Antibody to *Pneumocystis carinii* Protects Rats and Mice from Developing Pneumonia

Marilyn S. Bartlett, William C. Angus, Margaret M. Shaw, Pamela J. Durant, Chao-Hung Lee, Juan M. Pascale, and James W. Smith

Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Indiana, and National Institutes of Health, Bethesda, Maryland

Received 23 June 1997/Returned for modification 2 October 1997/Accepted 10 November 1997

Well-proven mouse and rat models were used to show that polyclonal antisera to *Pneumocystis carinii* protect against *P. carinii* pneumonia. Antibodies were obtained from animals that were allowed to recover from severe *P. carinii* pneumonia after immunosuppression had been stopped and then were given a booster injection of *P. carinii* from the same animal species. Mice immunosuppressed with corticosteroids or antibodies to L3T4* supersupercells (which are comparable to CD4 cells of humans) and transtracheally inoculated with mouse *P. carinii* did not develop *P. carinii* pneumonia if they were passively immunized with antisera, while mice immunosuppressed and inoculated by identical procedures but not given antibodies developed severe infections. Rats immunosuppressed with corticosteroids and inoculated with rat *P. carinii* had less severe infections if they were given rat anti-*P. carinii* antisera. The polyclonal antisera developed in mice provided greater protection for the mice than the polyclonal rat antisera did for the rats; however, the potencies and compositions of the antisera were not quantitated and probably differed. Since both rats and mice can be protected from *P. carinii* infections with polyclonal antisera, it may be possible to develop vaccines that will elicit protective antibodies in humans.

*Pneumocystis carinii* is a leading cause of pneumonia in patients with immune deficiencies, including individuals infected with human immunodeficiency virus and those receiving chemotherapy for malignancies or for transplantation. Although antimicrobics are available for treatment and prophylaxis of *P. carinii* pneumonia (20), many patients have adverse reactions or fail to respond to the most effective antimicrobial agents (28). In addition, even with prophylaxis, *P. carinii* continues to be a major cause of illness (23, 24). If a vaccine could be developed that would prevent infections from developing, or reduce infections so that they would be mild or occur later in the course of AIDS, it would be of great clinical utility.

The role of host defenses in preventing the development of *P. carinii* pneumonia is not well defined. Since individuals with human immunodeficiency virus develop *P. carinii* pneumonia when their CD4 cell counts fall below 200, cell-mediated immunity has been considered the most important host defense. However, reports of *P. carinii* pneumonia in children often describe hypogammaglobulinemia or agammaglobulinemia as predisposing factors (7, 19, 23, 24, 30). The role of antibodies in preventing or controlling disease has not been well explained, although a study by Gigliotti and Hughes (13) re-

P. carinii

infections, immunosuppressed animals were transtracheally inoculated as described previously (1, 4, 5). Briefly, BALB/c mice were immunosuppressed and transtracheally inoculated with mouse *P. carinii*, and virus-free Sprague-Dawley rats from Harlan Colony 202, Indianapolis, Ind., were immunosuppressed and transtracheally inoculated with rat *P. carinii*. Prior to inoculation, mice were immunosuppressed for 14 days with monoclonal antibody from clone GK1.5, which is directed to L3T4* cells (comparable to human CD4 cells) (8), in one study and with dexamethasone for 10 days at 1.2 mg/kg of body weight/day in the second study. Prior to inoculation, the rats were immunosuppressed with dexamethasone at 0.36 mg/kg/day for 7 days. Mice were transtracheally inoculated with 10⁶ mouse *P. carinii* organisms in 0.05 ml of saline, and rats were transtracheally inoculated with 10⁵ rat *P. carinii* organisms in 0.2 ml of saline; the wounds were closed with clips.

