Identification of Extrapulmonary *Pneumocystis carinii* in Immunocompromised Rats by PCR

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Pneumocystis carinii has been shown to cause extra-alveolar infections in humans, but the lack of a reproducible animal model has hindered the elucidation of mechanisms of *P. carinii* dissemination. In the present study, PCR and the immunosuppressed rat model were used to gain further insight into the dissemination of *P. carinii* organisms in extrapulmonary (EP) tissues. Primer sequences specific to major surface glycoprotein (MSG) and dihydrofolate reductase (DHFR) were used to detect *P. carinii* in lungs and EP tissues. Sprague-Dawley rats were grouped into two classes: one group included rats that had primary episodes of pneumocystosis and the other group included rats that had undergone treatment for *P. carinii* infection and that had second episodes of pneumocystosis. PCR analysis with MSG primers with tissues obtained from both groups of rats showed the presence of *P. carinii* DNA in adrenal tissue, bone marrow, blood, and heart, kidney, liver, lymph node, spleen, and thyroid tissues. Reverse transcriptase-PCR (RT-PCR) analysis was carried out with DHFR primers with lung, spleen, heart, kidney, and liver tissues from both groups of rats. Only those tissues that showed a positive PCR result and hybridization signal for the MSG gene were used for the RT-PCR experiments. RT-PCR analysis showed that the *P. carinii* DHFR gene is actively transcribed in these tissues, thereby indicating the presence of viable *P. carinii* organisms in EP tissues. Our observations suggest that *P. carinii* dissemination is influenced by factors other than *P. carinii* chemotherapy and that heavy organism load and destruction of lung tissue may contribute to the dissemination of *P. carinii*. The study provides an animal model that can be used for further investigations of the causes of EP pneumocystosis.

Pneumocystis carinii is a frequent opportunistic fungal pathogen which causes pneumonia in patients with immunosuppressive disorders (9). The rate of *P. carinii* infections has risen dramatically over the past several years with the advent of immunosuppressive therapies and infection with human immunodeficiency virus (HIV) (26, 36). Although *P. carinii* is believed to be an extracellular pathogen found mostly in the alveolar spaces, there is a growing body of evidence showing the presence of *P. carinii* in extrapulmonary (EP) tissues in humans (5, 8, 12, 18, 19, 30, 31, 33). Currently, the presence of *P. carinii* organisms in EP tissues is often described only at postmortem examination. Despite an increase in the incidence of EP *P. carinii* infection in HIV-positive patients, the true prevalence of EP infection among patients has not been determined, nor has the impact of these infections on the response to antipneumocystis treatments been defined (30). There is a definite need for further studies in this area in order to get a better understanding of the mechanisms of *P. carinii* dissemination.

In the present study, we used PCR and the immunosuppressed rat model to gain further insight into *P. carinii* dissemination in EP tissues. In one of our previous studies (16), the genome of a clone, clone B12.2, was shown to be highly repetitive and specific in hybridizing only to the genome of *P. carinii* organisms from rats but not to those of other pulmonary pathogens. Nucleotide sequence analysis revealed that the genome of this clone has homology with major surface glycoprotein (MSG) genes of *P. carinii* organisms from rats (15, 35). A pair of oligonucleotide primers were designed from the ge-

nome of clone B12.2. These primers specifically amplified an expected 560-bp region of the MSG gene of *P. carinii* organisms from rats. The detection limit of the PCR assay was approximately 10 *P. carinii* organisms. Using the sensitive PCR assay, we identified *P. carinii* DNA in EP tissues of rats with primary episodes of *P. carinii* infection as well as in rats that had undergone *P. carinii* chemotherapy for primary episode of pneumocystosis and that had recurrent *P. carinii* infections. Reverse transcriptase (RT)-PCR (RT-PCR) analyses of cDNA prepared from total RNA isolated from EP tissues of infected rats showed that the dihydrofolate reductase (DHFR) gene is actively transcribed in the EP tissues tested. Our observations suggest that factors other than *P. carinii* chemotherapy play a role in extra-alveolar tissue invasion by *P. carinii* isolates. It is possible that *P. carinii* organisms gain access to EP organs only after large numbers of the organisms are found in the lung and sufficient destruction of the lung tissue has occurred. To our knowledge, this is the first study to successfully use PCR to identify *P. carinii* organisms in EP tissues other than blood in rats and to provide some insights into the dissemination of *P. carinii* in the immunosuppressed rat model.

