Comparison of Corticosteroid- and L3T4+ Antibody-Immunosuppressed Mouse Models of Pneumocystis carinii Pneumonia for Evaluation of Drugs and Leukocytes

Marilyn S. Bartlett,1* William L. Current,2 Attilio Orazio,1 Nancy L. Bauer,1 Richard S. Neiman,1 Sherry F. Quiener,1 and James W. Smith1

Indiana University School of Medicine1 and Eli Lilly & Co.,2 Indianapolis, Indiana

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An immunologically immunosuppressed mouse model of Pneumocystis carinii pneumonia using antibody developed by Dialynas et al. (Immunol. Rev. 74:29–55, 1983) directed to L3T4+ T cells (referred to as L3T4+ antibody) was compared with a corticosteroid-immunosuppressed mouse model. Corticosteroid- or L3T4+ antibody-immunosuppressed BALB/c mice transtracheally inoculated with P. carinii developed severe infections within 5 weeks after inoculation and responded to treatments with an echinocandin B analog, LY302146, or trimethoprim plus sulfamethoxazole so that they had decreased numbers of P. carinii cysts and trophozoites. L302146 appeared to be more effective in L3T4+ antibody-immunosuppressed mice than in dexamethasone-immunosuppressed mice. Leukocyte populations in lungs of both mouse models during development of infection and during treatment were compared by using immune cell-specific staining. Lungs of L3T4+ antibody-immunosuppressed mice had many more cells detected with pan-B antibody and pan-T antibody than dexamethasone-immunosuppressed mice, and the lungs of successfully treated mice had about the same numbers of macrophages as those of nonimmunosuppressed uninfected mice. The immunologically immunosuppressed model will allow study of cytokines and other immune modulators alone and in combination with drugs.

Historically, Pneumocystis carinii pneumonia has been the initial disease manifestation in more than 60% of AIDS patients in North America and has ultimately occurred in up to 85% of them (8, 22). Widespread implementation of prophylaxis for P. carinii pneumonia has been successful in lowering its incidence; however, it remains an important disease. Even after widespread use of prophylaxis, more deaths occurred in the United States in 1991 from P. carinii pneumonia than from any other reportable infectious disease (18). Thus, a major goal for biomedical research is better diagnosis, prevention, and treatment of P. carinii pneumonia. To meet this goal, more reliable and predictive animal models are needed.

Most animal models of P. carinii pneumonia have relied on corticosteroid-induced immunosuppression to activate latent P. carinii infections in Sprague-Dawley rats (13, 19–21, 25, 33). The utility of these animal models is often limited by variability in frequency and severity of latent P. carinii infections, and as a consequence, the time required to develop heavy infection and severity of infection also varies from animal to animal. P. carinii isolates from different sources also show variability in karyotype and may differ in other more subtle and as-yet-unidentified ways (10). Latently infected non-virus-free animals immunosuppressed to develop heavy P. carinii infections often develop secondary infections that render many unusable. To circumvent these problems, Bartlett et al. (2), using virus-free and P. carinii-free rats, developed a transtracheally inoculated rat model; Boylan and Current (6) modified this model by an intratracheal inoculation. The use of specific-pathogen-free animals, sterile food, and bacterium- and fungus-free P. carinii inocula results in a low incidence of secondary infections, especially if animals are maintained in isolation. These models consistently produce severe P. carinii pneumonia within 5 weeks after inoculation of laboratory-maintained strains of P. carinii and have been used for 7 years in evaluating compounds for anti-P. carinii activity (1). Drugs currently moving into clinical trials have been identified with these models (4). All rat models to date have used immunosuppression with corticosteroids.

