Latent *Pneumocystis carinii* Infection in Commercial Rat Colonies: Comparison of Inductive Immunosuppressants plus Histopathology, PCR, and Serology as Detection Methods

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Histopathologic evaluation combined with a period of immunosuppression has been the standard procedure for detection of *Pneumocystis carinii* in commercial rat colonies. Variation in induction regimens and in the sensitivity of detection methods may result in underreporting of the presence of *P. carinii* in breeding colonies or delay its detection. In the present study, methylprednisolone and cyclophosphamide were evaluated for the ability to induce *P. carinii* infection in rats from an enzootically infected commercial barrier colony. The presence of *P. carinii* was detected by histopathologic methods and by amplification of a targeted region of the *P. carinii* thymidylate synthase gene by PCR over the 8-week study period. Sera taken from rats prior to either induction regimen were evaluated for the presence of *P. carinii*-specific antibodies by the immunoblotting technique. Few significant differences in ability to induce organism burden or in histopathology were observed between the two immunosuppressive regimens. However, a dramatic loss of weight over the study period was observed in rats treated with methylprednisolone but not in rats treated with cyclophosphamide. Although histopathologic changes attributable to *P. carinii* did not appear before 2 weeks with either immunosuppressant, the presence of the organism in these animals was detected by immunoblotting and PCR. Cyst scores and the intensities of the histopathologic lesions increased during the study period, but the number of rats exhibiting evidence of *P. carinii* infection did not change after week 3. These results suggest that use of the PCR method on postmortem lung tissue of rats without prior induction regimens or identification of anti-*P. carinii* antibodies in antemortem serum samples is a sufficiently sensitive method for detection of the presence of a *P. carinii* carrier state in rodent breeding colonies.

Diagnosis of intercurrent but asymptomatic and histopathologically cryptic infection of laboratory rodents by *Pneumocystis carinii* has historically required chronic treatment with immunosuppressants to depress the hosts’ immune systems, permitting resident or environmentally acquired immunosuppressants to depress the hosts’ immune systems, *Pneumocystis carinii* has historically required chronic treatment with cyclophosphamide for the ability to induce *P. carinii* infection in rats from an enzootically infected commercial barrier colony. The presence of *P. carinii* was detected by histopathologic methods and by amplification of a targeted region of the *P. carinii* thymidylate synthase gene by PCR over the 8-week study period. Sera taken from rats prior to either induction regimen were evaluated for the presence of *P. carinii*-specific antibodies by the immunoblotting technique. Few significant differences in ability to induce organism burden or in histopathology were observed between the two immunosuppressive regimens. However, a dramatic loss of weight over the study period was observed in rats treated with methylprednisolone but not in rats treated with cyclophosphamide. Although histopathologic changes attributable to *P. carinii* did not appear before 2 weeks with either immunosuppressant, the presence of the organism in these animals was detected by immunoblotting and PCR. Cyst scores and the intensities of the histopathologic lesions increased during the study period, but the number of rats exhibiting evidence of *P. carinii* infection did not change after week 3. These results suggest that use of the PCR method on postmortem lung tissue of rats without prior induction regimens or identification of anti-*P. carinii* antibodies in antemortem serum samples is a sufficiently sensitive method for detection of the presence of a *P. carinii* carrier state in rodent breeding colonies.

In the cost-benefit context of diagnosis, prolongation of the inductive period beyond a point enabling confident diagnosis becomes counterproductive.

In the present study, specific parameters of the stress test were evaluated in an effort to establish optimal induction and detection methods for the presence of *P. carinii*. A large, commercial, gnotobiologically derived, barrier-maintained breeding colony of Fischer 344 (F344) inbred rats previously known to have an almost 100% incidence of clinically silent, latent *P. carinii* infection was selected as the study population (13). The high incidence of the organism in this colony helped to ensure that observed differences were due to experimental manipulations and not to sporadic infection levels. Only adolescent males in the 200- to 250-g weight range (corresponding to the 8- to 10-week age group) were used, permitting other parameters of the stress test to be manipulated while holding the age-weight-sex invariant. Two of the most widely used immunosuppressants, methylprednisolone and cyclophosphamide, were evaluated for progression of organism burden and pathological lesions associated with the infection in the rats’ lungs over an 8-week period. In addition, targeted amplification of a region of a putative single-copy gene of *P. carinii*, thymidylate synthase (TS), by PCR was compared to a standard histopathologic scoring system to determine the most effective method for detection of the presence of *P. carinii*. Serologic detection of antibodies specific for rat *P. carinii* by the immunoblotting technique was explored as a potential nonin-
vase means for predicting progression to infection or a P. ca-
rinii-free state.

