Production of Monoclonal Antibody against *Pneumocystis carinii* by Using a Hybrid of Rat Spleen and Mouse Myeloma Cells

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It has been difficult to obtain pure *Pneumocystis carinii* antigen either from cultures or from infected lungs for use in producing a specific antibody against *P. carinii*. This report describes an approach toward producing a monoclonal antibody that bypasses the antigen purification steps. *P. carinii* infection was developed in Sprague-Dawley rats by the method of immunosuppression with cortisone. The infected lungs were homogenized, and the homogenate was used to immunize Sprague-Dawley rats. Rat spleen cells were then fused with SP2/0 mouse myeloma cells. Hybridoma clones were screened for antibody production against *P. carinii* by immunoperoxidase staining techniques and by enzyme-linked immunosorbent assay, using as antigens homogenates of normal rat lung, homogenates of *P. carinii*-infected rat lung, and harvests of *P. carinii* grown with WI-38 cells. Out of six hybridoma clones obtained that produced antibodies against *P. carinii*, one was able to produce ascitic fluid. This monoclonal antibody reacted with two *P. carinii* antigens with masses of about 35,000 and 65,000 daltons in *P. carinii*-infected lungs and three proteins with masses of about 35,000, 65,000, and 110,000 daltons in *P. carinii* that was harvested from a WI-38 cell culture.

Pneumocystis carinii, a common cause of subclinical infections in healthy people, also causes severe infections in malnourished infants and immunocompromised patients, such as those receiving transplants or therapy for lymphoreticular malignancies, and persons with acquired immunodeficiency syndrome or certain congenital abnormalities.

Currently, a diagnosis of pneumonia caused by *P. carinii* is made by the morphological demonstration of the organism in lung material obtained by using invasive procedures. The serological determination of antibody titer against *P. carinii* in the serum of a patient is of little value (6). Methods for antigen detection such as counterimmunoelectrophoresis have been described, but their usefulness is questionable (3, 8). There is a need for noninvasive, accurate diagnostic methods.

A reason for the poor performance of immunodiagnostic methods has been the lack of high-titer antibodies specific to *P. carinii* because of the difficulty in obtaining *P. carinii* antigens. The major source of antigen has been organisms (predominantly cysts) purified from infected lungs by using techniques such as Ficoll-Hypaque (9) or Percoll (M. S. Gradus, Ph.D. thesis, the University of Oklahoma, Oklahoma City, 1982) gradients. Although the enrichment of organisms can be achieved by using gradients, the purity of the preparations has been poor, and the treatment may have altered the antigens. The limited growth of *P. carinii* in cell cultures makes it difficult to obtain large amounts of *P. carinii*, and it is not certain that the organisms are identical to those found in vivo. In addition, there is some contamination by culture cells.

This study reports the production of monoclonal antibody against *P. carinii* in which the *P. carinii* antigens did not require purification. Infected rat lung was used to immunize rats, and hybridomas were developed in a rat-mouse fusion.

MATERIALS AND METHODS

Animals and chemicals. Sprague-Dawley rats and BALB/c mice were obtained from Harlan Laboratory, Indianapolis, Ind. WI-38 cells were purchased from Flow Laboratories, Inc., McLean, Va. Cortisone acetate was obtained from Merck Sharp & Dohme, West Point, Pa. *Staphylococcus aureus* cells, protein A, bovine serum albumin (BSA), and other common chemicals were provided by Sigma Chemical Co., St. Louis, Mo. Rabbit anti-rat immunoglobulin G (IgG) and peroxidase-conjugated rabbit anti-rat IgG were purchased from Jackson ImmunoResearch Laboratories, Inc., Avondale, Pa. Na¹²⁵I was obtained from Amersham Corp., Arlington Heights, Ill. Iodo-beads were supplied by Pierce Chemical Co., Rockford, Ill.

Development of *P. carinii* infections in rat lungs. Sprague-Dawley rats were injected with cortisone acetate for 8 weeks to elicit pneumocystis infection (2). Infected lungs were removed aseptically, and lung impression smears were examined to confirm the presence of *P. carinii*. Lungs that were heavily infected with *P. carinii* were frozen at -70°C until use. The infected lung tissue was used to inoculate tissue cultures, to immunize rats, and to prepare *P. carinii* organisms.