MATERIALS AND METHODS

Induction of *P. carinii* pneumonitis. A rat model similar to that originally described by Frenkel et al. (13) and Hughes et al. (14) was used. Briefly, viral antibody-positive male Sprague-Dawley rats weighting 225 to 250 g were obtained from Sasco Laboratories (Madison, Wis.). After 7 days of acclimation, all of the rats were immunosuppressed by injection of methylprednisolone acetate (Depomedrol; The Upjohn Co., Kalamazoo, Michigan) at 4 mg/week for 8 to 16 weeks (6). To exacerbate *P. carinii* pneumonia, malnutrition was induced by administration of a low-protein (8%) diet (ICN Biochemicals, Inc., Cleveland, Ohio). Drinking water was supplemented with tetracycline (1 mg/ml) to suppress secondary bacterial infections.

Ten rats were sacrificed at the end of 8 weeks of immunosuppression, a time when there was considerable weight loss, hair loss, crusty nose, and heavy breathing, all of which were suggestive of pneumocystosis. These animals, designated

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group I, were used to determine the presence of *P. carinii* DNA in lung and EP tissues. In addition, four rats each were sacrificed at 0, 2, 4, 6, and 8 weeks of immunosuppression to monitor the time course of *P. carinii* dissemination.

In another series of experiments, 12 rats were immunosuppressed for 8 weeks and were then treated with trimethoprim (TMP) and sulfamethoxazole (SMZ) (50 mg of TMP and 250 mg of SMZ per kg of body weight; Sigma, St. Louis, Mo.), which were administered continuously in the drinking water for 3 weeks. At the end of the treatment regimen, two rats were sacrificed to monitor the level of *P. carinii* infection in their lungs. The remaining rats were immunosuppressed for an additional 8 weeks. Only 5 of the 10 animals survived the second immunosuppression regimen. The surviving rats were sacrificed to test for the presence of *P. carinii* organisms in lung and EP tissues and made up group II.

Rat tissues. At various time intervals as described above, infected rats and four viral antibody-negative, *P. carinii*-free rats (area R10; Charles River Laboratories, Raleigh, N.C.) were anesthetized by using a combination of ketamine (ketamine hydrochloride injection USP; 100 mg/ml; Aveco Co., Inc., Fort Dodge, Iowa) and xylazine (20 mg/ml; Mobay Corp., Shawnee, Kans.). The chest cavity was opened, and blood was collected by cardiac puncture. Portions of lung, heart, spleen, liver, and kidney tissues were collected on dry ice, and the portions were immediately stored at -70°C for isolation of RNA from rats belonging to groups I and II. The remaining portions of lung, heart, spleen, liver, and kidney tissues along with adrenal, mesenteric lymph node, and thyroid tissues were rinsed thoroughly in phosphate-buffered saline (PBS) until no blood was visually detected, and tissues were collected in PBS for DNA isolation. Bone marrow was collected from femur bones by cutting off the ends of the bones and flushing PBS through the bones several times. Extreme care was taken to avoid cross contamination between tissues at the time of sample collection by using separate pairs of scissors and forceps to cut out each organ. *P. carinii* organisms were isolated from infected lung tissues as described previously (2). In brief, lung homogenates were washed once with 10 ml of saline by centrifugation at $300 \times g$ for 5 min. The supernatant was collected and the erythrocytes were lysed by resuspending them in 10 ml of 0.85% ammonium chloride for 5 min at 37°C . The samples were then washed two additional times with saline and filtered through 10 and 5 μm -pore-size filters. A 5- μl aliquot was taken from each sample and was dried on a microscope slide. The numbers of cysts per animal were determined from preparations stained with toluidine blue, and the numbers of trophozoites were determined from Diff Quik-stained preparations (16). In the rats used in the present study, cysts and trophozoites were present at a ratio of approximately 1 to 10. All *P. carinii* preparations were streaked onto both blood agar and Sabouraud dextrose agar plates to check for bacterial and fungal contaminants. Any sample containing detectable contaminants was not used for the present study.

