Pneumocystis carinii Antigen Detection in Rat Serum and Lung Lavage

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We developed a modified double-antibody sandwich enzyme-linked immunosorbent assay (ELISA) that detected relatively low concentrations of known Pneumocystis carinii antigen added to buffer or rat sera. Artificial immunization-derived polyclonal rabbit anti-P. carinii antibody was used on the solid phase to capture the antigen. Infection-derived (after P. carinii pneumonia) polyclonal rat anti-P. carinii antibody or a mixture of five murine monoclonal antibodies was used as the antigen detector antibody. Rabbit anti-rat immunoglobulin G antibody or goat anti-mouse immunoglobulin G antibody conjugated to alkaline phosphatase was used as the final antibody. After standardization and optimization of the various reactants in this ELISA system, approximately 53 ng of known P. carinii antigen per ml suspended in phosphate-buffered saline–Tween 20 buffer or 210 ng of antigen per ml suspended in normal rat serum diluted 1:4 could be detected. In addition, an indirect ELISA for P. carinii antibody measurement was developed, using as the antigen a soluble supernatant from a sonicated preparation of Percoll-purified whole cysts and trophozoites to coat the solid phase. Limited studies with sera from a small number of caesarian-obtained, barrier-sustained rats from Charles River Breeding Laboratories, Inc., and the National Institutes of Health and sera from normal and heavily infected rats indicated that the caesarian-obtained, barrier-sustained rats had negligible levels of antibody. The normal and heavily infected rats had variable antibody titers. A significantly high level of P. carinii antigenemia was detected in only 2 (11%) of 18 heavily infected rats. Extensive studies of the P. carinii pneumonia rat model with the ELISA did not reveal significant serum P. carinii antigenemia during the acute stage of infection. However, soluble P. carinii antigen was detected by the ELISA and Western blot assays in the supernatant of lavage fluid after centrifugation to sediment intact organisms. As expected, P. carinii antigens were detected by these assays in the lavage pellet recovered after centrifugation. In conclusion, the antigen assay used in this study detected P. carinii antigen in lung lavage but failed to detect P. carinii antigen in rat serum during the acute phase of infection.

In recent years, pneumocystosis has been recognized as a serious, life-threatening infection in immunosuppressed hosts. In a detailed epidemiologic study, Walzer et al. (51) found that the highest rates of Pneumocystis carinii pneumonia occurred in patients with leukemia. High rates also occur in patients with lymphomas, primary immune deficiency diseases, and organ transplants (7) and in bone marrow transplant patients (35). Recent reports (10, 13, 40) emphasize that P. carinii pneumonia has become a significant cause of morbidity and mortality in patients diagnosed with the acquired immune deficiency syndrome.

At present, the most reliable and sensitive diagnostic procedure for P. carinii pneumonia involves microscopic visualization of the organism in lung tissue or aspirate material. This is obtained by open lung or percutaneous biopsy or other invasive procedures that place additional risks on patients who already suffer from pulmonary distress. The measurement of antibody response by indirect fluorescent antibody tests (38, 41) and enzyme-linked immunosorbent assays (ELISAs) (32) as a diagnostic or prognostic tool has not proven of great value.

The detection of circulating antigens is of diagnostic value in certain fungal (34), bacterial (28, 47), Toxoplasma (2, 3, 6, 9, 25, 50), Schistosoma (1, 14, 15, 26, 37), filarial (16, 21, 23, 39), and Toxocara (4) infections. P. carinii antigenemia has been reported by Pifer and co-workers in P. carinii pneumo-

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MATERIALS AND METHODS

Source of rats. Unless otherwise indicated, the rats used in this study were Sprague-Dawley male rats obtained from Sasco, Omaha, Nebr. In one base-line antigenemia-antibody study, two additional sources of Sprague-Dawley rats were used. Caesarian-obtained, barrier-sustained, and virus-antibody-free (COBS/VAF) rats were purchased from Charles River Breeding Laboratories, Inc., Wilmington, Mass. COBS rats were also obtained from the National Institutes of Health (NIH).