Growth of *P. carinii* in tissue culture. *P. carinii*-infected rat lungs were used to inoculate monolayers of WI-38 cells. The *P. carinii* were propagated as described previously (1). Organisms were harvested from culture supernatant fluid by centrifugation at 10,000 × g for 15 min. The pellet, which contained the organisms, was washed twice with Hanks balanced salt solution lacking Ca²⁺ and Mg²⁺. The organisms were then suspended in the solution. A smear was stained with Giemsa stain, and the number of organisms was determined by light microscopy.

Immunization of rats. *P. carinii*-infected Sprague-Dawley rat lungs were homogenized in 5 ml of Hanks balanced salt solution by using a TenBroeck tissue grinder. The homogenate was mixed with an equal volume of complete Freund adjuvant, and 1 ml of this emulsion was injected intraperito-

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neally into 100-g female Sprague-Dawley rats. A booster injection was given 10 days later with homogenate of infected rat lung emulsified with incomplete Freund adjuvant. Subsequent injections of antigen were given biweekly. Rats were bled from the tail vein 2 months after the initial injection, and the sera were stored at -70°C until use.

Detection of specific antibodies by using the dot-blot technique. Tissue and cell antigens were homogenized and sonicated before use. The protein concentration of each antigen preparation was adjusted to 10 mg/ml with phosphate-buffered saline (PBS). Antigens were spotted separately onto nitrocellulose paper. The nitrocellulose paper was then incubated with a solution containing 3% BSA in PBS for 2 h to eliminate nonspecific adherence of reactants added later. The nitrocellulose paper was then transferred to tubes containing antiserum diluted in PBS containing 3% BSA. After 2 h of incubation, the nitrocellulose was washed with PBS then incubated with *S. aureus* protein A labeled with ^{125}I in PBS containing 3% BSA. The nitrocellulose paper was then washed and subjected to autoradiography.

ELISA. The enzyme-linked immunosorbent assay (ELISA) was used to evaluate rat antisera and monoclonal antibodies. Test antigens, in 0.2 M NaHCO_3 (pH 9.6) solution, were adsorbed onto enzyme immunoassay microtiter plates (Costar, Cambridge, Mass.) for 2 h at 37°C . The wells were then washed with PBS containing 0.05% Tween 20 (PBST). PBST containing 3% BSA (50 μl) was added to each well to block further binding sites. Dilutions of antibody (50 μl) were then added to each well, and the mixture was incubated for 2 h at 37°C with shaking. The wells were then washed with PBST. Horseradish peroxidase-conjugated rabbit anti-rat IgG was diluted 1:3,000 in PBST containing 3% BSA, and 100 μl were then added to each well. After 1 h of incubation, the wells were washed with PBST and 100 μl of the substrate solution was added. The substrate solution contained 0.4% *o*-phenylenediamine and 0.02% hydrogen peroxide in phosphate citrate buffer (pH 5.0). After 1 h of incubation, the reaction was terminated by the addition of 100 μl of 1 N HCl solution, and the results were read using an enzyme immunoassay spectrometer (model EL 307; Bio-Tek Instruments, Inc. Shelburne, Vt.).

Immunoperoxidase staining of *P. carinii*. Immunoperoxidase staining was used to evaluate monoclonal antibodies and was performed by using the modified procedure of Nadji and Morales (7). Impression smears prepared from *P. carinii*-infected or normal human or rat lungs were first fixed with 3% H_2O_2 in methanol for 30 min. After washing, the smears were incubated with normal human or rat serum, and then the test antibody. The smears were then washed and peroxidase-conjugated rabbit anti-rat IgG was applied to the smears. After washing, the slides were placed in 0.05% 3,3'-diaminobenzidine containing 0.01% H_2O_2 for 20 min, washed, and examined by light microscopy.