DNA isolation. DNA was isolated from lungs and EP tissues of *P. carinii*-free rats and *P. carinii*-infected rats. Extreme care was taken to avoid contamination of the EP tissues with material from infected lung tissues during DNA isolation by processing the EP tissues at times different from the times at which lung tissues were processed and by using fresh materials, solutions, and pipettes at every step. The EP tissues were rinsed thoroughly in PBS to get rid of as much blood as possible. Approximately 1 g of tissue was homogenized completely in 10 ml of PBS and was centrifuged at $1,700 \times g$ for 10 min, and the supernatant was discarded. The pellet was resuspended in 5 ml of buffer containing 10 mM Tris (pH 8.0), 20 mM EDTA, and 140 mM NaCl. Tissue homogenates were incubated at 37°C for 30 min in 10% sodium dodecyl sulfate and 50 mg of proteinase K (Sigma) per ml; this was followed by 1 h of incubation at 55°C . RNA was degraded with 5 μl of 20 mg of RNase (Boehringer Mannheim, Indianapolis, Ind.) per ml at 37°C for 90 min. The samples were then subjected to phenol-chloroform extraction. The resulting DNA was precipitated with ethanol and was dissolved in $0.1 \times$ Tris-EDTA (TE) buffer. To isolate DNA from heparinized blood, erythrocytes were lysed with 0.85% ammonium chloride (pH 7.5) for 5 min at 37°C , and the preparation was centrifuged at $1,700 \times g$ for 10 min. The resulting cell pellet was used to isolate DNA.

RNA isolation and cDNA synthesis. Total cellular RNA was isolated from the lungs of *P. carinii*-free rats and from the lungs, livers, hearts, kidneys, and spleens of rats in groups I and II by the protocol of Chomczynski and Sacchi (4). The tissues were ground exhaustively with a cold mortar and pestle. Total cellular RNA was isolated by phenol-chloroform extraction and was incubated with 1 U of RNase-free DNase (Stratagene, La Jolla, Calif.) per μg for 30 min at 37°C to remove contaminating DNA.

For cDNA synthesis, 3 μg of total cellular RNA was incubated at 42°C for 1 h in a total volume of 20 μl of a reaction mixture containing 50 mM Tris (pH 8.3), 75 mM KCl, 3 mM MgCl_2 , 0.4 μg of random hexamers (Gibco, Gaithersburg, Md.), 10 mM (each) deoxynucleotide triphosphates, 20 U of placental RNase inhibitor (Gibco), and 200 U of Moloney murine leukemia virus RT (Gibco). The samples were then heated at 94°C for 5 min, diluted to a final volume of 100 μl with 0.1% diethylpyrocarbonate-treated water, and stored at -70°C .

PCR and RT-PCR. Standard protocols to minimize DNA contamination during PCR and RT-PCR were followed, including the use of negative controls and the handling of DNA standards in a separate workplace (10). A standard PCR included DNA from the lung of an infected rat as a positive control and normal DNA from the lung of a healthy, *P. carinii*-free rat as a negative control. For RT-PCR, negative control reactions included one with cDNA from a *P. carinii*-free rat lung, one with a sample that had not undergone reverse transcription, and one from which the nucleic acid template was omitted in order to confirm

that the PCR products were not due to contamination. To assess the integrity of the cDNA, primers for the β -actin gene were used. PCR was performed in a 20- μl volume of a reaction mixture containing 10 ng of DNA or 15 μl of cDNA (RT-PCR), 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl_2 , 10 mM (each) the deoxynucleotide triphosphates, and 1.25 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). Ten picomoles of oligonucleotide primers for DNA amplification was used. The sense primer was primer P5 (5'-CTCAAGTTCTGCACACTCTCC-3') and the antisense primer was primer P6 (5'-TGCAAGGACTTTGAGGAGAA-3'). For cDNA amplification DHFR (25) and β -actin (32) primers were used. The cycling parameters for P5-P6 primers included 35 cycles of denaturation at 94°C for 1 min, annealing at 65°C for 1 min and 30 s, and extension at 72°C for 2 min. The annealing temperatures for the DHFR and β -actin primers were 55 and 60°C , respectively. Twenty-microliter volumes of the amplification reactions were analyzed by electrophoresis on 1.4% agarose gels containing 1 μg of ethidium bromide per ml, and the products were sized by comparison with *EcoRI*-*HindIII*-digested bacteriophage lambda DNA or ϕ X174 DNA digested with *HaeIII* molecular weight markers. The gels were blotted, and PCR amplicons were transferred to Zeta-Probe GT nylon membranes (Bio-Rad, Richmond, Calif.) (28).

For testing the sensitivity of the PCR assay with the P5 and P6 primers, liver homogenate from a *P. carinii*-free rat was seeded with homogenates of infected rat lungs with organism load equivalents of 0, 10, 20, 40, 80, 150, 300, 600, 1,500, and 3,000. DNA was isolated from the different dilutions, and the final pellet was resuspended in 5 μl of $0.1 \times$ TE buffer. PCR was performed on each preparation as described above.

