JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1987, p. 480–484
0095-1137/87/030480-05$02.00/0

Vol. 25, No. 3

Improved Rat Model for Studying *Pneumocystis carinii* Pneumonia

MARILYN S. BARTLETT,[1] SHERRY F. QUEENER,[2]* MARGARET A. JAY,[1] MICHELE M. DURKIN,[1] AND JAMES W. SMITH[1]

Departments of Pathology[1] and Pharmacology and Toxicology,[2] Indiana University School of Medicine, Indianapolis, Indiana 46223

Received 14 July 1986/Accepted 2 December 1986

Sprague-Dawley rats treated for 8 weeks with cortisone acetate (25 mg per rat twice weekly) were immunosuppressed to variable degrees. A total of 55% lost over 12% of their initial body weight, had cortisol concentrations in serum more than five times greater than those of the controls, and had markedly depressed ratios of helper to non-helper T cells, in both the spleen and peripheral blood. Animals that gained weight during immunosuppression had cortisol concentrations in serum only three times higher than those of the controls, had normal ratios of helper to non-helper T cells in the spleen, and had only modestly reduced T-cell ratios in peripheral blood. The degree of *Pneumocystis* pneumonia was evaluated in impression smears and sections of lungs taken from immunosuppressed rats. *Pneumocystis* infections were more severe in the rats that showed the greatest weight loss. Weight change during immunosuppression may therefore be used as a reliable means for predicting the degree of *Pneumocystis* infection in living rats. This protocol allows the selection of uniformly infected rats for studies assessing drug therapy of *Pneumocystis* pneumonia.

Sprague-Dawley rats, when immunosuppressed by adrenocortical steroids, spontaneously develop *Pneumocystis carinii* infection. This rat model, originally described by Frenkel et al. (4), has been used to study the pathogenesis and pathology of infection with *P. carinii* (10), as well as to provide a source of organisms for other studies or for culture (1). In addition, the model has been used for evaluating the effectiveness of various drugs for prophylaxis or therapy of *Pneumocystis* pneumonia (6, 9).

Using the model to screen additional agents for therapy of *Pneumocystis* pneumonia, we found that great variability in the severity of *Pneumocystis* infection among immunosuppressed rats from the same colony made the evaluation of drug effects difficult. We herein report that weight change during immunosuppression can be used to separate rats into groups with uniform infection. By minimizing the variation in severity of infection within groups of animals, the power of the rat model to reveal statistically significant drug effects is improved.

MATERIALS AND METHODS

Female Sprague-Dawley rats were purchased from Harlan Laboratories and Cox Laboratories (both in Indianapolis, Ind.) and from Charles River Breeding Laboratories, Inc., Wilmington, Mass.). Rats from Harlan Laboratories were studied most extensively, with pilot studies done on rats from Cox Laboratories and Charles River Laboratories. All rats were fed a regular diet and housed one per cage. Rats weighing between 100 and 200 g received 25 mg of cortisone acetate subcutaneously twice weekly for 8 to 16 weeks. Rats weighing over 200 g received 40 mg, and those weighing less than 100 g received 18 mg on the same schedule. Tetracycline was added to drinking water to achieve a dose of 15 mg per rat per day. Rats were weighed weekly during the immunosuppression period.

Two groups of 42 and 63 rats from Harlan Laboratories were studied. Rats in these groups had initial weights of 131.9 ± 1.2 and 139.1 ± 0.6 g, respectively; they were housed in an open room. The mean biweekly dose of cortisone acetate in these animals was 185 mg/kg of body weight. Two animals in each group were lost in housing accidents before the end of the study; data from these rats are not included in Table 1.

Five rats from Charles River Laboratories were originally housed in barrier cages in which the air into the cage was filtered. After 8 weeks of immunosuppression with weight gain and no clinical evidence of infection with *P. carinii*, two of these rats were transferred to the open room which contained the Harlan rats. Five rats from Cox Laboratories were housed in open cages in an isolation room.