MATERIALS AND METHODS

Animals. The study was organized into two separately staged experiments. In the first stage, randomly selected groups of 24 and 27 rats were used to produce P. carinii infection. In the second stage, 81 rats, weighing 150 to 200 g, were used to produce P. carinii infection and co-infect with one of three immunosuppressants to initiate the inductive stage. The first group of 24 rats was divided into the following groups: 8 groups of 3 rats each, housed identically to the first group, except that one group of 3 rats was subjected to terminal sample collection procedures (described below) on the day of arrival to constitute a preinduction baseline.

Immunosuppressive regimen. The first group of 24 rats was divided into two groups of 12 rats each. One group was dosed with methylprednisolone acetate (Depomedrol; Upjohn, Kalamazoo, Mich.) (4 mg/kg of body weight given once per week by intraperitoneal injection) and, in parallel, the rats in the other four cages were dosed with cyclophosphamide monohydrate (Sigma Chemical Co., St. Louis, Mo.) (33 mg/kg of body weight given once per week by intraperitoneal injection) and, in parallel, the rats in the other four cages were dosed with methylprednisolone acetate (Depomedrol; Upjohn, Kalamazoo, Mich.) (4 mg/kg given once per week by subcutaneous injection) (9). The animals were weighed weekly when removed for immunosuppressive treatment (or for terminal procedures), and their clinical condition was evaluated by inspection. One cage of three rats dosed with each immunosuppressant was withdrawn from the first stage for terminal procedures at the end of 4, 5, 6, and 9 weeks of induction, and, for the second stage, at the end of 1, 2, 3, and 4 weeks of induction. The fourth group was replicated to confirm comparability and continuity of inductive effects between the two stages.

Terminal procedures. Each animal was anesthetized with CO2 for blood sample collection by cardiac puncture and then euthanatized by overdose with anesthetic. A necropsy was performed on each rat in the study. The right anterior lung lobe was collected and frozen at -70°C for DNA extraction prior to PCR determinations. The large single left pulmonary lobe was excised and fixed in 10% neutral buffered formalin for histologic preparations. Histologic sections were made by standard methods for paraffin-embedded blocks, cut at a thickness of 5 μm, and stained alternatively with hematoxylin and eosin for assessment of pulmonary inflammatory changes and for the presence of P. carinii cyst forms, or with Grass and methenamine silver (GMS) for scoring of P. carinii cyst forms and inflammatory changes. There were no significant findings based on the gross examination of the lungs from the study rats. Histological evaluation of the pulmonary sections prepared as described above was evaluated at the end of each week by a veterinary pathologist blinded to the immunosuppressant group and the week of the study. The hematolyn- and-eosin-stained sections were used to score overall pulmonary inflammatory changes and were scored separately for the occurrence of lesions attributable to the effects of the immunosuppressant regimen. The results of each histologic evaluation were compared with the normative growth curve for each group of three rats, take at weekly intervals until the scheduled termination, are summarized in Table 1. Student’s t test was performed to determine the level of significance for each product moment determined. A Wilcoxon rank sum test was used to compare the two immunosuppressive regimens for each histologic parameter in footnote a to Table 1. Cochran’s test was used to compare PCR and serology as methods for the detection of P. carinii.