Preparation of antigen. A soluble P. carinii antigen preparation, obtained by centrifugation of a sonicated mixture of Percoll-purified whole cysts and trophozoites, was used in the ELISA. To obtain the soluble antigen, minced rat lung from immunosuppressed rats was digested as described by Walzer et al. (53), with some modifications (17). Digestion was carried out in a solution of collagenase, hyaluronidase, DNase, and Hanks balanced salt solution, followed by a series of washes to remove the enzymes. Control antigen from normal rat lung (NRL) was prepared in a similar manner, except that the preparation was not fractionated on a Percoll gradient because too little protein was recovered when this was done. The NRL preparation often contained 10^7 to 10^8 cysts per g of lung, whereas the soluble antigen preparation was derived from immunosuppressed rats and contained 10^4 to 10^5 cysts per g of lung. Trophozoite counts usually were 10-fold higher than cyst counts in the soluble antigen preparations. Cyst and trophozoite counts were done on toluidine blue O- and Giemsa-stained preparations, respectively, as described previously (11, 24).

The lavage source of whole cysts and trophozoites was prepared as previously described (18). This antigenic preparation was used in the Western blotting (immunoblottning) procedures. Lavage specimens were also obtained in a similar manner from selected animals being monitored for serum antigenemia.

Protein assays were performed as previously described (5).

Preparation of antisera. Rabbit serum was obtained from four male New Zealand White rabbits immunized with the Percoll-purified mixture of whole trophozoites and cysts. Preimmune serum also was collected from these rabbits and stored at −20°C. Each rabbit was immunized subcutaneously with 5 × 10^6 whole cysts and trophozoites (not subjected to sonication) emulsified in incomplete Freund adjuvant and boosted 2 weeks later with the same immunogen. A final immunogen boost was given at 4 weeks without the adjuvant. Sera were collected 3 days after the final boost. The immunization-derived rabbit serum was fractionated into the gamma globulin-rich fraction by ammonium sulfate precipitation (8) and stored at −20°C.

The gamma globulin-rich fraction of two immunized rabbits had antibody against rat immunoglobulin G (IgG) as detected by ELISA. To remove this activity, 25 mg of rat IgG (Sigma Chemical Co.) was covalently bound to CNBr-activated Sepharose 4B (Pharmacia Fine Chemicals), and the rabbit gamma globulin was passed over the column twice. Regeneration of the column between absorptions was accomplished as recommended by the manufacturer. This procedure removed all detectable rabbit anti-rat IgG in the gamma globulin as measured by ELISA.

Sera were obtained from three male Sprague-Dawley rats before and after recovery from induced P. carinii pneumonia. Pneumonia was induced by giving corticosteroid therapy (25 mg of cortisone acetate, twice weekly) and low (8%) protein diet ad libitum to 10 rats for 6 to 8 weeks or until the animals had lost approximately 30% of their body weight and had difficulty breathing. Seven of these rats were sacrificed and used for antigen preparation. Lung digests revealed an average of ≈10^7 P. carinii cysts per g of lung in these rats. About 3 months after cessation of corticosteroid therapy and restoration of normal diets, the sera were collected from the remaining three rats. At this point, the three rats were gaining weight and appeared fully recovered. No cysts were seen on stained lung impression smears. The ELISA antigen titer of the rat sera against soluble P. carinii antigen before disease induction was 1:50 to 1:200. Three months after cessation of immunosuppression, the ELISA antigen titer of the pooled sera, hereafter referred to as infection-derived sera, was 1:3,200. All sera were aliquoted and frozen at −20°C.

Rat sera used for antibody and antigenemia measurements. Normal rat sera were collected from healthy 200- to 250-g male Sprague-Dawley rats. The ELISA antibody titer against the soluble P. carinii digest was 1:50 to 1:200. Lung digests from these animals typically revealed 10^7 to 10^9 cysts per g of lung in some but not all of these animals. Sera from rats with P. carinii pneumonia were collected after rats were treated with cortisone acetate and given a low-protein diet for 6 to 8 weeks. Lung digests typically revealed 10^5 to 10^7 cysts per g of lung in rats treated this way. The presence of cysts was also verified by histological examination of lung tissue sections. Sera were also collected from 200- to 250-g male Caesarian-obtained, barrier-sustained (COBS/VAF) and COBS (NIH) (75 to 100-g) male Sprague-Dawley rats immediately upon arrival from Charles River Lakeview, Newfield, N.J., or NIH, respectively. The ELISA for antibody revealed titers of <1:50 to 1:50, but lung digest assays typically revealed 10^7 to 10^8 cysts per g of lung tissue. In a large-scale rat study, serum was collected weekly from rats undergoing various regimens of immunosuppression and recovery from immunosuppression. These sera were stored at −20°C until tested.