To evaluate the severity of infection, rats were anesthetized with ketamine hydrochloride and sacrificed by exsanguination. The lungs were removed aseptically, impression smears were prepared, portions were fixed in Formalin for histology, and additional portions were frozen and subsequently cultured as previously described (1). Both impression smears and sections were stained with a rapid methenamine silver stain by the procedure of Brinn (2), with the modification that the chromic-acid step was done at room temperature for 10 min rather than in a microwave oven. Positive *Pneumocystis* control slides were included with each run. Impression smears were also stained with Giemsa stain; sections were also stained with hematoxylin and eosin.

Slides were examined as unknowns by two examiners, and their readings were combined for composite scores. The severity of infection was determined on a scale of 1 to 4+, with 4+ representing more than 10 organisms per 1,000× field, 3+ representing 1 to 10 organisms per field, 2+ representing 2 to 9 organisms in 10 fields, 1+ representing 1 organism in 10 fields, and 0 representing no organisms seen. The numbers of both trophozoites and cysts were evaluated from the Giemsa stains, but only the numbers of cysts could be evaluated from the silver stains.

From selected rats, blood was collected in preservative-free heparin, and the spleens were collected in RPMI 1640 for T-cell studies. The cortisol level in serum was determined by using GammaCoat radioimmunoassay (Travenol-

* Corresponding author.

TABLE 1. Changes in body weight of rats during immunosuppression[a]

Wt change (no. of rats)	Mean initial body wt (g) ± SEM	Mean wt change[b] (g) ± SEM
Expt 1		
Loss of >15 g (21)	133.1 ± 1.3	−23.6 ± 1.0
Loss of 0–15 g (9)	132.4 ± 1.9	−7.2 ± 1.1
Gain (10)	130.0 ± 3.1	+12.5 ± 2.3
Expt 2		
Loss of >15 g (36)	139.9 ± 0.9	−23.6 ± 0.8
Loss of 0–15 g (15)	138.1 ± 0.9	−9.2 ± 1.0
Gain (10)	137.7 ± 1.4	+5.8 ± 1.5

[a] The animals were Sprague-Dawley female rats from Harlan Laboratories.
[b] Weight changes were calculated after 8 (experiment 1) or 9 (experiment 2) weeks of immunosuppression.

Genentech Diagnostics, Cambridge, Mass.). Leukocyte counts and differentials were determined on a Coulter Counter (model Plus IV; Coulter Electronics, Inc. Hialeah, Fla.).

For analysis of the T-cell subsets, approximately 10^6 mononuclear cells from either the spleen or peripheral blood were obtained by using Histopaque 1077 (Sigma Chemical Co., St. Louis, Mo.) density gradients. The cells were incubated with 10 μl of the appropriate antibody (Pan T, Helper T, or Non-helper T; Accurate Chemical and Scientific Corp., Westbury, N.Y.) for 30 min at 4°C. After two washes with phosphate-buffered saline–2% fetal bovine serum, the wash solution was aspirated, and 200 μl of goat anti-mouse immunoglobulin-fluorescein isothiocyanate (Coulter Clone; Coulter Immunology, Hialeah, Fla.) was added. After a 30-min incubation on ice, the cells were washed thrice with phosphate-buffered saline–2% fetal bovine serum, and the wash solution was removed by aspiration. One milliliter of cold resuspension medium was added, and the fluorescence was read on a fluorescence-activated cell sorter (FACS; Becton Dickinson and Co., Cockeysville, Md.) with a 100-μm orifice, 0.71-mA current, and 500 V on photomultiplier for fluorescence 1.0 (green). Background fluorescence or autofluorescence was subtracted using 10^6 unstained cells.

Data are reported as the mean ± the standard error of the mean here and in the tables.