**Development of mouse antisera.** The animals developed infections for 10 weeks, at which time severe infections had developed; immunosuppression was stopped, and the animals were allowed to recover. During the second week after the cessation of immunosuppression, the animals were injected intraperitoneally with 0.1 ml of solubilized *P. carinii* prepared as follows. Heavily infected lung tissue was ground in phosphate-buffered saline (10 mg/ml) and centrifuged slowly (300 × g) to remove large lung debris. The supernatant, containing approximately 10⁷ organisms, was centrifuged at 5,000 × g for 10 min, and the pellet was suspended in 0.1 ml of 8 M urea with 10 mg of dithiothreitol/ml in water, solubilized at 4°C overnight, and diluted 1:10 in phosphate-buffered saline. After an additional 17 days, the animals were anesthetized and exsanguinated by cardiac puncture and sera were evaluated for antibody by enzyme-linked immunosorbent assay (ELISA) by a method that was developed to detect *P. carinii* in cultures and in animals (9). Briefly, ELISAs were performed in time with corticosteroids, assessing the contributions of host defenses in the course of disease was not possible because corticosteroids suppress all lymphocytes, including T and B lymphocytes, and influence phagocytosis and inflammation. Inoculated animal models, used in this study, develop severe infections more rapidly and reproducibly than latently infected animals (1, 4). By using them, it has been possible to more clearly determine the effects of drugs on infections (2, 3), study immune responses during the development of infections (5), and test the usefulness of antisera in providing protection. This report describes the use of polyclonal antisera from convalescent animals that had received a booster injection to prevent or diminish infections in these well-established models.

**MATERIALS AND METHODS**

**Animal models.** To develop *P. carinii* infections, immunosuppressed animals were transtracheally inoculated as described previously (1, 4, 5). Briefly, BALB/c mice were immunosuppressed and transtracheally inoculated with mouse *P. carinii*, and virus-free Sprague-Dawley rats from Harlan Colony 202, Indianapolis, Ind., were immunosuppressed and transtracheally inoculated with rat *P. carinii*. Prior to inoculation, mice were immunosuppressed for 14 days with monoclonal antibody from clone GK1.5, which is directed to L3T4* cells (comparable to human CD4 cells) (8), in one study and with dexamethasone for 10 days at 1.2 mg/kg of body weight/day in the second study. Prior to inoculation, the rats were immunosuppressed with dexamethasone at 0.36 mg/kg/day for 7 days. Mice were transtracheally inoculated with 10⁶ mouse *P. carinii* organisms in 0.05 ml of saline, and rats were transtracheally inoculated with 10⁵ rat *P. carinii* organisms in 0.2 ml of saline; the wounds were closed with clips.

**Development of mouse antisera.** The animals developed infections for 10 weeks, at which time severe infections had developed; immunosuppression was stopped, and the animals were allowed to recover. During the second week after the cessation of immunosuppression, the animals were injected intraperitoneally with 0.1 ml of solubilized *P. carinii* prepared as follows. Heavily infected lung tissue was ground in phosphate-buffered saline (10 mg/ml) and centrifuged slowly (300 × g) to remove large lung debris. The supernatant, containing approximately 10⁷ organisms, was centrifuged at 5,000 × g for 10 min, and the pellet was suspended in 0.1 ml of 8 M urea with 10 mg of dithiothreitol/ml in water, solubilized at 4°C overnight, and diluted 1:10 in phosphate-buffered saline. After an additional 17 days, the animals were anesthetized and exsanguinated by cardiac puncture and sera were evaluated for antibody by enzyme-linked immunosorbent assay (ELISA) by a method that was developed to detect *P. carinii* in cultures and in animals (9). Briefly, ELISAs were performed in

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, MS A-128, Indiana University School of Medicine, 635 Barnhill Dr., Indianapolis, IN 46202-5120. Phone: (317) 274-5767. Fax: (317) 278-2018. E-mail: mbartlet@iupui.edu.
TABLE 1.  P. carinii infection scores with various treatments