Probe. The amplicons obtained by using primer pair P5-P6 and DHFR from a heavily infected rat lung were recovered from the agarose gels by electroelution (22) and were used as probes in hybridization experiments. The amplicons were labelled by using random prime labelling (Boehringer Mannheim) with [α - ^{32}P]dCTP (3,000 Ci/mmol) and were purified by filtration on a G-50 Sephadex column (Pharmacia, Uppsala, Sweden). Membranes containing the transferred DNA were then hybridized with the appropriate ^{32}P -labelled probe. Membranes were prehybridized for 3 h in a hybridization buffer containing $6 \times$ SSC ($1 \times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 45% (vol/vol) deionized formamide, 100 mg of herring sperm DNA per ml, and 0.5% sodium dodecyl sulfate. They were then hybridized with the ^{32}P -labelled *P. carinii* probe overnight at 42°C in a hybridization incubator. The membranes were washed by treating them twice with $5 \times$ SSC-0.1% sodium dodecyl sulfate for 20 min at 42°C and twice in $0.5 \times$ SSC-0.1% sodium dodecyl sulfate for 30 to 60 min at 42°C . The membranes were exposed to Kodak X-Omat LS film with two intensifying screens at -70°C for 17 h.

RESULTS

Sensitivity of the PCR assay. Primers P5 and P6 designed from genomic clone B12.2 were specific in amplifying a 560-bp region of the MSG gene of *P. carinii* organisms from rats. The sensitivity of the PCR assay was tested quantitatively by seeding homogenates of *P. carinii*-free rat liver with known quantities of *P. carinii* nuclei (cysts and trophozoites). As shown in Fig. 1, samples containing as few as 10 *P. carinii* organisms per PCR mixture efficiently amplified a 560-bp product, demonstrating the high degree of sensitivity of the assay. The ability of primers P5-P6 to detect minute quantities of *P. carinii* DNA provides a means of identifying the presence of *P. carinii* DNA in EP tissues of infected rats.

Detection of *P. carinii* DNA in rats with primary pneumocystosis. In the first series of experiments, DNA isolated from the lungs of 10 rats with primary episodes of *P. carinii* infection were subjected to the PCR assay with the P5 and P6 primers. These rats had pulmonary infection with *P. carinii*, as demonstrated by conventional staining of the *P. carinii* organisms isolated from the lungs (range, 2.8×10^7 to 3.6×10^8 *P. carinii* nuclei per lung) and a positive PCR amplification of infected lung DNA. EP tissues from these rats were tested for the presence of *P. carinii* organisms by PCR. The identities of the PCR amplicons from lung and EP tissues were confirmed by Southern hybridization of the gel blots by using the 560-bp MSG gene of *P. carinii* from rats as a probe. The distributions of *P. carinii* organisms in EP sites of the rats with primary episodes of pneumocystosis (group I) are detailed in Table 1. The data in Table 1 indicate that *P. carinii* DNA was detected in adrenal, heart, kidney, liver, lymph node, spleen, and thyroid tissues and in bone marrow and blood. In this group of rats, the

FIG. 1. Detection limits of *P. carinii* DNA by the PCR and hybridization assay. (A) Ethidium bromide-stained gel. The number of organisms in an enriched preparation of *P. carinii* organisms isolated from the lungs of an immunosuppressed rat was quantitated histochemically by counting the sample repeated times. Homogenates of normal rat liver were seeded with dilutions of *P. carinii* containing 0, 10, 20, 40, 80, 150, 300, 1,500, and 3,000 organisms, and then the genomic DNA was isolated and used in PCRs (lanes c to k, respectively). The negative control sample included 10 ng of rat liver genomic DNA (lane b), and the positive control sample was 10 ng of genomic DNA isolated from the lungs of a heavily infected rat (lane l). Lane a, ϕ X174-*Hae*III marker. The PCR products were run on a gel and were subsequently transferred to a nylon membrane for hybridization. (B) Southern hybridization of the gel in panel A by using the 32 P-labelled 560-bp MSG sequence of *P. carinii* organisms from rats as a probe.

most common EP site of *P. carinii* infection was the lymph node. No *P. carinii* DNA was found in the lungs of *P. carinii*-free rats immunosuppressed for up to 8 weeks ($n = 2$).