RESULTS

Two large groups of age- and weight-matched rats from Harlan Laboratories were studied independently. The initial mean body weights of these rats were similar (131.9 ± 1.2 and 139.1 ± 0.6 g). Over half of these rats (n = 57) lost more than 15 g over the 8- or 9-week immunosuppression period,

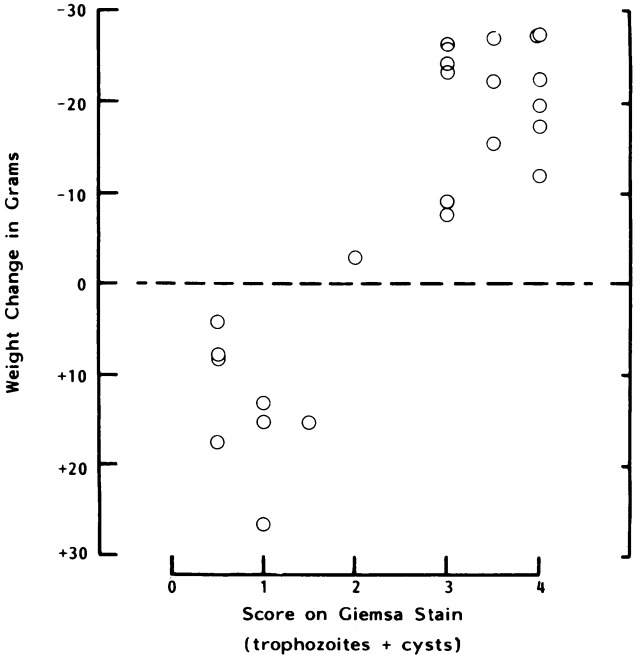

FIG. 1. Correlation of weight change after 9 weeks of immunosuppression with degree of *P. carinii* infection, as measured by Giemsa staining of lung impression smears taken after 11 weeks of immunosuppression. The animals are described in Table 2. Eight of the samples scored from +3 to +4 were also tested for viable organisms by culturing the lung. All grew *P. carinii*.

24 animals lost from 0 to 15 g, and 20 animals gained weight. Weight loss was most rapid during the first few weeks of immunosuppression; by week 6, body weight stabilized in most animals. Rats that gained weight tended to gain steadily during the period of observation. The initial weights did not correlate with the weight lost during immunosuppression (Table 1).

Twenty-four rats were randomly selected from experiment 1 for morphologic evaluation. The severity of infection, as evaluated by Giemsa stains of lung impression smears and methenamine silver stains of impression smears and sections, is shown in Table 2. The numbers of *Pneumocystis* organisms showed an inverse correlation with weight loss during immunosuppression (Fig. 1). The lungs from the rats described in Table 2 and Fig. 1 were used as inocula for cultures. Organisms were successfully cultured from all of the seven tested animals that lost >15 g; organisms from one of the eight tested animals that gained weight grew slightly in culture, and the rest failed to grow.

The cortisol concentration in serum in samples taken at sacrifice was determined in 10 rats, including those that lost

TABLE 2. Relationship between weight loss and severity of infection[a]

Wt change (no. of rats)[b]	Mean initial wt (g) ± SEM	Mean wt change (g) ± SEM	Severity of infection score[c]		
			Impression smears		Sections ($AgNO_3$)
			Giemsa	$AgNO_3$	
Loss of >15 g (12)	132.9 ± 7	−23.3 ± 3.9	3.5 ± 0.4	3.6 ± 0.7	3.4 ± 0.6
Loss of <15 g (4)	129.5 ± 6	−7.8 ± 3.8	3.0 ± 0.8	2.6 ± 0.7	3.0 ± 1.0
Gain (8)	130.7 ± 8	+13.5 ± 6.9	1.1 ± 1.0	1.8 ± 0.6	2.1 ± 0.9

[a] The animals described in this table were randomly selected from experiment 1 (Table 1).
[b] Mean weight changes were measured after 9 weeks of immunosuppression.
[c] Severity of infection scores were calculated from scores assigned by two independent evaluators (see Materials and Methods).