ELISA for antibody and antigen. The indirect ELISA for antibody determinations was performed as described previously (18). The modified double-antibody sandwich (MDAS) ELISA for antigen detection was performed with reagents, buffers, and amounts identical to those used for the indirect ELISA (18). The only modifications of rabbit gamma globulin (capture antibody) on the solid phase instead of soluble P. carinii digest or NRL antigen. The sample to be assayed for P. carinii antigen was added and incubated for 2 h at 25°C. The plates were shaken dry, washed four times with phosphate-buffered saline (PBS)-Tween 20 buffer and shaken dry again. The detector antibody was either polyclonal infection-derived rat serum or a mixture of five murine monoclonal antibodies (MABs) (18). Detector antibody was diluted in PBS-Tween 20 buffer, added to each well in a 50-μl volume, and incubated at room temperature in a humid chamber for 2 h. The plates were shaken dry and washed four times in PBS-Tween 20 before the addition of the conjugated antibody against rat or mouse immunoglobulin. Negative control wells included (i) coating buffer instead of rabbit gamma globulin, (ii) PBS-Tween 20 instead of antigen, (iii) PBS-Tween 20 instead of detector antibody, (iv) PBS-Tween 20 instead of conjugated antibody, and (v) normal rat serum instead of detector antibody. These negative control wells were distributed throughout the plates to give the maximum number of negative controls on each plate. Each sample was measured in duplicate, and the zero equilibration for reading absorbance on test samples.
was performed on wells which were coated with gamma globulin and PBS–Tween 20 instead of antigen and then the appropriately diluted detector and conjugated antibody. This procedure eliminated any background activity of the detector and conjugated antibodies. Circulating immune complexes were measured in each sample by elimination of the detector antibody step as outlined by Losonsky et al. (30). In this test, the alkaline phosphatase-labeled anti-rat IgG would bind the Fc portion of the immune complex previously bound by the rabbit antibody, indicating the presence of immune complex formation. The sensitivity level of a given antigen detection assay was defined as the amount of added antigen which gave an absorbance value that was two or more standard deviations greater than the mean of the negative control. This criterion established the 95% confidence interval of statistical significance for observed differences between experimental values.

**Large-scale rat study protocol.** Groups of rats were subjected to different initial durations (2, 4, or 6 weeks) of immunosuppression. A second immunosuppression of 4-week duration was given to each group. Recovery (rest) periods of 9-week duration followed each immunosuppression. The protocol, hopefully, simulated the different levels of immunosuppression and recovery that might occur in human patients. A group of 82 male 200- to 225-g Sprague-Dawley rats were subdivided into four groups (Fig. 1). Groups 1, 2, and 3 consisted of 16 rats each. Group 4 was the control group and consisted of 34 rats. All rats were numbered, and even-numbered and odd-numbered animals were bled on alternate weeks. Rats in group 1 received corticosteroid and a low (8%)–protein diet regimen for 2 weeks, rested for 9 weeks (normal diet with no steroid), received the immunosuppressive regimen again for 4 weeks, rested for 9 weeks, and were sacrificed. Rats in group 2 received the immunosuppressive regimen for 4 weeks, rested for 9 weeks, received the immunosuppressive regimen again for 4 weeks, rested for 9 weeks, and were sacrificed. Rats in group 3 received the immunosuppressive regimen for 6 weeks, rested for 9 weeks, received the immunosuppressive regimen again for 4 weeks, rested for 9 weeks, and were sacrificed. Group 4 control animals were given a normal diet and no immunosuppression. Weight loss or gain was recorded weekly for all animals in the study. Two rats from each group were sacrificed at the beginning of each period of immunosuppression and at the termination of the immunosuppression. The right lung of these rats was fixed for histological studies; the left lung was lavaged lightly (5 ml) with PBS, and the lavage was used in antigen detection studies.