Detection of *P. carinii* DNA in rats with recurrent pneumocystosis. It was of interest to determine if multiple episodes (two episodes in this case) of pneumocystosis facilitate the dissemination of *P. carinii* organisms to EP tissues. Since antibiotic therapy with TMP-SMZ drastically reduces the number of organisms (27) and remains the mainstay of therapy for the treatment of human pneumocystosis (14), 12 rats with established primary pneumocystosis were treated with TMP-SMZ (50 mg of TMP and 250 mg of SMZ) for 3 weeks. At the end of 3 weeks, no *P. carinii* organisms were detected in the lungs or EP tissues by microscopic visualization of stained preparations ($n = 2$). By PCR, no *P. carinii* DNA was detected in EP tissues other than lungs, in which the intensity was greatly

reduced following treatment. Only 5 of the 10 rats immunosuppressed for an additional 8 weeks following treatment survived. The majority of the tissues tested from the animals that survived this recurrent infection showed positive amplification and hybridization signals for *P. carinii* DNA (Table 1, group II). Because of the difficulty in obtaining lymph node tissues, they were not studied for this group of rats. A representative gel electrophoresis profile of amplified *P. carinii* DNA from EP organs of a rat that had a second episode of pneumocystosis is shown in Fig. 2. Even though we used equal amounts of DNA in the PCR, some EP tissues showed greater signals (Fig. 2, lane g). The reason for this is not known, but it may mean that there was a larger amount of *P. carinii* DNA in that preparation.

RT-PCR of *P. carinii* cDNA in rats with primary and recurrent pneumocystosis. To test whether circulating *P. carinii* DNA in EP tissues represents viable *P. carinii* organisms, RT-PCR of heart, kidney, lung, spleen, and liver cDNA obtained from rats in group I was performed. Only those lung and EP tissues that showed a positive PCR result and hybridization signal for the MSG gene were used in the DHFR RT-PCR analysis. To confirm that amplifiable cDNA was obtained, β -actin primers (32) that amplify a 620-bp fragment were used (Fig. 3A). Since MSG primers were designed from a genomic clone and did not amplify *P. carinii* cDNA, a different set of primers that amplify a 273-bp coding region of the DHFR gene of *P. carinii* organisms from rats (25) was used to test for the presence of viable *P. carinii* organisms in EP tissues. The intensities of the RT-PCR amplicons with the DHFR primers were extremely faint on agarose gel electrophoresis (data not shown), but the signals became detectable only after Southern hybridization (Fig. 3B). These observations demonstrate the presence of viable *P. carinii* organisms in EP tissues.

Kinetic study. To determine at what times following initial immunosuppression that *P. carinii* DNA could be detected in lung and EP organs, tissues were collected from rats at 0, 2, 4, 6, and 8 weeks of immunosuppression and their DNAs were subjected to PCR and hybridization. *P. carinii* DNA was detected in lungs as early as 2 weeks postimmunosuppression (Fig. 4A and B). These signals gradually increased in intensity with time of immunosuppression. *P. carinii* DNA was detected in lymph nodes at 6 weeks of immunosuppression (Fig. 4C and D). At 8 weeks of immunosuppression, *P. carinii* DNA was detected in other EP tissues with the exception of blood and bone marrow (Fig. 4E and F). Control, nonimmunosuppressed rats showed no evidence of *P. carinii* DNA in either their lungs or their EP tissues (data not shown).

TABLE 1. DNA amplification and hybridization analysis of *P. carinii* DNA in lungs and EP tissues of immunosuppressed rats

Group	No. of samples positive for <i>P. carinii</i> by amplification and hybridization of MSG gene in:									
	Adrenal tissue	Bone marrow	Blood	Heart tissue ^a	Kidney tissue ^a	Liver tissue ^a	Lung tissue ^a	Lymph node tissue	Spleen tissue ^a	Thyroid tissue
I ($n = 10$) ^b	6	6	4	7	8	9	10	10	6	2
II ($n = 5$) ^c	2	5	5	5	3	5	5	NA ^d	5	5

^a RT-PCR analysis was carried out with DHFR primers with lung, spleen, heart, kidney, and liver tissues. Only those tissues that showed a positive PCR result and hybridization signal for the MSG gene were used for the RT-PCR experiments. RT-PCR analysis showed that the *P. carinii* DHFR gene is actively transcribed in these tissues, thereby indicating the presence of viable *P. carinii* organisms in EP tissues.

^b Tissues were collected from rats with primary pneumocystosis. In group I rats, the number of *P. carinii* organisms in lungs ranged from 2.8×10^7 to 3.6×10^8 *P. carinii* nuclei per lung.

