V under 42 mA of constant current with a Mini-PROTEAN II Cell (Bio-Rad, Richmond, Calif.). Bromophenol blue in the loading buffer was used as a marker.

After SDS-PAGE, the antigens were transferred to Immobilon-P polyvinylidene difluoride (PVDF) transfer membranes (Millipore, Bedford, Mass.) with a semidry transfer unit (Bio-Rad) (17). The proteins were transferred for 30 min at 20 V; the current was adjusted to the size and number of gels being transferred (length \times width \times 5.5 = milliamperes). Before transfer, the gels were equilibrated in Towbin buffer (25 mM Tris, 192 mM glycine, 20% methanol [pH 8.3]) for 15 to 20 min. Each gel had the antigens loaded in the same order on each half of the gel, and after the transfer the Immobilon-P paper was cut in half. Half of the paper was stained with Coomassie blue protein stain (50% methanol, 0.5 g of Coomassie blue [Sigma, St. Louis, Mo.]), while the other half was used for the Western blot analyses. For the Western blots, the PVDF membrane was blocked in 3% (wt/vol) bovine serum albumin (BSA) in a mixture of PBS and 0.02% sodium azide at room temperature for 1 h. After the PVDF membrane was washed in PBS with 500 μ l of Tween 20 per liter and 0.02% sodium azide three times for 5 min each time, the PVDF membrane was incubated for 1 h at room temperature in the first antibody solution. In each case, the first antibody was rat, ferret, or mouse convalescent-phase antiserum. All antibody solutions were diluted to their final concentration in 0.3% BSA in PBS. After this incubation, the PVDF membrane was washed as described above. The PVDF membrane was incubated with the secondary antibody, which was conjugated with alkaline phosphatase (for rat and mouse antisera) for 1 hour at room temperature. Both anti-rat immunoglobulin G (IgG) and anti-mouse IgG antibodies made in goats were obtained from Sigma. The second antibody to the ferret convalescent-phase antisera is described below. After this incubation, the PVDF membrane was washed twice in PBS as before and washed for 5 min in substrate buffer (10 mM diethanolamine, 0.5 mM MgCl₂ [pH 9.5]). The PVDF was incubated with the substrate, which contained nitroblue tetrazolium (NBT) and bromochloroindolyl phosphate (BCIP), in the dark for 5 to 15 min. The NBT and BCIP were from Bio-Rad.

For immunoblotting, the *P. carinii* antigens from rats, ferrets, and mice were tested simultaneously against each antibody. Convalescent-phase rat antisera were used at a dilution of 1:200, while the convalescent-phase mouse anti-