**RESULTS**

**Indirect ELISA standardization.** Soluble *P. carinii* antigen was used to coat the solid phase for antibody measurement. The optimal concentration of the antigen used to coat the solid phase was 2.5 μg of protein per ml with a constant concentration of detector antibody (1:10 dilution of rat infection-derived serum) and constant dilutions (either 1:500, 1:750, or 1:1,000) of conjugate (alkaline phosphatase-linked anti-rat IgG). A 1:750 dilution of conjugate was chosen for routine assays because it gave an absorbance value of approximately 1.0 when used with 1:100 dilution of infection-derived rat serum as the detector antibody. Negative control serum absorbance values were near zero in all determinations.

**MDAS ELISA standardization.** Western blotting analysis was used to determine the major antigenic components in whole digests of *P. carinii* recognized by the artificial immunization-derived rabbit gamma globulin and the infection-derived rat sera. This was done to ascertain that the capture antibody (rabbit immunization-derived antibody) could capture *P. carinii* molecules that subsequently would be recognized by the detector antibody (rat infection-derived antibody). A prominent component at approximately 116 kilodaltons (kDa) (Fig. 2, lanes c and d) was detected by both antibodies. Both antibodies also recognized an antigenic component at approximately 50 to 55 kDa. Absorption studies (data not shown) indicated that antibodies in the rabbit and rat sera were directed against the same antigens. Based on these data, the gamma globulin from rabbits immunized against *P. carinii* whole cysts and trophozoites was used as the capture antibody in the MDAS ELISA. To determine reactivity of the rabbit antibody with soluble *P. carinii* digest or NRL, the solid phase was coated with either antigen. The antigen was reacted with rabbit anti- *P. carinii* serum or gamma globulin or rabbit preimmune serum. Alkaline phosphatase-conjugated goat anti-rabbit IgG was used as the final antibody. Two of our immunized rabbits had no
detectable antibody in an ELISA assay against rat IgG. The other two rabbits, immunized with another lot of whole *P. carinii* antigen, did have antibody against rat IgG. The antibody against rat immunoglobulin was reduced to nondetectable levels after passage through an immunoaffinity column of Sephadex 4B beads coupled to rat IgG as described in Materials and Methods.

To determine the proper concentration of gamma globulin to coat the solid phase, various concentrations of gamma globulin were coated on the solid phase and tested for the ability to capture various concentrations of soluble *P. carinii* digest. A 1:100 dilution of pooled infection-derived rat sera was used as the detector antibody. Pre-*P. carinii* pneumonia sera of the same dilution (1:100) was used as a negative detector antibody control. Rabbit anti-rat IgG conjugate was used as the final antibody. A gamma globulin concentration of 6 ng of protein per ml gave close to maximal absorbance values regardless of antigen concentration. We chose this concentration of rabbit gamma globulin to serve as the capture antibody for coating the solid phase.

A standard curve of reactivity of the soluble antigen diluted in PBS-Tween 20 buffer with the established parameters of the MDAS in place showed that the system detected as little as 53 ng of soluble antigen per ml in PBS-Tween 20 buffer. Since this assay was developed for detecting antigens in rat sera, standard curves of reactivity of the *P. carinii* soluble antigen in undiluted and normal rat sera diluted 1:4 were determined. In undiluted normal rat serum and normal rat serum diluted 1:4, the sensitivities were 300 and 210 ng/ml, respectively.

A pool of five murine MAb's with different epitope specificities (18) were also used as the detector antibody in the MDAS ELISA. The standard curve of reactivity in the MDAS with pre-*P. carinii* pneumonia rat sera and antitrophozoites. Each strip was stained with amido black (lanes e and f) or blotted with infection-derived rat serum (lane d) or immunization-derived rat gamma globulin (lane c). *P. carinii* antigens on nitrocellulose strips in lanes a and b were reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG (lane a) or rabbit anti-rat IgG (lane b) only and served as conjugate controls. Lane f contained molecular size markers.

FIG. 2. Western blotting results of the rabbit gamma globulin and infection-derived conjugated goat preparations containing *P. carinii* whole cysts and trophozoites. Each strip was stained with amido black (lanes e and f) or blotted with infection-derived rat serum (lane d) or immunization-derived rat gamma globulin (lane c). *P. carinii* antigens on nitrocellulose strips in lanes a and b were reacted with alkaline phosphatase-conjugated goat anti-rabbit IgG (lane a) or rabbit anti-rat IgG (lane b) only and served as conjugate controls. Lane f contained molecular size markers.