RESULTS

The animals in this study were evaluated weekly for weight loss, histopathologic changes in the lungs, organism burden, and the presence of P. carinii DNA in the lungs by PCR as induction progressed over the 8-week period. The weight loss experienced by the rats in the groups treated with methylprednisolone was found to be statistically different than the weight gain of the rats receiving the alternative immunosuppressant therapy, cyclophosphamide. The mean body weights for each group of three rats, taken at weekly intervals until the scheduled termination, are summarized in Fig. 1. A similarly slowed weight loss occurred with all methylprednisolone-treated groups, beginning after the first inductive injection (r = 0.99, P < 0.001). In contrast, all groups treated with cyclophosphamide adhered essentially to the normative growth curve for F344 male rats (r = 0.99; P < 0.001) (25). As discussed below, the observed weight loss was attributed to the corticosteroid treatment and not to the effects of progressive pneumocystosis. There were no significant findings based on the gross appearance of the lungs during necropsy dissection.

Pulmonary changes within the rat lungs detected by histology are summarized in Tables 1 and 2. The criteria for assessment are found in footnote a to Table 1. In the rats sampled prior to induction, some background inflammation was ob-
TABLE 1. Histopathologic lesion and cyst scores and Western immunoblot and PCR results in rats during methylprednisolone induction

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Weeks of induction</th>
<th>Pulmonary histopathology</th>
<th>Western immunoblotting</th>
<th>PCR (no. positive/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Overall inflammation</td>
<td>Pneumocystis lesions</td>
<td>Preinduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>GMS cyst scorea</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(no. positive/no. tested)</td>
<td></td>
</tr>
<tr>
<td>40–42</td>
<td>1</td>
<td>0.66</td>
<td>0.33</td>
<td>0.00 (0/3)</td>
</tr>
<tr>
<td>43–45</td>
<td>2</td>
<td>1.00</td>
<td>1.00</td>
<td>1.33 (3/3)</td>
</tr>
<tr>
<td>46–48</td>
<td>3</td>
<td>1.00</td>
<td>0.33</td>
<td>0.66 (0/3)</td>
</tr>
<tr>
<td>49–51</td>
<td>4</td>
<td>1.00</td>
<td>1.00</td>
<td>2.00 (3/3)</td>
</tr>
<tr>
<td>13–15</td>
<td>4</td>
<td>1.00</td>
<td>1.00</td>
<td>1.33 (3/3)</td>
</tr>
<tr>
<td>16–18</td>
<td>5</td>
<td>1.33</td>
<td>1.66</td>
<td>2.00 (3/3)</td>
</tr>
<tr>
<td>19–21</td>
<td>6</td>
<td>0.66</td>
<td>1.00</td>
<td>1.33 (3/3)</td>
</tr>
<tr>
<td>22</td>
<td>Died</td>
<td>NDb</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>23–24</td>
<td>8</td>
<td>1.50</td>
<td>1.50</td>
<td>3.00 (2/2)</td>
</tr>
<tr>
<td>Total avg</td>
<td></td>
<td>1.02</td>
<td>0.94</td>
<td>1.54 (20/23)</td>
</tr>
</tbody>
</table>

a Histopathologic grading scales are as follows. Overall pulmonary inflammations (hematoxylin and eosin sections): 0, no discernible inflammation; 1, inflammation affecting less than 25% of examined tissue; 2, inflammation affecting 25 to 50% of examined tissue; 3, inflammation affecting 50 to 75% of examined tissue; 4, inflammation affecting over 75% of examined tissue. Pneumocystis lesions (hematoxylin and eosin sections): 0, no microscopic evidence of Pneumocystis infection; 1, multifocical poorly defined areas of inflammation consistent with Pneumocystis infection; 2, one or two well-defined areas of alveolar filling highly suggestive of Pneumocystis infection; 3, more than two well-defined areas of alveolar filling highly suggestive of Pneumocystis infection. Pneumocystis forms (Gomorri's methenamine silver sections): 0, no cysts seen; 1, rare single cysts seen at a rate of less than 1 per 10 40 fields; 2, 1 to 10 cysts per 10 40 fields; 3, 10 to 100 cysts per 10 40 fields; 4, >100 cysts per 10 40 fields.
b Means of groups of three rats.