Several mouse models also have been described. An inoculated mouse model reported by Shellito et al. (27) provided a non-steroid-immunosuppressed model that used monoclonal antibody directed to L3T4+ cells, which are comparable to human CD4+ cells, as described by Dialynas et al. (11); however, that model was not very useful for compound evaluation since only 60% of the animals developed P. carinii infection and only after 12 weeks. A mouse model that used the same antibody immunosuppression was used to evaluate the effects of aerosolized gamma interferon (5), but the study evaluated only P. carinii cysts in the lungs even though tissue-invasive and intra-alveolar organisms are predominately trophozoites (23). Neither study compared antibody-induced immunosuppression with corticosteroid-immunosuppression, nor did either of them evaluate inflammatory cell populations in the lungs. Studies of immune function have been carried out in severe combined immunodeficient (SCID) mice that were naturally infected with P. carinii and then reconstituted with spleen cells (17), but the method is cumbersome and has variability in incidence and intensity of infection among mice. Naturally infected SCID or nude mice have variable severities of infection, and it takes many weeks or months for groups of animals to develop heavy infections (28, 30, 31). In addition, SCID mice may develop infections with bacteria, yeasts, and viruses (30). One mouse model relied upon transmission of P. carinii.
carinii by housing infected mice with uninfected mice and used dexamethasone as the agent of immunosuppression (26).

The transtracheally inoculated dexamethasone-immunosuppressed mouse model reported by Bartlett et al. (3) has proven useful for evaluation of drugs because infections are consistently heavy and develop quickly; however, the large doses of dexamethasone, 1.2 mg/kg of body weight per day, greatly affect immune responses. Also, corticosteroids stabilize lysosomal membranes, suppress the inflammatory response, and interfere with processing and presentation of antigen by macrophages. Not only do corticosteroids produce nonspecific immunosuppression by reducing lymphocyte and other inflammatory cell populations, but they also act as catabolic agents, causing severe weight loss and debility.

None of the previously reported models based on depletion of specific components of the immune system have developed consistently heavy P. carinii pneumonia. In this paper we report the use of the monoclonal antibody directed to the L3T4+ T-helper/inducer (T) cells (referred to as L3T4+ antibody) in a mouse model that consistently develops heavy infections of P. carinii within 5 weeks after transtracheal inoculation with inocula from frozen stores of banked P. carinii-infected mouse lung tissue. This model is compared with the corticosteroid-immunosuppressed mouse model for the evaluation of drugs. In addition, we evaluated differences in cell populations in the lungs of control and drug-treated, P. carinii-infected animals immunosuppressed with either dexamethasone or L3T4+ antibody.

MATERIALS AND METHODS

To produce and use the L3T4+ antibody, we used methods reported by Gomez et al. (15), Ershler et al. (12), Stull et al. (29), Shellito et al. (27), and Furney et al. (14). Difficulties in obtaining quantities of antibodies from culture (15), irradiated mice (12), or CBA/J mice (29) turned our attention to the use of thymectomized mice (14), and we ultimately used nu/nu mice. Cells from clone GK1.5 were purchased from the American Type Culture Collection and were also kindly provided by I. M. Orme.

Acute pulmonary infection. GK1.5 cells cultured in 75-cm² tissue culture flasks (Corning) in Dulbecco modified Eagle medium (Sigma, St. Louis, Mo.) plus 20% fetal calf serum (Intergen) at 35°C in 5% CO₂ were grown to confluency, and then 2 × 10⁶ to 10 × 10⁶ cells were injected intraperitoneally into each Pristane (Sigma)-primed nu/nu nude (Charles River). Ascites developed in 4 to 14 days; fluid was collected and centrifuged at 200 × g for 10 min, and the supernatant was frozen at −70°C.

Ascites purification. All ascitic fluids were checked for immunoglobulin G by enzyme-linked immunosorbent assay (ELISA) prior to antibody purification. The fluid was titrated by using twofold dilutions and primary antibody, alkaline phosphatase-tagged anti-rat immunoglobulin G (Sigma) diluted in phosphate-buffered saline (PBS)-0.02% azide to 1:1,000. The plates were incubated with substrate (1 mg of p-nitrophenyl phosphate [Sigma] per ml) in substrate buffer and read at 405 nm on an ELISA reader.