FIG. 3. Typical standard curve of reactivity in the MDAS ELISA with rabbit anti-*P. carinii* as the capture antibody. The detection antibody was either a pool of five MAbs against *P. carinii* or polyclonal infection-derived rat serum (POST-PCP rat sera). Anti-bovine leukemia virus (POLY-MAbs) MAbs (BLV-MAbs) or sera collected from normal rats (PRE-PCP rat sera) were used as detection antibody controls. Anti-mouse or -rat IgG conjugated to alkaline phosphatase was used as the final antibody.

MAbs and post-*P. carinii* pneumonia rat sera (positive controls) are shown in Fig. 3. The sensitivity of the pooled MAbs as the detector antibody when the soluble *P. carinii* digest was diluted in PBS-Tween 20 buffer was similar to that obtained with the infection-derived sera. However, at higher concentrations of soluble digest the MAb detector mixture was considerably more reactive than the polyclonal rat infection-derived serum.

Base-line antibody and antigenemia measurements. Preliminary experiments were carried out to determine the baseline reactivity of the antibody and antigenemia assays in individual rats. The antibody levels, as measured by the indirect ELISA on 18 male Sprague-Dawley caesarian-obtained, barrier-sustained (COBS/VAF Plus), COBS (NIH) Sprague-Dawley, 18 normal male Sprague-Dawley, and 18 heavily infected male Sprague-Dawley rats (week 9 of immunosuppression), are shown in Fig. 4. Antigenemia levels of these four groups of rats are shown in Fig. 5. Only 2 (11%) of 18 rats in the heavily infected group showed a significantly high level of antigenemia. In this test system 2,000 ng of soluble antigen per ml created, on the average, an *A540* value of 1.55. Lung digests were done on all rats from all four groups to ascertain infection intensity. COBS rats typically had $10^7$ to $10^8$ cysts per g of lung, normal rats had $10^4$ to $10^6$ cysts per g of lung, and rats in the heavily infected group had...
10⁸ to 10⁹ cysts per g of lung. Thus the assay failed to detect consistently significant antigenemia in sera taken from rats at the peak of immunosuppression (9 weeks).

A pilot study conducted before the large-scale rat study also did not reveal antigenemia during various phases of acute disease induction. However, we found antigenemia levels rose after recovery. In this study the antigenemia assays were done on sera from six rats before immunosuppression, during 9 weeks of immunosuppression and during 9 weeks of recovery from immunosuppression. Six control rats were also included as part of the pilot study. The immunosuppression and recovery regimens and sera collection schedules were identical to those described above for the large-scale rat study in Materials and Methods. Since the pilot and large-scale studies gave similar results, only the more extensive large-scale rat study data are described below.

**Large-scale rat study.** The protocol used in the large-scale study is described in Materials and Methods and illustrated in Fig. 1. Serum was collected for antigenemia and antibody assays, as indicated. Rats in groups 1, 2, and 3 lost weight during bouts of immunosuppression and gained weight during recovery periods. The group 4 control rats increased steadily in mean body weight from 240 to 450 g.

The antigenemia patterns of the four groups of rats are shown in Fig. 6. These data represent the mean absorbance in the MDAS ELISA measuring antigenemia in a 1:4 dilution of serum with infection-derived rat serum as the detector antibody. The absorbance levels dropped during both immunosuppression periods and rose during both recovery periods. A similar pattern but with lower absorbance values was seen with the pooled MAb detector system to measure antigenemia in a 1:4 dilution of serum (Fig. 7). Surprisingly, the control rats exhibited a marked rise in absorbance values in serum samples assayed 15 weeks into the study.

The mean reciprocal IgG antibody titers to the soluble *P. carinii* antigen of the control and test rats are shown in Fig. 8. The mean antibody titer of the control rats rose steadily over the entire course of the large-scale rat study. The titers of the test rats tended to remain lower during immunosuppression, followed by a rise in antibody titer during recovery periods. IgM levels (data not shown) remained at the ≤1:50 level throughout the study.

Because the pilot study results suggested the likelihood that no serum antigenemia would be detected, lavage specimens were collected for the antigenemia assay from two rats...

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**FIG. 4.** Reciprocal IgG antibody titers in 18 COBS/VAF, 18 COBS (NIH), 18 normal, and 18 heavily infected Sprague-Dawley rats as measured by the indirect ELISA.