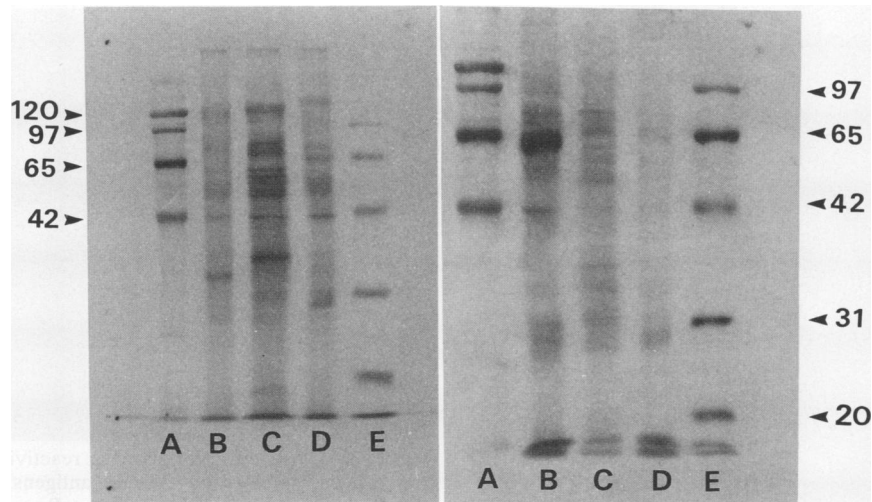


FIG. 1. (Left) Coomassie blue protein stain of *P. carinii* antigens. Lanes A and E, molecular mass markers (in kilodaltons); lane B, rat *P. carinii* antigen preparation; lane C, ferret *P. carinii* antigen preparation; lane D, mouse *P. carinii* antigen preparation. (Right) Coomassie blue protein stain of uninfected antigens. Lanes A and E, molecular weight markers (in kilodaltons); lane B, uninfected-rat antigen preparation; lane C, uninfected-ferret antigen preparation; lane D, uninfected-mouse antigen preparation.

sera and convalescent-phase ferret antisera were used at 1:100 (these dilutions gave optimal reactions). When rat and mouse antisera were used, the second antibodies were those described above. However, there was no commercially available anti-ferret IgG antibody, so a third antibody was used in this blot. The first antibody was the convalescent-phase ferret antiserum. The second antibody was anti-ferret IgG which was made in a rabbit (New Zealand White; Johnson) that was immunized with ferret IgG which had been eluted from a protein A column (Pierce, Rockford, Ill.). The third antibody was goat anti-rabbit IgG (Sigma). Each convalescent-phase antiserum was also tested against the uninfected control antigens prepared as described above.

RESULTS

The half of the PVDF membrane stained with Coomassie blue showed differences in protein staining of *P. carinii* antigens from rats, ferrets, and mice (Fig. 1). A major band at about 120 to 130 kDa can be seen in all *P. carinii* antigen lanes, whereas in the uninfected antigen lanes this band is not present (Fig. 1). Although the molecular weights differ slightly among rat, ferret, and mouse *P. carinii* antigen preparations, they may represent very similar proteins (7). The staining patterns suggest that the higher-molecular-weight proteins are *P. carinii* related, since they are present only in the *P. carinii* antigens and not in antigens prepared from uninfected animals.

The rat *P. carinii* antigen preparation was recognized only by antibodies present in the rat convalescent-phase antisera (Table 1; Fig. 2 to 4, lanes A). These results suggest that the epitopes on the rat *P. carinii* antigen are specific to *P. carinii* from rats, since no cross-reaction was demonstrated. The uninfected-rat antigen had one band that was consistently recognized by rat and mouse convalescent-phase antisera (data not shown). This band (66 kDa) was probably not *P. carinii* related, since it was present in the uninfected antigen.

The ferret *P. carinii* antigen was recognized by the ferret convalescent-phase antisera but not the mouse or rat convalescent-phase antisera (Table 1). Ferret convalescent-phase antisera recognized antigens of 70 kDa or less in the ferret *P. carinii* antigen (Fig. 2 to 4, lanes B). This indicates that the 120-kDa glycoprotein, which is seen in the Coomassie blue stain of the ferret *P. carinii* antigen preparation and not in the normal ferret preparation, was not immunoreactive with this antiserum. Of the uninfected antigen preparations, only the ferret and mouse preparations showed any reaction with the ferret convalescent-phase antisera (data not shown). However, since the ferret had a latent *P. carinii* infection, these reacting bands may still be related to low numbers of organisms present. Control experiments were done to determine whether any of the banding was due to the rabbit anti-ferret or the goat anti-rabbit antibodies used, but no banding was seen when any of the antibodies was left out of the antibody sandwich. However, a clean, uninfected ferret

TABLE 1. Molecular masses of bands seen when *P. carinii* antigens were blotted with convalescent-phase antisera

Species from which antisera were obtained	Strength of reaction ^a of <i>P. carinii</i> antigen of indicated molecular mass (kDa) from indicated species														
	Rat			Ferret				Mouse							
	120	45	40	70	65	45	40	120	100	70	60	50	45	40	25
Rat	++	+++	+	-	-	-	-	+	-	++	-	+	+	-	+
Ferret	-	-	-	++	++	+	+	-	-	-	++	-	+	+	+
Mouse	-	-	-	-	-	-	-	+++	+	-	+	+	+	+	-

^a Scoring system: +, weakly positive; ++, moderately positive; +++, very strongly positive.