Antibodies were purified from the ascites by ammonium sulfate precipitation. The precipitate was dialyzed at 4°C overnight with two changes of PBS and then centrifuged at 3,000 × g for 30 min to remove any remaining debris. The protein concentration was determined by measuring the A₂₅₀. The solution was cultured to assess the degree of contamination with bacteria, and low numbers of bacilli were detected. The antibody was stored at −20°C.

Immunosuppression of mice. Female BALB/c mice, 6 to 8 weeks old and free from P. carinii, were obtained from Harlan Sprague-Dawley, Indianapolis, Ind. In initial studies, doses of 0.1 and 0.5 mg of the purified antibody solution were given twice weekly by intraperitoneal injection. Infection of mice showed that the low dose, 0.1 mg, was sufficient to allow infection to develop, although the mean score for severity was somewhat lower than that of the mice receiving the higher dose. Subsequent studies used 0.2 mg of purified antibody given twice a week. After 1 week, leukocytes were evaluated in Giemsa-stained blood films to monitor lymphocyte depletion. When lymphocyte depletion was greater than 50% (14 days), the mice were transtracheally inoculated with 10⁵ P. carinii trophozoites from banked frozen inoculum (3). BALB/c mice obtained at the same time from the same supplier were immunosuppressed with dexamethasone at 1.2 mg/kg/day in drinking water, evaluated for lymphocyte depletion, and transtracheally inoculated as described above.

Treatment of mice. The anti-P. carinii drugs used in this study were chosen because of their different modes of action and because their activity may be directed to different life cycle forms of P. carinii. The combination drug trimethoprim-sulfamethoxazole (TMP/SMX) inhibits folate metabolism and is active against both trophozoite and cyst forms of P. carinii. LY302146, from Eli Lilly & Co., is a semisynthetic analog of echinocandin B, a compound with known antifungal and anti-P. carinii activity (9). As is the case with other compounds in the echinocandin B class, LY302146 is an inhibitor of fungal (1,3)-β-d-glucan synthase and appears to cause rapid destruction of P. carinii cysts. Its effects on trophozoite forms have not been well studied.

In the first preliminary study, dexamethasone- and antibody-immunosuppressed mice were evaluated for host response and development of P. carinii infection during immunosuppression and during treatment with LY302146 at 2.0 mg/kg/day by intraperitoneal injection and TMP/SMX at 50/250 mg/kg/day in drinking water. Each group also received tetracycline (0.5 g/liter) in drinking water to prevent bacterial infection. The numbers of lymphocytes and macrophages in lungs were evaluated prior to any treatment and at 4 days and 2 and 3 weeks after treatment and compared with numbers for untreated mice and immunosuppressed but untreated mice. Animals were sacrificed by exsanguination, and lungs either were perfused, fixed in formalin, prepared for sectioning in paraffin blocks, sectioned, and stained with hematoxylin and eosin or were perfused with a 1:2 dilution of TissueTek and saline, frozen, and used for frozen sections (study 2). Slides from paraffin or frozen sections were stained with antibodies directed to different antigens, as follows: B cells, monoclonal CD45R (clone RA3-6B2), Gibco, Gaithersburg, Md., at a 1:2 dilution; T cells, monoclonal CD3 (clone YCD3-1), Gibco, at a 1:2 dilution; cytotoxic/suppressor T cells, monoclonal CD8a (clone 53-6.7), Gibco, at a 1:2 dilution; helper/inducer T cells, monoclonal CD4 (clone YTS191.12), Gibco, at a 1:2 dilution; monocytes/macrophages, lysozyme (polyclonal), Dako, Santa Barbara, Calif., at a 1:400 dilution; proliferating macrophages monoclonal to proliferating cell nuclear antigen, PCNA (clone PC10), Dako, at a 1:80 dilution. Impression smears of lung samples were stained with Giemsa azure B (Harleco, Gibbstown, N.J.) and with a modified methenamine-silver nitrate stain which uses a microwave oven (7).