**FIG. 5.** Antigenemia levels (A₄₀₅) of 18 COBS/VAF, 18 COBS (NIH), 18 normal, and 18 heavily infected Sprague-Dawley rats as measured by the MDAS ELISA.

**FIG. 6.** Mean antigenemia levels in the test and control rats in the large-scale rat study. The A₄₀₅ of a 1:4 dilution of serum was measured in the MDAS ELISA with infection-derived rat serum as the detector antibody.

**FIG. 7.** Mean antigenemia levels in the test and control rats of the large-scale rat study. The A₄₀₅ of a 1:4 dilution of serum was measured in the MDAS ELISA with pooled MAbs as the detector antibody.
each before immunosuppression, after 2, 4, and 6 weeks of the initial suppression, after 9 weeks of recovery, after the second (4 weeks) immunosuppression, and after the final 9 weeks of recovery in the large-scale study. Control animal lavages were also done at intervals throughout the study. Light lavage specimens were centrifuged (10,000 × g for 15 min), a portion of the resulting pellet was stained, and the number of cysts was determined (Fig. 1). Cyst counts of the control rats revealed no cysts at 1, 2, and 4 weeks of the large-scale study. In group 1, no cysts were detected after 2 weeks of initial immunosuppression, but 4 weeks after the second immunosuppression, 5.6 × 10⁶ cysts per ml were detected. No cysts were detected at the conclusion of each recovery period. Group 2 rats showed 1.2 × 10⁶ cysts per ml after the first 4 weeks of immunosuppression and 2.5 × 10⁶ cysts per ml after the second immunosuppression. Group 3 rats had 1.9 × 10⁶ cysts per ml after the first 6 weeks of immunosuppression and 2.0 × 10⁶ cysts per ml after the second immunosuppression. No cysts were detected at the conclusion of each recovery period in any of the immunosuppressed rats.

Histological studies (Gormori silver and hematoxylin and eosin stains) revealed no detectable pathology due to P. carinii in rats before the study. After 2 weeks of treatment, group 1 rats showed minor lung changes. After 4 weeks of immunosuppression, group 2 rats showed significant alveolar honeycomb formation with some cysts. Group 3 rats, after 6 weeks of immunosuppression, showed many cysts and significant honeycomb formation. At the end of each recovery period, each group showed no evidence of prior P. carinii pneumonia. The control rats at each sampling period showed no evidence of P. carinii pneumonia as measured histologically.

The antigen detection ELISA, with pooled MAbS as the detector, revealed significant absorbance activity in lavage specimens collected during the acute phase of P. carinii infection. Fresh lavage specimens were centrifuged at high speed (10,000 × g for 15 min) to sediment all trophozoites and cysts. Antigen could be detected by the MDAS ELISA with pooled MABs as the detector in the lavage supernatant (free of P. carinii organisms) and in the pellet that contained the intact organisms. The antigen activity was still present in the pellet after it was washed to remove vestiges of supernatant. These data strongly suggested that P. carinii antigen could be detected in the lavage specimen either as soluble antigen or in the whole organisms. However, there was considerable variability in absorbance values (antigenemia) from different rats, and no correlation could be made between length of immunosuppression or number of cysts and the absorbance value. In retrospect we believe the light lavage resulted in considerable variability in the quality of the samples recovered from the lungs. Also, the focus on monitoring by cyst counts ignored the likely changes in trophozoite populations during immunosuppression and recovery phases.

To confirm the presence of antigen in the lavage supernatant and pellet, lavage collected from three additional rats immunosuppressed for 6 weeks were analyzed by Western blotting. After centrifugation at 2,000 × g for 10 min, the lavage pellet was washed twice at 2,000 × g for 10 min and suspended in buffer. A portion of the initial supernatant was centrifuged a second time at 10,000 × g for 15 min, and the supernatant was recovered for analysis. Three control rats taken at week 28, a time when no organisms were detected in the lavage (Fig. 1), were similarly processed. The washed pellet and high-speed centrifuged supernatant from immunosuppressed and control rats were subjected to Western blot analysis as previously described. Pooled MABs were used as the detector antibody, and comparison was made with whole P. carinii digest antigen, which contains known P. carinii molecules that have been described and characterized earlier (19). The lavage supernatant, lavage pellet, and whole P. carinii digest antigens (Fig. 9, lanes E, F, and G, respectively) showed similar antigen banding patterns. The major antigen bands at 110 to 116 kDa and 50 to 55 kDa can be
seen. Additional bands are seen in the lavage supernatant in the 70-kDa and 90-kDa range that are also seen in the whole *P. carinii* digest. Lanes A and B contained conjugate controls (detector antibody omitted) for lavage supernatant and lavage pellet, respectively, from the immunosuppressed animals. Lane C and D contained negative controls for lavage supernatant and lavage pellet, respectively, harvested from normal rats (week 28). A faint 66-kDa band was detected in the lavage supernatant controls (lane A and C), which suggests the band was nonspecific for *P. carinii*.