* ND, not done.

erved, although there were no lesions or silver-staining forms suggestive of the presence of significant numbers of *P. carinii*. Since little is known about the first stages of *P. carinii* infection or colonization in the host, it is possible that this inflammation was due to the presence of small numbers of *P. carinii* undetected by the silver stain or to the presence of trophic forms of *P. carinii* that do not stain with the silver stain. Another possible explanation for the mild inflammatory reaction is the presence of an unidentified microbial population or idiopathic host process. Rats treated with methylprednisolone (Table 1) manifested lesions attributable to pneumocystosis, and silver-staining cyst forms typical of *Pneumocystis* were detected after the first week of induction, as was also the case for the cyclophosphamide-treated series (Table 2). There was a significant trend for the intensity of lesions attributable to pneumocystosis (cyclophosphamide, *r* = 0.56 and *P* < 0.01; methylprednisolone, *r* = 0.65 and *P* < 0.01) and for the number of silver-staining cyst forms (cyclophosphamide, *r* = 0.45 and *P* < 0.05; methylprednisolone, *r* = 0.55 and *P* < 0.01) to increase between weeks 2 and 8 for both immunosuppressants. No significant differences in the efficiencies of the immunosuppressants in facilitating histopathologic diagnosis by inducing overt expression of disease were demonstrated in the groups of rats evaluated in this study. Additionally, the results indicate that there was no further increase in the efficiency of detection of histopathologically positive animals after the third week of induction with either immunosuppressant. In all, 20 of 23 (87%) rats induced with methylprednisolone were found histopathologically positive for pneumocystosis, whereas 16 of 23 (70%) rats were similarly detected when induced with cyclophosphamide. The seeming difference between the effects of the immunosuppressants was probably due to some variability in the incidence of natural infection between groups, although we chose this particular colony to minimize such differences. The dramatic body weight loss observed with all groups treated with methylprednisolone was interpreted as a direct effect of the corticosteroid rather than of pneumocystosis, as the incidences of *P. carinii* within all of the groups were quite similar and the weight loss was profound in only the methylprednisolone-treated animals. We acknowledge that some slight differences in the abilities of the immunosuppressants to provoke histopathological lesions may be uncovered by the use of larger groups of animals, but it was the strategy of the present studies to use small numbers of animals for the purpose of cost efficiency in the commercial facility setting.

The results of the PCR analysis showed this technique to be useful for diagnostic detection, especially during the first 2 weeks of induction. Two of the three untreated rats were detected as positive by PCR, and four of six rats were detected as
positive after 1 week of induction (two of three in each immunosuppressant series) (Tables 1 and 2). Thus, six of nine rats were found positive by PCR under circumstances that would have resulted in false negatives if diagnosis were based on histopathologic results alone. This discrepancy was abolished by the end of the second through eighth weeks, since the results of histopathology and PCR were equivalent, 20 of 23 versus 19 of 23, respectively, with methylprednisolone and 16 of 23 versus 17 of 23, respectively, with cyclophosphamide. Thus, the chief advantage of PCR lay in the detection of P. carinii before microscopically observable recrudescence of the organisms.

The serologic results were quite significant, in that 37 of 49 (75.5%) preinduction samples were positive for antibodies to P. carinii as detected by immunoblotting. The frequency of serologic positives in the terminal samples, 38 of 46, or 82%, was not diminished by the immunosuppressive regimen. Serologic results in general paralleled those of PCR, and for both immunosuppressants combined, there were no significant differences (Cochran’s test; *P > 0.05*) between the detection rates of terminal serology and PCR (about 83%). Results for the nine rats in the untreated group and those given a single week of immunosuppressant treatment were revealing: although all of the rats were histopathologically negative, six of nine were serologically positive and six of nine were positive by PCR. However, the correlation between the methods was not particularly high (three of nine were PCR positive but serologically negative, and likewise, three of nine were serologically positive but PCR negative).

There were some discrepant results among PCR, immunoblot serology, and histopathology. In the cyclophosphamide series, PCR failed to detect 1 of 17 rats known to be positive by histopathology, and likewise, in the methylprednisolone series, 4 of 23 rats were positive by histopathology but undetected by PCR. Reevaluation of the lungs by resampling of the lung tissue followed by homogenization prior to digestion improved the sensitivity of the procedure, resulting in positive amplification of three of the four samples previously negative by PCR. These data suggest that the sensitivity of this PCR method depends on the method of tissue sampling, and they imply that the organisms may be focally distributed within the colonized or infected animal lung.