In study 2, mice in groups of 10 were immunosuppressed with either dexamethasone or L3T4+ antibody and then were treated with LY302146 or TMP/SMX as described above or were left untreated. The evaluation of infectivity and cell populations was as described below.
Immunohistochemical staining. Cryostat sections of mouse lung tissue (4 μm thick) were attached to poly-l-lysine-covered slides and fixed in acetone for 15 min at room temperature. To block nonspecific staining, slides were transferred to PBS, treated with H₂O₂ for 4 min, and covered with normal goat serum for 20 min. Blocked sections were incubated overnight with the following primary antibodies: CD3 (YCD3) for T cells, CD45R (RA3-6B2) for B cells, CD4 (YTS191.12) for helper/inducer cells, CD8 (53-6.7) for cytotoxic/suppressor cells, polyclonal anti-lysozyme antibody for macrophages and monocytes, and PCNA for proliferating macrophages. Slides were then stained with a biotin-conjugated goat anti-mouse antibody for 30 min (Kirkegaard & Perry Laboratories, Gaithersburg, Md.) followed by peroxidase-conjugated streptavidin for 30 min (Kirkegaard & Perry Laboratories). The enzyme was developed with 3,3'-diaminobenzidine (Sigma). Cells were quantified on areas measuring at least 5 mm² and counted at a magnification of ×400 in at least 10 fields with an ocular grid (24). Neutrophils were identified morphologically. Fields with the highest cellular density were selected. These included all foci of consolidation of alveoli, areas containing vacuolated macrophages, and all perivascular and peribronchial inflammatory infiltrates. In study 2, the first two mice of each group were used for cell type evaluations.

Determination of infection. In study 1, infection levels in mice were evaluated over time post-*P. carinii* inoculation and after 4, 14, and 21 days of drug treatment. In study 2, infection levels were evaluated after 3 weeks of drug treatment. Untreated infected control mice were evaluated at the same time points as treated mice. Impression smears of lung portions were made at the time of sacrifice and evaluated by microscopic examination of Giemsa and silver stains according to the following roughly logarithmic scale: >100 organisms per 1,000× field = 5+; 1 to 100 organisms per field = 4+; 1 to 10 organisms per field = 3+; 2 to 9 organisms in 10 fields = 2+; 1 organism in 10 or more fields = 1+; and no organisms in 50 fields = 0 (1). In 3 years of use with mouse models, this scale for Giemsa-stained samples has yielded scores of 4.4 ± 0.1 for untreated controls and 0.1 ± 0.1 for TMP/SMX-treated mice (means ± standard errors).

RESULTS

In the first study, which evaluated development of infection and leukocyte cell populations over time, we found that there was greater proliferation of neutrophils and lysozyme-positive macrophages in the more heavily infected animals. Untreated mice immunosuppressed with L3T4⁺ antibody had approximately 260 macrophages and 125 neutrophils per 400× field, and untreated mice immunosuppressed with dexamethasone had approximately 190 macrophages and 210 neutrophils per 400× field. There were fewer lysozyme-positive macrophages and there were fewer proliferating macrophages detected by PCNA staining in mice immunosuppressed with L3T4⁺ antibody and treated with LY302146 than in the LY302146-treated mice immunosuppressed with dexamethasone. LY302146-treated mice immunosuppressed with L3T4⁺ antibody had about 150 macrophages and 40 neutrophils per 400× field, while LY302146-treated mice immunosuppressed with dexamethasone had about 110 macrophages and 128 neutrophils per 400× field. Uninoculated mice had few macrophages detected and a low number of cells reactive to pan-T antibody. Fewer organisms were found in the LY302146-treated animals that were immunosuppressed with the L3T4⁺ antibody than in the dexamethasone-immunosuppressed animals. Mean scores of infection for stained impression smears were 0.0 for the LY302146-treated L3T4⁺ antibody-immunosuppressed mice (n = 3), compared with 3.0 for the LY302146-treated dexamethasone-immunosuppressed mice (n = 2), 4.1 for untreated control mice (n = 2) immunosuppressed with L3T4⁺ antibody, and 4.2 for untreated control mice immunosuppressed with dexamethasone. Control mice developed infections of comparable severities. Mice treated with LY302146 and immunosuppressed with antibody had very few organisms after 2 weeks of treatment, while those given the same treatment but immunosuppressed with dexamethasone had initial reductions in numbers of organisms (Giemsa score, 0.6 at 2 weeks) but at sacrifice (3 weeks of treatment) still had significant infections, as seen in Fig. 1. On the basis of the results of this preliminary study, the larger second study was performed.