TABLE 3. Cortisol concentrations in plasma from normal and immunosuppressed rats[a]

Wt change (no. of rats)	Mean initial wt (g)	Mean wt change (g)	Cortisol concn in serum (μg/dl)
Loss of >15 g (4)	136 ± 3.0	−23.2 ± 2.1	11.1 ± 0.9
Gain (3)	139 ± 3.5	+4.6 ± 2.6	5.9 ± 1.2
Normal controls (3)	138 ± 3.0		2.2 ± 0.2

[a] Normal controls did not receive cortisone injections. All others received the standard doses of cortisone acetate. Plasma concentrations were measured 96 h after the last injection and represent trough concentrations. All animals except the normal controls were randomly selected from experiment 2 (Table 1).

weight and those that gained weight during immunosuppression (Table 3). All rats that were immunosuppressed maintained cortisol levels well above those of the untreated controls. Those rats that lost weight had significantly higher cortisol levels than those of the rats that gained weight. The cortisol values reported in Table 3 were from animals 96 h postinjection with cortisone. Animals were regularly injected at 72- to 96-h intervals; these values therefore repesent the lowest cortisol concentrations expected in these rats.

Immune function was assessed by measuring the populations of T-cell subsets both in the spleen and in peripheral blood from the same 10 immunosuppressed and normal rats as those used for cortisol determinations (Table 4). Animals that lost weight during immunosuppression showed markedly depressed proportions of helper cells, as well as overall suppression of leukocyte populations and diminished proportions of lymphocytes. In contrast, animals that received the same dose of cortisol but gained weight during immunosuppression had about the same proportions of helper cells in the spleen as normal rats that had not received cortisol; the proportion of helper cells in peripheral blood was lower than normal in these rats.

Pilot studies suggested that Sprague-Dawley rats from Cox Laboratories or Charles River Laboratories failed to develop severe *Pneumocystis* pneumonia during immunosuppression (Table 5). All of the rats from Charles River Laboratories, although they had moderate numbers of *Pneumocystis* cysts on silver stains, had few trophozoites on impression smears and had gained weight during the period of immunosuppression. All the rats from Cox Laboratories had high numbers of cysts (3 to 4+) detected in silver stains, and two of the five had significant numbers (3+) of trophozoites. None appeared ill at the time of sacrifice after 16 weeks of immunosuppression. *Pneumocystis* cultures of two rats from Charles River Laboratories and three rats from Cox Laboratories showed no growth.

DISCUSSION

The rat model of *Pneumocystis* infection developed by Frenkel et al. (4) has been modified by various investigators. Some workers have administered dexamethasone in drinking water for immunosuppression, whereas others have administered cortisone acetate subcutaneously. Diets have ranged from normal diets (23% protein) to low-protein diets (8% protein). Starting weights, ages, and sexes of the animals have also varied.

Most workers have reported that after immunosuppression for about 8 weeks, rats spontaneously develop *Pneumocystis* infection and appear ill but that some rats do not develop clinical disease or even morphologic evidence of infection (13). Some may die of bacterial infection during immunosuppression, despite treatment with antibiotics in drinking water. Several investigators have noted variability in susceptibility to *Pneumocystis* infection in different strains of rats or in the same strain from different suppliers or from the same supplier at different times. Although Sprague-Dawley rats are used most often in the United States, Wistar rats are used most often in Japan (11, 15), in part because the available Wistar rats are more susceptible to pneumocystis than the available Sprague-Dawley rats are. Even with Sprague-Dawley rats of similar size given the same immunosuppressive agent and regular diet, the degree of infection that developed varied. In three studies using 200-g rats immunosuppressed with dexamethasone given in drinking water at similar doses, the percent infected varied from 50% at 8 weeks (3) to 90 to 100% at 6 weeks (7) and 100% at 10 weeks (8).