**DISCUSSION**

A primary goal of this study was to define the parameters of the immunosuppressive stress test sufficiently to establish guidelines enabling confident diagnosis of rat populations harboring P. carinii organisms. Under the conditions of this study, i.e., given the rat strain (F344), age, and sex, these parameters were determined to be as follows. (i) Methylprednisolone (4 mg/kg/week) and cyclophosphamide (33 mg/kg/week) functioned equivalently in forcing expression of histopathologically confirmed pneumocystosis in ~80% of the rats in the study. (ii) A 2-week induction period was sufficient to enable histopathologic detection of at least two rats in a group of three, thereby establishing the presence of P. carinii in the rat colony. There was no further increase in the frequency of histopathologic confirmation after the third week of induction. (iii) Immunoblotting of sera from the 49 rats used in these studies showed reactivity to P. carinii antigens in 38 rats (77.5%) prior to any immunosuppressive therapy. There were no significant increases or decreases in serologic detection by virtue of induction. (iv) Amplification of a region of the P. carinii TS gene by PCR permitted detection of an apparent carrier state in all groups of rats, including those surveyed on the day of arrival, those in a noninduced group, and the terminal samples from rats receiving a single dose of either immunosuppressant. As with immunoblotting detection, a positive PCR did not require immunosuppressive induction to detect P. carinii carriers.

The two drugs used as immunosuppressants in the study have very different mechanisms of action (5, 11). Methylprednisolone is a glucocorticoid with profound effects on the immune system, especially lymphocyte number and function, and on almost every organ system. Thus, chronic administration of glucocorticoids leads to skeletal muscle wasting, resulting in reduction of body mass, as observed for the rats in this study. The finding of weight loss in rats (and mice) undergoing corticosteroid immunosuppression for expression of pneumocystosis has been previously reported (3, 14, 32) and has been used to gauge the progression of the disease process in many laboratories. In contrast, cyclophosphamide is a DNA-alkylating agent, the effects of which result primarily in inhibition of DNA synthesis, particularly in proliferating cells. Cachexia has not been reported for this drug. The weight gain (or noninterference with normative growth) of the rats in this study during immunosuppression with cyclophosphamide appears to be a new finding, since previous studies in which cyclophosphamide was used to express latent agents have not followed animal weights during induction (6, 26, 29).

An 8- to 10-week induction period has been a standard recommendation for detection of P. carinii in commercial rodent colonies (15), a time period used to maximize expression of

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**TABLE 2. Histopathologic lesion and cyst scores and Western immunoblot and PCR results in rats during cyclophosphamide induction**

<table>
<thead>
<tr>
<th>Animal no.</th>
<th>Weeks of induction</th>
<th>Pulmonary histopathology</th>
<th>Western immunoblotting</th>
<th>PCR (no. positive/no. tested)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Overall inflammation</td>
<td>Pneumocystosis lesions</td>
<td>GMS cyst score</td>
<td>Preinduction</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(no. positive/no. tested)</td>
<td></td>
</tr>
<tr>
<td>28–30</td>
<td>1</td>
<td>0.66</td>
<td>0.33</td>
<td>0.00 (0/3)</td>
</tr>
<tr>
<td>31–33</td>
<td></td>
<td>2.00</td>
<td>1.33</td>
<td>0.66 (2/3)</td>
</tr>
<tr>
<td>34</td>
<td>Died</td>
<td>ND</td>
<td>ND</td>
<td>ND</td>
</tr>
<tr>
<td>35–36</td>
<td>3</td>
<td>1.00</td>
<td>0.50</td>
<td>1.00 (2/2)</td>
</tr>
<tr>
<td>37–39</td>
<td>4</td>
<td>1.33</td>
<td>1.00</td>
<td>1.66 (3/3)</td>
</tr>
<tr>
<td>1–3</td>
<td></td>
<td>2.33</td>
<td>2.00</td>
<td>2.33 (3/3)</td>
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<tr>
<td>4–6</td>
<td></td>
<td>1.33</td>
<td>1.66</td>
<td>1.33 (2/3)</td>
</tr>
<tr>
<td>7–9</td>
<td></td>
<td>2.33</td>
<td>2.33</td>
<td>1.33 (3/3)</td>
</tr>
<tr>
<td>10–12</td>
<td></td>
<td>1.33</td>
<td>0.33</td>
<td>0.33 (1/3)</td>
</tr>
<tr>
<td>Total or avg</td>
<td>1.54</td>
<td>1.10</td>
<td>1.08</td>
<td>19/23</td>
</tr>
</tbody>
</table>