In the second study, mice in groups of 10, immunosuppressed and treated with LY302146 or TMP/SMX, had the infection scores shown in Fig. 2. Giemsa stain scores include both trophozoite and cyst forms, while silver stain scores are for cyst forms only. These data along with data obtained in the preliminary studies demonstrated that untreated control mice immunocompromised by L3T4⁺ monoclonal antibody treatment developed heavy consistent infections similar to those in mice immunosuppressed with high-dose dexamethasone (3.7 ± 0.3 is not statistically different from 4.3 ± 0.2 [Table 1]). The two studies taken together suggested that LY302146 might be more effective in clearing *P. carinii* from the lungs of mice immunosuppressed with the L3T4⁺ antibody than the lungs of mice immunosuppressed with dexamethasone; the differences are marked in the preliminary study with small numbers of mice and barely miss being statistically significant in study 2 (Table 1). As expected, high-dose TMP/SMX was highly effective in eliminating *P. carinii* from the lungs of mice immunosuppressed with L3T4⁺ antibody or with dexamethasone.

Inflammatory cell populations in lungs of infected mice after...
FIG. 2. Mean *P. carinii* infection scores of mice in groups of 10 that were untreated (control) or treated with LY302146 (LY-46) or TMP/SMX and were immunosuppressed with L3T4+ antibody or dexamethasone (study 2). Infection scores are logarithmic representations of the number of trophozoites and cysts in Giemsa-stained (A) and methenamine-silver nitrate-stained (B) impression smears of lung. Mean scores (X) ± standard errors are given above the bars for each group. n, number of animals in group.

3 weeks of treatment were compared with those of untreated immunosuppressed control mice as shown in Table 1. L3T4+ antibody-immunosuppressed mice had a variable number of cells reactive to CD3+ antibody, mostly arranged in perivascular and peribronchial infiltrates as well as numerous B cells. Heavily infected mice had many neutrophils. Dexamethasone-immunosuppressed mice had almost no cells reactive to T or B antibodies. More inflammatory cells were seen in the more heavily infected animals. In both TMP/SMX- and LY302146-treated mice in which *P. carinii* had been almost eliminated, there were fewer macrophages detected by lysozyme staining and fewer proliferating macrophages detected by PCNA activity than in the untreated immunosuppressed control mice. In the dexamethasone-immunosuppressed untreated mice there were many macrophages and neutrophils, suggesting that depletion of lymphocytes by the corticosteroid immunosuppression does not prevent accumulation of these cells in infected lungs. Mice immunosuppressed with L3T4+ antibody

### TABLE 1. Infection scores and cell counts for treated and untreated mice immunosuppressed with L3T4 antibody or dexamethasone