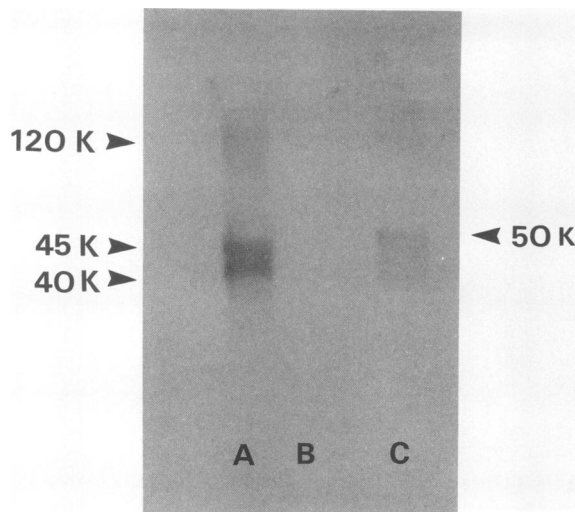


FIG. 2. Western blot showing reactivity of convalescent-phase rat antisera with *P. carinii* antigens. Lane A, rat *P. carinii* antigen preparation; lane B, ferret *P. carinii* antigen preparation; lane C, mouse *P. carinii* antigen preparation. Arrowheads indicate molecular masses in kilodaltons.

lung antigen control was not available, so we used lung tissue from a non-immune-suppressed ferret.

The mouse *P. carinii* antigen was recognized by convalescent-phase antisera from all three species (Table 1; Fig. 2 to 4, lanes C). Rat and ferret convalescent-phase antisera reacted differently to mouse *P. carinii* antigen. The ferret antisera bound to the low-molecular-mass proteins, recognizing bands at 60, 45, 40, and 25 kDa in the mouse antigen (Fig. 3, lane C); by comparison, ferret antisera reacted to 70-, 60-, 45-, and 40-kDa bands from ferret *P. carinii* (Fig. 3, lane B). In contrast, rat antisera bound 120-, 70-, and 50-kDa bands in the mouse *P. carinii* antigen (Fig. 2, lane C), but these antigens were unrecognized by ferret antisera. Both mouse and rat antisera bound to high- and low-molecular-

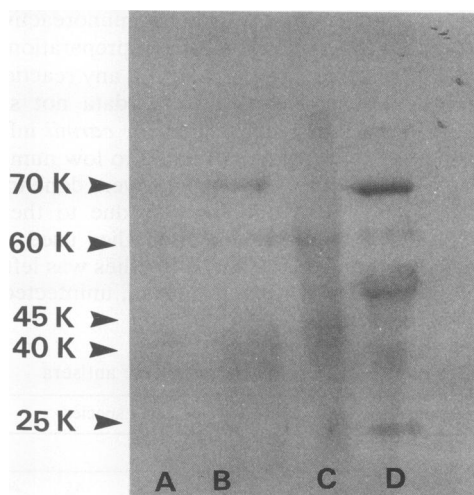


FIG. 3. Western blot showing reactivity of convalescent-phase ferret antisera with *P. carinii* antigens. Lane A, rat *P. carinii* antigen preparation; lane B, ferret *P. carinii* antigen preparation; lane C, mouse *P. carinii* antigen preparation, lane D, molecular mass markers (shown in kilodaltons at left).