**DISCUSSION**

This study showed considerable overlap in ELISA antibody titers between normal and heavily infected rats (Fig. 4). The COBS rat antibody titers were consistently clustered at the negative or nonsignificant level of antibody. However, the COBS rats used were relatively young (2 to 4 weeks old) compared with heavily infected rats (16 weeks old). In the large-scale study the antibody response against *P. carinii* increased with age (Fig. 8). We observed some increase in specific anti-*P. carinii* IgG antibody in the heavily infected rats, but there was considerable overlap between titers of normal and infected rat sera. In unpublished studies, we found that antibody titers tended to remain low during prolonged (8 to 9 weeks) immunosuppression but rose when the animals were allowed to recover for 2 to 3 months. Walzer et al. (52) observed an increase in serum IgG antibody titers with steroid tapering and clearance of the organism from the lungs, but suppression of IgG was observed during induction of *P. carinii* infection. These results indicate that antibody responses are not reliable indicators of *P. carinii* pneumonia status. Other workers (31, 32, 38, 41) have made similar conclusions.

The Western blot results of our studies (Fig. 2) showed that the immunization-derived rabbit gamma globulin detected major *P. carinii* antigenic components also recognized by the infection-derived rat antibody. Absorption studies indicated the rabbit and rat infection-derived antiserum against *P. carinii* contained antibody or antibodies against common epitopes. There were other common antigenic bands detected by the rat infection- and rabbit immunization-derived polyclonal antibodies other than the 116 and 50 to 55-kDa bands. The band at approximately 170 kDa appeared common to the two sources of antibodies. There also appeared to be some minor unique bands. However, these bands were not always present, whereas the 116- and 50- to 55-kDa bands were consistently present. Thus we concluded they were the dominant *P. carinii*-associated bands (19).

Based on these characteristics of the rabbit and rat antiserum, we chose to use the rabbit antiserum as a capture antigen because it had higher titer against *P. carinii*; most importantly, we had a sufficient quantity of it to use throughout the study. Also, preliminary studies (unpublished) indicated that when infection-derived rat antibody was used as the capture antibody there was no increase in the sensitivity of the system. We decided to use infection-derived rat antibody against *P. carinii* as the antigen detector antibody based on the rationale that antibody produced during recovery from clinical *P. carinii* pneumonia would be relevant to the infection-disease recovery process and that rat sera did not have the anti-rat antibody. When known soluble *P. carinii* digest antigen was diluted in PBS–Tween 20 buffer, the system detected as little as 53 ng of antigen per ml (Fig. 3). Slightly less (210 ng/ml) sensitivity was achieved when the antigen was placed in 1:4 normal rat serum (Fig. 3).

Using a much purer antigen preparation, Meckstroth et al. (34) detected 38 ng of *Candida albicans* antigen per ml of serum. *Schistosoma japonicum* antigen has been detected at 100-ng/ml concentrations in serum (44). Other parasitic antigen detection ELISA systems can detect as low as 31 ng of *Toxoplasma* antigen per ml (9) and 125 ng of *Schistosoma* antigen per ml (15). Thus, the sensitivity of our MDAS ELISA approached these values.

The loss of sensitivity that occurred when antigen was placed in serum (Fig. 3) could reflect problems associated with immune complexes or the interfering properties of complement and other serum proteins. Specific interference and steric interference or poor affinity of the detector antibody could reduce sensitivity. Sensitivity problems of these types have been reported by Yolken (54) with ELISAs. In our study, internal controls (described in Materials and Methods) were monitored for the presence of *P. carinii*-immunocomplexes. There was no evidence for an increase in the level of immune complexes at any point in the study.