<table>
<thead>
<tr>
<th>Drug and immunosuppressant*</th>
<th>Infection scoreb</th>
<th>Cell count (no./mm³)c</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>CD3 cells</td>
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<tr>
<td></td>
<td></td>
<td>Mouse 1</td>
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<tr>
<td>None</td>
<td></td>
<td>3.7 ± 0.3</td>
</tr>
<tr>
<td>Dex</td>
<td></td>
<td>4.3 ± 0.2</td>
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<tr>
<td>L3T4</td>
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<tr>
<td>LY302146</td>
<td></td>
<td>1.4 ± 0.3</td>
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<tr>
<td>Dex</td>
<td></td>
<td>0.6 ± 0.1</td>
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<tr>
<td>L3T4</td>
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<td></td>
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<tr>
<td>TMP/SMX</td>
<td></td>
<td>0.1 ± 0.1</td>
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<tr>
<td>Dex</td>
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<tr>
<td>L3T4</td>
<td></td>
<td>0</td>
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</table>

* Dex, dexamethasone.

b Infection scores were evaluated in Giemsa-stained impression smears from each of 10 mice in each group. *P* values for the comparison of each experimental set to its own control were all <0.0001 (analysis of variance, unpaired t test). The *P* value was 0.12 for the comparison of untreated controls in the dexamethasone and L3T4 sets; the *P* value for the comparison of LY302146 treatment in the dexamethasone and L3T4 sets was 0.05, but the standard deviations in these two sets of data were not appropriately distributed, invalidating this parametric test. By the nonparametric Mann-Whitney test, these values were not significantly different (*P* = 0.12).

c Counts were made on separate lung sections from two mice for each cell type. CD4 staining showed only a few positive cells in all specimens (results not shown).

⁴ Very weak staining observed.

⁵ NA, datum not available.
had many more B cells, cells reactive to pan-T antibody, and cytotoxic/suppressor T cells than dexamethasone-immunosuppressed mice.

**DISCUSSION**

The immune status of the immunologically immunosuppressed mouse model is more analogous to the immune status of AIDS patients, who have depleted CD4 cells, than is the immune status of the dexamethasone-immunosuppressed mouse or rat model. Although the rat model has most often been the means of evaluating compounds for efficacy against P. carinii and has been predictive of utility in the treatment of human P. carinii pneumonia, the antibody-immunosuppressed model may have some distinct advantages. Our data suggest that LY302146, a cyclic lipopeptide of the echinocandin class of antifungal agents (9), may be more effective in inhibiting the proliferation of P. carinii in mice immunosuppressed with antibody directed to the L3T4+ lymphocytes than in mice immunosuppressed with dexamethasone. Compounds which require or are more effective when there are intact host defenses other than CD4 cells may be better evaluated with the L3T4+ model. The similarity of host defenses in this model to those in AIDS may make it a better predictor of the utility of some drugs than models using high-dose corticosteroids which nonspecifically deplete lymphoid cells.

The immune cell-specific staining of lung samples of L3T4+ immunosuppressed mice showed that L3T4+ cells (helper/inducer) were depleted while other cell populations appeared to be similar to those of nonimmunosuppressed mice. The numbers of CD8 cells and B cells were greatest in untreated L3T4+ antibody-immunosuppressed infected mice and lower in animals successfully treated with either drug. In the corticosteroid model it was impossible to evaluate CD4, CD8, and B cells because of the marked lymphoid cell depletion, whereas the L3T4+ antibody-immunosuppressed model allowed evaluation of cell populations. In both immunosuppression groups numbers of macrophages were lower in successfully treated mice than in heavily infected controls. As has been reported for AIDS patients with severe P. carinii infection (16, 32), neutrophil numbers were higher in the lungs of severely infected animals and lower in the lungs of successfully treated mice.

The L3T4+ model, with its closer similarity to the immune status of AIDS patients, may be more discriminating than previous models; our data suggest that LY302146 may be more active against P. carinii in the L3T4+ model. This model may also be useful for evaluating effects on P. carinii infection of cytokines and other modulators that could not be studied in immunosuppressed models. The L3T4+ model may also be useful for studies of the mechanisms by which corticosteroids prevent worsening of clinical symptoms early in antimicrobial therapy of AIDS patients with severe P. carinii pneumonia.

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**REFERENCES**