^c Tissues were collected from five rats that had recurrent pneumocystosis. Following primary pneumocystosis, these animals were treated with TMP-SMZ (50 mg of TMP and 250 mg of SMZ) for 3 weeks and were then immunosuppressed for an additional 8 weeks to allow for recurrent *P. carinii* pneumonia. This group initially started with 10 animals, but 5 animals died during the second immunosuppression. Among the remaining rats, the numbers of *P. carinii* organisms in lungs ranged from 2.5×10^6 to 2.8×10^8 *P. carinii* nuclei per lung.

^d NA, not available.

FIG. 2. (A) Representative gel profile of amplified *P. carinii* DNA by using the primer pair P5-P6 from EP tissues of a rat that was immunosuppressed for 8 weeks, treated with TMP-SMZ (50 mg of TMP and 250 mg of SMZ) for 3 weeks, and again immunosuppressed for an additional 8 weeks. Lanes: a, *EcoRI-HindIII*-digested bacteriophage lambda DNA marker; b, liver tissue; c, spleen tissue; d, kidney tissue; e, adrenal tissue; f, thyroid tissue; g, heart tissue; h, bone marrow; i, blood; j, lung tissue; k, positive control DNA from heavily infected rat lung tissue; and l, negative control DNA from *P. carinii*-free rat. (B) Southern hybridization blot of the gel in panel A by using the ^{32}P -labelled 560-bp MSG sequence of *P. carinii* organisms from rats as a probe.

DISCUSSION

Although a significant increase in the incidence of EP pneumocystosis has been reported in humans during the last few years (5, 8, 12, 18, 19, 30, 31, 33), numerous basic questions regarding the host-parasite relationship have not been answered. We have studied the dissemination of *P. carinii* organisms using the immunosuppressed rat model and PCR. Primers were designed from a MSG gene sequence of *P. carinii* organisms from rats in order to detect the organism in the EP tissues of infected rats by PCR. A search of the GenBank database for complementary sequences of primers by using the GCG software program (Genetics Computer Group, Madison, Wis.) revealed data that showed 100% homology with MSG genes of *P. carinii* organisms from rats (15, 35). No homologous sequences were found for other fungi or bacteria, indicating the excellent specificities of the primers. Furthermore, hybridization of the PCR amplicon to the chromosomes of *P. carinii* organisms from rats showed that this sequence is highly repetitive and is distributed in most of the *P. carinii* chromosomes. The detection limits of the PCR assay were 100 fg of *P. carinii* DNA (equivalent to approximately 10 *P. carinii* organisms). The increased sensitivity of the PCR assay allowed us to detect *P. carinii* DNA in lung tissue at as early as 2 weeks of immunosuppression and in adrenal, heart, kidney, lymph node, liver, spleen, and thyroid tissues and bone marrow and blood at 8 weeks of immunosuppression. Interestingly, RT-PCR analysis of cDNA prepared from total cellular RNA isolated from spleen, heart, kidney, and liver tissues of *P. carinii*-infected rats showed that the housekeeping gene DHFR is actively transcribed in these EP tissues, thereby suggesting that *P. carinii* DNA in the EP tissues represents live *P. carinii* organisms.

It remains to be determined, however, whether *P. carinii* organisms are transported to EP sites within phagocytic cells or are spread through the lymphatic system. Since *P. carinii* DNA was found in lymph node, spleen, and liver tissues and bone marrow, there is a good probability that *P. carinii* is spread

through the reticuloendothelial system (3). In agreement with our findings, Denis et al. (7), using the immunofluorescence assay, were able to detect the presence of *P. carinii* organisms in the heart, spleen, liver, and kidney tissues of 5 of 9 (55%) Wistar rats and in the heart, liver, and brain tissues of 2 of 10 (20%) outbred white mice after 9 to 12 weeks of immunosuppression with methylprednisolone acetate. By the PCR assay, EP *P. carinii* infection was reported in various organs from 2 weeks postinfection (20), but the journal article was later retracted (21), stating that previously reported positive results may have been due to contamination of the samples. In our study, extreme care was taken to avoid cross contamination of samples. A major concern was contamination with blood which may be positive for *P. carinii*, even though we washed the various tissues extensively to remove as much blood as possible. The fact that we identified *P. carinii* in the EP tissues of six immunosuppressed rats which did not show *P. carinii* organisms in their blood (Fig. 4) supports our findings that the identification of *P. carinii* organisms in EP tissues most likely is not due to contaminating blood. In contrast to our findings, Evans et al. (11) were not able to identify *P. carinii* dissemination in the liver, spleen, or kidney tissues of Sprague-Dawley rats immunosuppressed for 6 weeks with hydrocortisone acetate, using nested PCR with mitochondrial rRNA primers. This could have been due to a low level of sensitivity of the assay (5.9 pg or 590 *P. carinii* organisms) or the degree of *P. carinii* burden in the animals at 6 weeks of immunosuppression. We found *P. carinii* DNA in the lungs and lymph nodes at 6 weeks of immunosuppression (Fig. 4), but by 8 weeks of immunosuppression most other tissues were also positive. In an additional series of experiments, *P. carinii*-infected rat lungs and EP tissues were analyzed for the presence of *P. carinii* organisms after the cessation of methylprednisolone acetate immunosuppression. *P. carinii* DNA signals that appeared in EP tissues at 8 weeks of immunosuppression became undetectable 2 weeks after the cessation of immunosuppression. In agreement with previous reports (27, 34), *P. carinii* DNA in lung tissue persisted as long as 8 weeks after the termination of steroid treatment.