*a Histopathologic scoring is described in note a to Table 1. *b Means of groups of 3 rats. *c ND, not done.
pneumocystosis for purposes unrelated to detection of latent infection (2, 3, 22, 32). Data from the present study suggest that a 2-week induction period should be sufficient to histopathologically support or reliably refute the existence of the latent carrier state in sample groups drawn from breeding colonies.

Serologic diagnosis by the immunoblotting technique was quite effective in detecting antibodies indicative of exposure to \textit{P. carinii} in all groups of rats prior to induction. The presence of antibodies to \textit{P. carinii} antigens in nonimmunosuppressed rats has been reported extensively in the literature (4, 7, 20, 31) but has not been routinely accepted as a criterion for the expected development of \textit{P. carinii} pneumonia, since the presence of antibodies does not always correlate with development of pneumocystosis and their absence does not guarantee the lack of infection (7). Our study shows that >75% of a subpopulation of rats taken from an enzootically infected animal colony (representing about 5% of the male nonbreeding population) were seropositive to \textit{P. carinii}. Thus, in such a setting, random sampling of animal sera for the presence of anti-\textit{P. carinii} antibodies would be useful to qualitatively assess the presence of \textit{P. carinii} within a large commercial rat colony.

There are, nonetheless, several factors that hinder endorsement of serology as a generally useful diagnostic tool in the commercial setting at the present time. Chief among them is the practical problem of the lack of availability of validated testing reagents (antigens) in bulk and in sustained production to support high-volume testing programs. There are theoretical problems as well, which relate to documented demonstrations of genetic (hence, immunologic) diversity of putative \textit{P. carinii} strains and species among host species (4, 17, 30), as well as evidence for genetic and antigenic diversity of \textit{P. carinii} distributed within a given host species (8–10, 27), which argues against the designation of a universal antigen preparation.

There are two important considerations favoring development of a diagnostic serologic tool. First, the test could be performed on individual experimental rats without requiring their sacrifice, prior to a large-scale animal study or purchase, using a relatively noninvasive blood collection procedure. Second, it is conceivable that an antigen preparation suitable for the survey of \textit{P. carinii} within a particular mammalian species (e.g., the rat) will become available as the repertoire of the surface antigens is more fully defined and common epitopes are identified (17–19, 24).

Although the animals were histopathologically negative, \textit{P. carinii} ampicloids were produced by PCR from the lungs of six of nine terminally collected samples from noninduced rats and from those that received a single immunosuppressant dose. While suggestions that PCR could be used to detect the latent carrier state in noninduced rodents have been made previously (16, 23), most studies have not focused on the assay as a means to detect the presence of the organism in large-scale commercial rat colonies. Although our study used a single-copy gene as the target for PCR, it is likely that the sensitivity of detection would be increased by the use of a multicopy gene target, such as the mitochondrial large-subunit rRNA gene (13, 28).

The case for diagnostic screening of rodent colonies for latent \textit{P. carinii} carriers by PCR of lung samples taken from rats in noninduced sample groups is so compelling on the basis of time, availability of reagents, economy, and accuracy as to strongly endorse this technique as the method of choice with single or multiple genes as targets. The major drawback to a PCR-based assay is the requirement for postmortem tissue, as bronchoalveolar lavage and needle biopsies are difficult to perform on small laboratory animals with a high survival rate.

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REFERENCES