*P. carinii* antigenemia has been detected by CIE in humans (35, 41, 46) and rats (42). Recently Jarowenko et al. (27) reported that a latex particle agglutination test could be used to successfully detect *P. carinii* antigenemia. However, other workers have reported low sensitivity (33) or failure to detect antigenemia (22, 36). In our preliminary base-line study (Fig. 5) the MDAS ELISA detected antigenemia in only 2 of 18 heavily immunosuppressed rats with obvious *P. carinii* pneumonia. To clarify these discrepancies we monitored temporally the *P. carinii* antibody and antigenemia patterns before and during induction and during recovery from steroid-induced *P. carinii* pneumonia in the rat model with hopes that additional information would be obtained that shed light on the significance of any antigenemia patterns which might occur. A pilot study (data not shown) involving six immunosuppressed and six normal control rats showed no detectable antigenemia during the acute phase of disease, but apparently antigenemia did rise during recovery. The more extensive large-scale study confirmed our pilot study observations that antigenemia was not detectable during acute disease but seemed to rise during recovery periods (Fig. 6 and 7). However, the control rats (given no immunosuppression) in the large-scale study also exhibited a rise in antigenemia during the same time period. The fact that antigenemia rose in a similar manner in both experiments suggests that the observation was real. However, except for the similarity in aging and ages of the animals, we have no satisfactory explanation for significance of the observation.

The inability to detect *P. carinii* antigen in rat serum may be due to a defective test system. However, the ability of the system to detect relatively minute quantities of known *P. carinii* antigen suggests the test was functional and sensitive. Also, the system detected *P. carinii* antigens in lavage fluid collected from rats with clinical pneumocystosis (see below). The absence of *P. carinii* antigen in rat serum could be due to the inaccessibility of this pulmonary pathogen to the bloodstream (sequestered because of the tight adherence of organism to the host type I pneumocytes) or the lack of *P. carinii* virulence factors interfering against phagocytosis by host monocytes. Recently, other workers (13, 20) reported dissemination of *P. carinii* to multiple organ systems in humans; this supports the concept of lymphatic and hematogenous routes of spreading. However, dissemination seems to be a rare event and apparently does not occur regularly. To date, serum antigenemia has been reported primarily in infections where the location of the pathogen in

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**PNEUMOCYSTIS CARINII ANTIGEN DETECTION**

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the body provides easy access of the pathogen or its antigens to the general circulation. The MDAS ELISA and Western blot with pooled MAbs as the detector detected *P. carinii* antigen in the lavage fluid collected during acute *P. carinii* pneumonia. Antigen also was detected in the pellet recovered after centrifugation. This was not surprising, because *P. carinii* cysts could also be demonstrated after smears of the pellet sediment were stained with toluidine blue O. However, the absorbance values (indicator of antigen concentration) were variable and did not correlate well with the number of cysts in the pellet. A number of factors might have contributed to lack of correlation. They include (i) the ability or inability of the organisms to remain tightly bound to capture antibody during subsequent washes in the MDAS ELISA procedure, (ii) significant variability in the trophozoite/cyst ratio, (iii) the possibility of steric hindrance in a system that captures intact organisms, and (iv) the possibility that the organisms or soluble *P. carinii* digest had already reacted in vivo with host antibody against *P. carinii* and had fewer reactive epitopes available for reaction with the capture antibody used in the MDAS ELISA. In retrospect, more consistent results may have been obtained if the lavage fluid had been sonicated sufficiently to solubilize the *P. carinii* before performing the antigenemia assay.

Of great interest was the detection of soluble *P. carinii* antigen in the lavage supernatant fluid after removal of organisms via centrifugation (Fig. 9). If *P. carinii* antigens can be demonstrated in lavage fluids that contain few, if any, cysts, then a useful diagnostic tool might be developed for diagnosing pneumocytosis before the acute phase of the disease. Further studies are necessary to confirm the specificities of the banding reactions found at 66 and 90 kDa. Although diagnostic techniques that require invasion of the lung are not desirable, the current controversy about the reliability of serum antigenemia assays demands further exploration of assays that use specimens collected from the lung. Further studies are underway to clarify factors affecting the antigenemia assay and characterize the kinetics of *P. carinii* antigens in lavage fluid and their relationship to the disease process in rats.

**LITERATURE CITED**