It was of interest to determine whether or not a difference in the dissemination of *P. carinii* organisms in EP tissues could be detected in animals with recurrent infection (second episode in

FIG. 3. (A) Representative gel profile of RT-PCR-amplified 620-bp rat β -actin fragment from the cDNA of a rat with primary pneumocystosis. (B) Southern blot hybridization of DHFR RT-PCR amplicons with a 273-bp *P. carinii* DHFR probe. Lanes: a, $\phi\text{X174-HaeIII}$ -digested molecular weight marker; b, negative control sample from which RT was omitted; c, negative control sample without template; d, lung cDNA; e, spleen cDNA; f, heart cDNA; g, kidney cDNA; h, liver cDNA and i, cDNA from *P. carinii*-free rat lung.

FIG. 4. PCR amplification of *P. carinii* DNA from EP tissues and lungs of rats at various weeks during first immunosuppression regimen. (A, C, and E) Gel electrophoresis profiles of PCR amplification products from rat tissues after 2, 6, and 8 weeks of immunosuppression, respectively. (B, D, and F) Southern hybridization of gels in panels A, C, and E, respectively, by using ^{32}P -labelled MSG sequence of *P. carinii* organisms from rats as a probe, respectively. Lanes: a, $\phi\text{X174-HaeIII}$ DNA marker; b, liver tissue; c, spleen tissue; d, kidney tissue; e, bone marrow; f, thyroid tissue; g, heart tissue; h, lymph node tissue; i, blood; j, lung tissue; k, *P. carinii*-free DNA as a negative control.

our case). In order to induce a recurrent infection, animals that had primary *P. carinii* infection were treated with TMP-SMZ for 3 weeks to allow them to recover. Following the treatment, they were immunosuppressed for an additional 8 weeks to induce a second episode of PCP. The animals (5 of 10) that survived this second immunosuppression regimen appeared to have an increase in the number of organs in which *P. carinii* organisms were found compared with the number found during the primary infection (Table 1). There was an increase in the numbers of organisms in all of the organs except adrenal and kidney tissues, as follows: blood, 100 versus 40%; spleen tissue, 100 versus 60%; thyroid tissue, 100 versus 20%; bone marrow, 100 versus 60%; heart tissue, 100 versus 70%; and liver tissue, 100 versus 90%. Because of the low number of animals that ended up being evaluated in this part of the study, it is difficult to determine the significance of these findings at this time. In humans, recurrent *P. carinii* infections have been cited to increase the risk of *P. carinii* dissemination from primary lung lesions mainly as a result of *P. carinii* prophylaxis with aerosolized pentamidine (30, 33). However, aerosolized pentamidine has been suggested to be a risk factor for the

dissemination of *P. carinii* organisms only in 50% of the patients with disseminated *P. carinii* infections. This is because several patients were not receiving aerosolized pentamidine at the time that their EP disease was diagnosed. Furthermore, cases of EP *P. carinii* infection have been reported in patients receiving systemic prophylaxis for PCP with TMP-SMZ (23, 30). Thus, it is not clear at this time whether localized or systemic treatment itself plays any role in *P. carinii* dissemination. We chose to use TMP-SMZ for the main purpose of allowing the animals to recover from *P. carinii* infection, which allowed us to induce a recurrent infection in the animals. It was not our goal in the present study to systematically test the effect of TMP-SMZ on *P. carinii* dissemination. The fact that *P. carinii* organisms were detected in EP tissues of animals with primary pneumocystosis and no prophylaxis led us to speculate that factors other than anti-*P. carinii* therapy may be responsible for *P. carinii* dissemination. These could be local pulmonary factors, systemic factors, or perhaps even variations in the invasiveness of different organisms. However, it would be of interest to determine if the level of *P. carinii* dissemination varied significantly in various groups of animals treated with different anti-*P. carinii* drugs administered by various routes. These studies could be performed with our established model.