<table>
<thead>
<tr>
<th>Animal Immunosuppression</th>
<th>Antiserum</th>
<th>Score ( \text{a} ) when treated with:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>At inoculation and at wk 2 and 4</td>
</tr>
<tr>
<td>Mouse L3T4</td>
<td>0.5 ± 0.2&lt;sup&gt;*&lt;/sup&gt;</td>
<td>1.2 ± 0.3&lt;sup&gt;**&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mouse Dexamethazone</td>
<td>0.3 ± 0.2&lt;sup&gt;***&lt;/sup&gt;</td>
<td>ND</td>
</tr>
<tr>
<td>Rat Dexamethazone</td>
<td>3.5 ± 0.3&lt;sup&gt;****&lt;/sup&gt;</td>
<td>2.3 ± 0.1&lt;sup&gt;****&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

* Giemsa stain score ± standard error. \( P \) values for differences between treated and untreated animals: *, \( P = 0.003; **, P = 0.023; ***, P = 0.000; ****, P = 0.006. ND, not done.
<sup>**</sup> TMP + SMX, trimethoprim plus sulfamethoxazole.

96-well Corning Easy Wash plates coated with purified mouse P. carinii antigens. The plates were incubated at 35°C, washed three times, blocked, washed, incubated with mouse sera, washed three times, incubated with anti-mouse immunoglobulin G (IgG) tagged with alkaline phosphatase, and washed three times. P-Nitrophenyl phosphate was then added, and the plates were held for color development for approximately 30 min. The plates were read at 405 nm on a Molecular Devices ELISA reader. In the serum pool from the first 16 mice, individual sera had optical densities of from 0.278 ± 0.001 to 0.453 ± 0.011 (normal mouse IgG was 0.20 ± 0.003). The second serum pool from 19 mice, the optical density range was from 0.283 ± 0.014 to 0.511 ± 0.005 and normal mouse IgG was 0.101 ± 0.003. Western blots were performed to define populations of antibodies. Classes of antibodies were determined and shown to be primarily IgG. The sera were pooled and used to treat mice.

Development of rat antisera. Rat antisera were developed in approximately the same manner as that used for mouse antiserum. Transtracheally-inoculated Sprague-Dawley rats developed P. carinii infections for 6 weeks; then the dexamethasone immunosuppression was stopped, and the rats were allowed to recover. At the end of the first week after dexamethasone was stopped, the rats were given solubilized rat P. carinii prepared in the same way as solubilized mouse P. carinii except that the pellet contained approximately 10<sup>6</sup> organisms and the final volume given was 0.3 ml per rat intraperitoneally. The rats were exsanguinated by cardiac puncture 2 weeks after the P. carinii injection, and the individual rat sera were evaluated by ELISA and Western blotting. The rat anti-P. carinii antibody was primarily IgG. Rat sera shown to have antibodies to rat P. carinii antigens were pooled.

Development and evaluation of infections. After inoculation, the animals were continued on immunosuppression for 6 weeks, allowing infections to become severe. The severity of infections was determined by scoring numbers of organisms in histologically stained impression smears of lung samples. Animals were anesthetized and exsanguinated by cardiac puncture, and their lungs were removed. A portion of the left lower lobe of each lung was used for the preparation of smears for Giemsa and methenamine-silver nitrate staining. The smears were examined under 1000× magnification. At least 100 fields were examined as unknowns by two experienced microscopists and scored according to the following roughly logarithmic scheme (organisms per representative 1,000× microscopic field): 5+, >100; 4+, 11 to 100; 3+, 1 to 10; 2+, 2 to 9 (in 10 fields); 1+, 1 (in 10 to 50 fields); and 0 (in >50 fields).

Evaluation of antisera for major surface glycoprotein specificity. The mouse serum pool that provided protection was used to blot a soda dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (18) gel of rat P. carinii recombinant major surface glycoprotein (MSG) (21). For controls, normal mouse serum, rat convalescent-phase serum, and normal rat serum were included in the blot. The mouse serum pool also was blotted against a mouse P. carinii antigen prepared from mostly trophozoite forms obtained by differential centrifugation according to a method developed by Merali and Clarkson (22). Individual rat serum samples were tested by ELISA and Western blotting, using as the antigen a rat P. carinii preparation of mostly trophozoite forms prepared by the same differential centrifugation method.