Production of monoclonal antibodies. Spleen cells were obtained from Sprague-Dawley rats that had been immunized as described above and booster injected with *P. carinii*-infected rat lung tissue 3 days before splenectomy. Erythrocytes were removed by brief hypotonic shock in distilled water. Approximately 2×10^8 spleen cells were fused with 2×10^7 SP2/0 murine myeloma cells by treatment with polyethylene glycol (4). Fused cells were then plated in 96-well plates and grown in hypoxanthine-aminopterin-thymidine medium. After 2 weeks of growth, supernatants of wells were assayed for the presence of antibodies against *P. carinii* by the ELISA and immunoperoxidase staining technique. Cultures that were positive for both reactions were

subcloned in soft agar. Single colonies were isolated and rescreened for the production of anti-*P. carinii* antibody.

To produce ascitic fluid, hybridoma cells were injected into the peritoneum of pristane-primed BALB/c mice. Ascitic fluid was then drained from the peritoneum by using an 18-gauge needle and stored at 4°C in 0.001% sodium azide or further purified by DE-52 column chromatography to isolate the IgG fraction. The concentration of the purified IgG was adjusted to 1 mg/ml with PBS.

Immunoprecipitation. Immunoprecipitation was used to characterize partially the antigens recognized by monoclonal antibodies. *S. aureus* cells containing protein A were used to precipitate the antigen-antibody complexes. After these cells were treated by boiling in 2% sodium dodecyl sulfate for 10 min and washing three times with PBS, 10% suspensions in PBS containing 3% BSA were made. Prior to use, the *S. aureus* cells were incubated with the unlabeled antigens to block nonspecific antigen binding sites. Antigens were iodinated with ^{125}I by using Iodo-beads (5). The labeled antigens (approximately 10^6 cpm, 80 μl each) were then incubated with 10 μl of monoclonal antibody at 37°C for 1 h and at 4°C overnight. After this incubation, 10 μl of rabbit anti-rat IgG (10 mg/ml) was added to each specimen. The reactions were incubated with occasional shaking at 37°C for 30 min and at 4°C for 2 h. Then 10 μl of the pretreated *S. aureus* cells was added, and the suspensions were incubated at 37°C for 1 h and at 4°C for 1 h with occasional shaking. After incubation the cells were pelleted. Pellets from each tube were suspended and washed four times in 1 ml of PBS. The washed pellets were suspended in 20 μl of protein solubilizer containing 2% sodium dodecyl sulfate, 10 M urea, 0.05% 2-mercaptoethanol, and 0.025% bromophenol blue. After the sample boiled for 10 min, the radioactivity of 1- μl portions of the solubilized specimens was determined by a gamma counter. Based on the 1- μl counts, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed by using comparable amounts of radioactivity per well. The gel was dried after electrophoresis by using a gel drier, and autoradiography was performed.

RESULTS

Production of monoclonal antibodies. A total of six Sprague-Dawley rats were immunized with a homogenate from *P. carinii*-infected lungs of Sprague-Dawley rats. At 12 weeks after the initial immunization, sera were collected from each rat and assayed for the presence of antibody against *P. carinii*, using the dot-blot technique. The result is illustrated in Fig. 1. As shown in the autoradiograph, darker spots were observed at the position where *P. carinii*-infected rat lung antigens (PCRL) and *P. carinii* harvested from WI-38 cell culture antigens (PC-WI) were spotted. No significant intensity of the spots was seen on normal rat lung (NRL) samples. These results were confirmed by ELISA, using the same antigens. The titers of the antisera against PCRL or PC-WI were determined to be approximately 1:10,000 and less than 1:100 against NRL. These results indicate that the immunized rats were producing antibodies specific to *P. carinii*. Although specific antibodies can be easily produced with this approach, the amount of antiserum obtainable is very limited. Therefore, attempts were made to produce monoclonal antibodies.