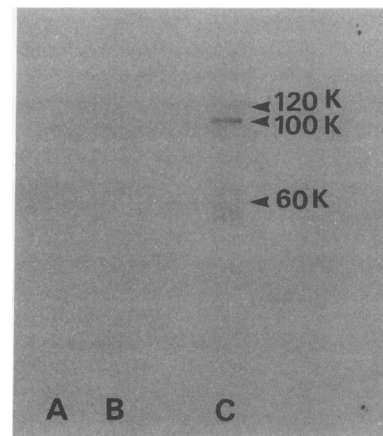


FIG. 4. Western blot showing reactivity of convalescent-phase mouse antisera with *P. carinii* antigens. Lane A, rat *P. carinii* antigen preparation; lane B, ferret *P. carinii* antigen preparation; lane C, mouse *P. carinii* antigen preparation. Arrowheads indicate molecular masses in kilodaltons.

mass proteins. There was no reaction between the normal mouse antigen and convalescent-phase antisera from rats or mice (data not shown). Only the 60-kDa band seen in the reaction with the ferret antisera was seen in both normal and *P. carinii*-infected mouse antigens. Other bands seen with the *P. carinii* mouse antigen were *P. carinii* related.

DISCUSSION

These banding patterns suggest that there are strain differences in *P. carinii* organisms infecting rats, ferrets, and mice. In contrast to findings by Kovacs et al. (11, 12) and Gigliotti et al. (7-9), who used monoclonal or hyperimmune antisera, and Walzer and Linke (19), who used convalescent-phase antisera, the 116- to 120-kDa glycoprotein was not the major band seen. In our studies with convalescent-phase antisera, we have found that sera from approximately 3 of 10 animals were strongly reactive to this glycoprotein, suggesting that in some strains it may not be highly immunogenic. Strains used by other workers probably differed from those we studied. Convalescent-phase antisera from all three species reacted with mouse *P. carinii* antigens, indicating that some antigens of mouse *P. carinii* are related to antigens of ferret *P. carinii* or rat *P. carinii*. However, neither the rat nor the ferret *P. carinii* antigen preparations were recognized by antisera from heterologous species. This suggests that the antigens in rat and ferret *P. carinii* are species specific, unlike the mouse antigens, or that rat and ferret antigens may be similar to the mouse *P. carinii* antigens but their antibody binding sites are blocked.

Gigliotti et al. (8) characterized a ferret *P. carinii* antigen (120 kDa) believed to be a surface glycoprotein and found the carbohydrates on this antigen to be very similar to those found on rat *P. carinii* antigen. If the carbohydrates played a major role in antigenicity, one would have expected to see some cross-reaction between rat and ferret *P. carinii* antigens. However, we did not observe any cross-reactions between the rat and ferret *P. carinii* antigens and antisera, even though many of the same protein bands were evident on the Coomassie blue-stained gel (Fig. 1). These results suggest that even though the carbohydrate compositions of the 120-kDa antigens are similar, the protein portion of the

antigen may be the more immunogenic portion, thus preventing cross-reaction.

Because mouse *P. carinii* antigen has not been characterized to the extent of rat and ferret antigens, it is difficult to determine the relationship of mouse *P. carinii* to rat and ferret *P. carinii*. Both rat and mouse antisera did react as described by Walzer and Rutledge (20). The mouse convalescent-phase antisera showed no reactivity with *P. carinii* from other species, while rat sera showed heterologous reactions with *P. carinii*. Gigliotti (7) reported a common epitope detected with a monoclonal antibody in *P. carinii* from rats, ferrets, and mice. Further analysis of the protein and carbohydrate properties of the major *P. carinii* antigens from rat, ferret, and especially mouse *P. carinii* may help in determining the relationships among rat, ferret, and mouse *P. carinii* antigens.

In summary, this study has demonstrated specific as well as shared antigens from rat, ferret, and mouse *P. carinii* by using convalescent-phase antisera from the same sources. Identification of host-specific antigens shows that there is variability of *P. carinii* infecting some species. Further characterization of these *P. carinii* antigens is needed.

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