We observed that *P. carinii* organisms were detected in the EP tissues of animals that had very heavy *P. carinii* burdens in their lungs. Perhaps *P. carinii* organisms gain access to EP organs only after large numbers of organisms are found in the lung and sufficient destruction of the lung tissue has occurred. Our observations are supported by the findings of Cohen and Stoeckle (5), who suggested that HIV-infected individuals may become predisposed to disseminated pneumocystosis after damage to the endothelial barrier in the lungs by successive episodes of pneumocystosis. Since *P. carinii* is rarely considered a potential etiological agent for a clinical sign or symptom outside the lung, the true prevalence of EP *P. carinii* infection in humans is unknown. It is also likely that there were cases of EP *P. carinii* infection in humans in the pre-AIDS era that remained undetected because of the lack of a sensitive assay for *P. carinii*.

The use of a noninvasive blood-based diagnostic assay for *P. carinii* dissemination in rats and humans has been suggested (17, 27). Using DHFR primers, Schluger et al. (24, 25) were the first to report *P. carinii* DNA in the sera of 4 of 15 rats that were in the 10th week of immunosuppression. Subsequently, Lipschik et al. (17), using mitochondrial rRNA primers in a nested PCR, reported more success in detecting *P. carinii* DNA in blood from infected rats (11 of 14) than in blood from HIV-positive patients with disseminated *P. carinii* infection (3 of 11). Using MSG primers, we detected *P. carinii* organisms in the blood of 4 of 10 rats that were immunosuppressed for 8 weeks and in the blood of 5 of 5 rats that had a second episode of pneumocystosis (Table 1). Furthermore, using the DHFR primers, Sepkowitz et al. (27) were able to correlate *P. carinii* DNA levels in sera with active pulmonary infection in 8 of 8 rats immunosuppressed for 10 weeks. However, Tamburrini et al. (29), using PCR amplification of the *P. carinii* DHFR gene, were unable to correlate the presence of *P. carinii* DNA in serum with active pneumonitis in HIV-infected patients and therefore suggested that the detection of *P. carinii* DNA in serum is not a good diagnostic marker for pneumocystosis in HIV-positive patients. In addition, Denis et al. (7), using the immunofluorescence assay, did not find any evidence of *P. carinii* organisms in blood but detected *P. carinii* organisms in other EP tissues from rats that were immunosuppressed for 9 to 12 weeks. Using mitochondrial rRNA primers, Evans et al. (11) were unable to detect *P. carinii* organisms in the blood or

EP tissues of rats at 6 weeks of immunosuppression. Recently, Atzori et al. (1) reported the detection of *P. carinii* DNA in the majority of serum specimens collected from AIDS patients with *P. carinii* pneumonia, before the initiation of chemotherapy, by using nested PCR and primers designed from the internal transcribed spacer of the rRNA genes. The reason for the different results obtained by PCR of blood samples is not known but may be due to the variability of *P. carinii* infection in different animals. It is possible that the organism either was no longer present at the time of sample collection or was present at levels below the detection limit of the assay. Tamburrini et al. (29) observed that the detection of *P. carinii* DNA in serum varied greatly with the time of sample collection, in particular, the time that anti-*P. carinii* chemotherapy was started. Technical aspects like primer selection, a double amplification, or the presence of PCR inhibitors in the serum samples could explain the different results. Nevertheless, *P. carinii* DNA amplification with serum may help to clarify important aspects of the pathophysiology of *P. carinii* infection. Thus, it is important that a large number of samples be tested with a variety of primers before blood-based diagnosis is considered a reliable indicator of *P. carinii* infection or dissemination.

In summary, we have demonstrated the utility of DNA amplification for the study of experimental *P. carinii* dissemination. In immunosuppressed rats, the presence of *P. carinii* DNA in EP tissues correlated with advanced *P. carinii* infection. Our observations support the concept of EP *P. carinii* dissemination in rats and present the possibility that EP *P. carinii* infection may develop in humans with primary acute cases of pneumocystosis, as was seen with rats. Thus, the sensitive PCR approach with EP tissue biopsy specimens could be used to determine *P. carinii* dissemination in humans. A better understanding of the mechanism(s) involved in EP *P. carinii* dissemination in both primary and recurrent *P. carinii* infections can be investigated by using the rat model.

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