Since PCRL-immunized rats produced antibody to *P. carinii*, the majority of the antibody-producing cells should be sensitized by *P. carinii* antigens. Therefore, the spleen cells of the immunized rats were harvested and fused with

FIG. 1. Determination of anti-*P. carinii* antibodies in Sprague-Dawley rats immunized with *P. carinii*-infected lung homogenate. Homogenates of NRL, PCRL, and PC-WI were used as the test antigens. Antigens were spotted onto nitrocellulose paper strips. The nitrocellulose paper strips were then incubated with 3% BSA and reacted with sera of immunized rats. After washing, the paper strips were reacted with ^{125}I -labeled *S. aureus* protein A in the presence of 3% BSA. The excess ^{125}I -labeled *S. aureus* protein A was washed off with PBS. Autoradiographs were then taken.

SP2/0 murine myeloma cells. The hybrids were grown and selected on hypoxanthine-aminopterin-thymidine medium. Of the total 960 wells of culture, 36 showed growth of the hybrids. Culture supernatants of these hybrids were assayed for the presence of antibodies to *P. carinii* by ELISA with PC-WI as the antigen. The 10 cultures that were positive for the assay were then subcloned in soft agar to isolate antibody-producing clones. Culture supernatants of the hybridomas thus isolated were again tested for antibody against *P. carinii* by ELISA, using PC-WI, and by immunoperoxidase staining techniques. By both methods, six clones were positive. Attempts were also made to produce ascitic fluid in mice with each of these six positive clones. Only one clone, designated as R13/3G4-6, caused ascitic fluid accumulation.

FIG. 2. Immunoprecipitation of *P. carinii* antigens by monoclonal antibody. Homogenates of NRL, PCRL, and WI-38 cells (WI) were used as the antigen. The antigens were sonicated and then iodinated with ^{125}I . Several reactions were performed: A, ^{125}I -labeled antigen plus monoclonal antibody plus *S. aureus* cells; B, ^{125}I -labeled antigen plus monoclonal antibody plus rabbit anti-rat IgG plus *S. aureus* cells; C, ^{125}I -antigen plus *S. aureus* cells. The antigen-antibody-*S. aureus* cell complexes were collected by centrifugation, washed, and boiled in sodium dodecyl sulfate-urea sample buffer. Autoradiography was performed after the polyacrylamide gel electrophoresis.

FIG. 3. Immunoperoxidase staining of *P. carinii*-infected rat lung section with the monoclonal antibody. Sections of *P. carinii*-infected rat lung were incubated with normal serum followed by the monoclonal antibody. The sections were washed, and the peroxidase-conjugated rabbit anti-rat IgG was then applied to the sections. After washing, the sections were incubated with 0.05% 3,3'-diaminobenzidine containing 0.01% H_2O_2 . The sections were then washed and examined by light microscopy.

The ascitic fluid was collected, and the IgG fraction was purified for further studies.

Characterization of the monoclonal antibody. The specificity of the monoclonal antibody R13/3G4-6 was characterized by ELISA, immunoperoxidase staining, and immunoprecipitation. ELISA was performed by using as antigens fetal calf serum, uninfected WI-38 cells, PCRL, NRL, normal human lung, and PC-WI. The monoclonal antibody reacted to PCRL and PC-WI at a titer of about 1:1,000,000, whereas no reaction to other antigens was observed.

To characterize partially which *P. carinii* antigens reacted with the monoclonal antibody, immunoprecipitation was performed. Homogenates of NRL, PCRL, WI-38, and PC-WI were labeled with ^{125}I . Performed were three types of reaction: (i) labeled antigen plus monoclonal antibody plus *S. aureus* cells; (ii) labeled antigen plus monoclonal antibody plus rabbit anti-rat IgG plus *S. aureus* cells; (iii) labeled antigen plus *S. aureus* cells. The antigen-antibody-*S. aureus* cell complexes were dissociated by boiling in protein solubilizer and electrophoresed on a 12.5% polyacrylamide gel. The resultant autoradiogram is shown in Fig. 2. No bands were observed in the NRL samples; however, two bands corresponding to proteins with molecular masses of approximately 35,000 and 65,000 daltons were present in the reaction of labeled PCRL antigen plus monoclonal antibody plus rabbit anti-rat IgG. These two bands were also seen with the PC-WI antigen. These results suggest that these two proteins are *P. carinii* antigens recognized by the monoclonal antibody. In the PC-WI sample, two additional bands were seen, perhaps because of the nonspecific binding of the *S. aureus* cells to IgG present in fetal calf serum. Appearing in the sample of labeled PC-WI plus monoclonal antibody plus rabbit anti-rat IgG plus *S. aureus* was one darker band that comigrated with the larger nonspecific protein (about 110,000 daltons). It is conceivable that a *P. carinii* protein of that size was recognized by the monoclonal antibody.