Treatment of animals with antisera. Immunossuppressed mice were given 60 μl of antiseraum pool, and rats were given 400 μl intraperitoneally. For the first mouse study, which used L3T4<sup>+</sup> antibody immunosuppression, antisera was given to one group of mice 1 day prior to P. carinii transtracheal inoculation and at 2 and 4 weeks postinoculation while antisera was given to a second group of mice only at 3 and 5 weeks after P. carinii inoculations. For the second mouse study, which used dexamethasone immunosuppression, antisera was given 1 day prior to transtracheal inoculation and at 2 and 4 weeks after inoculation. Immunossuppressed rats were given antiseraum 1 day prior to transtracheal inoculation with P. carinii and at 2 and 4 weeks after inoculation. For each of the above studies, there were 10 mice or 8 rats in each antiseraum-treated group, 10 mice or 8 rats inoculated at the same time as the antiseraum-treated groups but not given antiseraum, and 10 mice or 8 rats inoculated at the same time and treated with trimethoprim plus sulfamethoxazole at 50 and 250 mg/kg/day, respectively, in drinking water.

**DISCUSSION**

Polyclonal anti-P. carinii antisera can protect mice and rats from developing severe P. carinii infections. The mice immunossuppressed by antibody to L3T4<sup>+</sup> lymphocytes (comparable...
to CD4 lymphocytes of humans) and given mouse polyclonal antiserum to CD4 lymphocytes of humans) and given mouse polyclonal antiserum prior to inoculation were most effectively protected from *P. carinii* pneumonia. This antiserum pool was also effective when given after infections had begun to develop, at 3 weeks postinoculation. In dexamethasone-immunosuppressed mice, the antiserum was effective when given prior to inoculation. Rats were less well protected with the available rat antiserum pool than were mice with their antiserum pool. Still, rats that were treated with polyclonal antiserum at the time of inoculation had less severe infections than the rats that were not treated. The antiserum pool may not have been administered in sufficient quantity to provide protection, or the amounts of protective antibodies may have been less in the rat serum pool than in the mouse serum pool.

The mouse antiserum did not react with recombinant rat *P. carinii* MSG. Some investigators have suggested that MSG is a dominant antigen, and since a previous study had shown cross-reaction of antibodies to surface glycoproteins with mouse and rat *P. carinii* (6), pure recombinant MSG was used to see if the antiserum that provided protection contained antibodies directed to this antigen. The protective mouse antiserum had antibodies that reacted to mouse *P. carinii* antigens of 97, 85, and 55 KDa (Fig. 2) in addition to the 130-kDa (MSG) antigens.

Host responses to *P. carinii* have been studied in many systems in an effort to understand the organism’s pathogenic mechanisms and identify methods to prevent infections or reduce the complications of infection. Although *P. carinii* was identified as the cause of many serious outbreaks of disease in children in institutions following World War II (12), in children treated for leukemia (21, 25, 29), and in those with risk factors such as malnutrition (17) and immunosuppressive chemotherapies (23, 27), the successful use of trimethoprim plus sulfamethoxazole for prophylaxis in those shown to be at risk (16) discouraged studies of the organism and *P. carinii* pneumonia. Not until the advent of AIDS did strong interest in *P. carinii* pneumonia develop. Because patients with AIDS were immunosuppressed by the loss of CD4 lymphocyte function, the study of the role of humoral immunity in preventing *P. carinii* pneumonia has been largely ignored. The possibility of using passively transferred antiserum or a vaccine which could induce protective antibodies offers a new approach to the management of immunosuppressed patients who are at increased risk for *P. carinii* infections. (Experiments are in progress to test this hypothesis.) Identification of antibodies that afford protection and the antigens which stimulate their formation is ongoing. The goal is to identify a potentially effective immunogen so that soon after detection of HIV positivity, or in anticipation of transplantation, a vaccine could be given to allow antibodies to develop and thus decrease the likelihood of *P. carinii* pneumonia. Further studies are needed.

REFERENCES