Immunoperoxidase staining of *P. carinii*-infected rat lung tissue sections was performed to confirm further the specificity of the monoclonal antibody. Clusters of organisms were deeply stained with the antibody, whereas no staining of the lung tissue was observed, indicating that the mono-

FIG. 4. Immunoperoxidase staining of *P. carinii*-infected human lung smears. Impression smears of *P. carinii*-infected human lung were fixed with 3% H₂O₂ in methanol for 30 min. After washing, the smears were processed for immunoperoxidase staining as described in the legend to Fig. 3.

clonal antibody is indeed specific for *P. carinii* (Fig. 3). Immunoperoxidase staining was also performed on a smear of *P. carinii*-infected human lung (Fig. 4). A dark deposit was observed on the *P. carinii* cyst wall. This was absent in the negative controls in which ascites containing no antibody to *P. carinii* were used.

The specificity of the other five monoclonal antibodies was also examined by using the culture supernatants of the hybridomas. Of these clones, three reacted identically to the monoclonal antibody R13/3G4-6. The other two clones reacted with one protein present in the PCRL, but not in the PC-WI; however, they also reacted with one WI-38 cell protein of the same size (about 40,000 daltons). Whether this protein is a *P. carinii* antigen common to WI-38 cells is unknown and is currently being investigated.

DISCUSSION

This report has described a method to produce monoclonal antibodies against *P. carinii* that bypasses the cumbersome procedures for the isolation or enrichment of *P. carinii* organisms to be used as antigen. Sprague-Dawley rats were immunized with homogenates of *P. carinii*-infected lungs of Sprague-Dawley rats. Since isologous antigens may be recognized as self-antigens, the majority of the spleen cells of the immunized rat would be sensitized by *P. carinii* antigens and would produce antibodies to *P. carinii*. Therefore, a successful fusion of anti-*P. carinii* antibody-producing cells with mouse myeloma cells can direct the myeloma cells to produce specific antibodies against *P. carinii*. Of the six hybridomas which produced monoclonal antibody against *P. carinii*, one was able to produce ascites in the peritoneum of a mouse. This monoclonal antibody was shown to react with two *P. carinii* antigens present in the PCRL, and possibly three *P. carinii* antigens in the PC-WI. This unusual reaction may be caused by the presence of common antigenic determinants in different antigens, or the larger antigen may be a precursor for the smaller ones.

The inability of other hybridomas to generate ascites in mice may be because the hybridomas also contain rat genes other than those that control antibody production; thus, rat antigens are expressed, and the myeloma cells are recognized as foreign and destroyed. It is possible that whole-body irradiation of recipient mice might circumvent this problem. By using this approach, attempts are being made to produce ascites from the other five hybridomas.

In spite of this potential problem, the above procedures are useful for monoclonal antibody production against *P. carinii*. Other approaches might be to use *P. carinii*-infected mouse lungs in a mouse-mouse fusion or to perform a rat-rat fusion. However, we did not have ready access to *P. carinii*-infected mouse lungs or to the rat-rat system. The ability of the monoclonal antibody to react with human *P. carinii*, as demonstrated in the immunoperoxidase reaction (Fig. 4), could be potentially useful in developing a diagnostic test for *P. carinii* infection. Furthermore, studies aimed at strain differences between mouse, rat, and human *P. carinii* are now feasible. Antigenic variation, if any, at different stages of development can also be studied. The observation that the monoclonal antibody reacted with two antigens in infected rat lungs, but with three antigens in the cultured organism (Fig. 2), suggests that antigenic variation may exist in *P. carinii*, although further studies are required to verify this possibility. This monoclonal antibody may also help us in investigating the possible causes of growth limitation of *P. carinii* in tissue culture.

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