For testing new agents for therapy of *Pneumocystis* pneumonia, a uniformly infected animal model is desirable. After pilot studies with rats from various suppliers, female Sprague-Dawley rats weighing 130 to 140 g from Harlan Laboratories were selected. Animals received standard laboratory chow, because animals on low-protein diets tended to be more prone to bacterial infections during immunosuppression and because organisms from the latter grew less well in culture.

The studies of Sprague-Dawley rats from Harlan Laboratories reported here showed that the severity of infection varied greatly. The lung impression smears stained with Giemsa were believed to be the best indicator of severity of infection and predictor of growth in culture. Giemsa stains trophozoites which are not evident in silver stains and stains intracystic bodies, thus staining only viable cysts. Silver and other cyst-wall stains stain both empty and organism-containing cysts. We previously noted that the ability to culture *P. carinii* is dependent on the number of trophozoites in the inoculum (M. Bartlett, S. Medley, M. Durkin, J. Piskura, and J. Smith. Abstr. Annu. Meet. Am. Soc. Trop. Med. Hyg., abstr. no. 175, p. 135, 1984).

Previous studies on Lewis rats illustrated variability in the level of infection with *P. carinii* (13, 14), but no one has investigated the source of animal-to-animal variation or reported on a method to select matched animals from a variably infected population. Our study showed that groups of infected animals closely matched in the severity of *Pneumocystis* infection can be selected by using animals that lost more than 15 g during 8 to 9 weeks of immunosuppression. Most of these animals were able to survive an additional 4 weeks of immunosuppression for continuing studies. Mortality in this group could be further limited if animals that weighed less than 100 g were not used. Animals with mild weight loss (0 to 15 g) were still significantly infected but were less infected on average than the rats that lost more than 15 g were. Animals that gained weight during 9 weeks of immunosuppression were only mildly infected. These animals were not used for evaluation of therapy or as sources of organisms for culture but could be used for pilot studies to establish drug doses and toxicity.

The response of rats to cortisone acetate may be influenced by metabolic differences among the animals or by intrinsic differences in the immune systems. Cortisone concentrations in the blood of rats that gained weight during immunosuppression were significantly lower than the concentrations measured in rats that lost weight, suggesting a difference in the rates of cortisol metabolism in these two

TABLE 4. Subsets of lymphocytes in normal and cortisone-treated rats[a]

Wt change (no. of rats)	Mean ± SEM			
	Peripheral blood			Spleen H/NH[b]
	Total no. of leukocytes	% Lymphocytes	H/NH[b]	
Loss of >15 g (4)	$(1.4 \pm 0.1) \times 10^3$	57.8 ± 8.2	0.6 ± 0.04	0.3 ± 0.1
Gain (3)	$(1.5 \pm 0.2) \times 10^3$	78.5 ± 5.7	1.2 ± 0.2	0.6 ± 0.03
Normal controls (3)	$(4.8 \pm 0.5) \times 10^3$	89.0 ± 5.4	2.0	0.6 ± 0.1

[a] Viability of isolated lymphocytes ranged from 68 to 99% (mean, 90%). The rats were the same ones as those in Table 3.
[b] H/NH, Ratio of helper to non-helper cells.

groups. Differences between these two groups were also noted in the total leukocyte counts and in the percentage of lymphocytes. The ratios of helper to non-helper T cells from spleens were also different in the two groups. The results of these studies therefore suggest that those rats that lost more than 15 g during cortisone acetate administration were markedly more immunosuppressed than the animals that gained weight while receiving the same doses of cortisone acetate.

The reasons for variable susceptibility to *P. carinii* among and within strains of rats are unknown. This study showed correlation of cortisol levels with the degree of immunosuppression, which may at least partially explain the rat-to-rat variation within a given colony of one strain. The severity of *Pneumocystis* pneumonia correlated with the degree of immunosuppression, as measured by T-cell helper/non-helper ratios. Variable susceptibility among rat strains and colonies may also arise from variations in the pharmacokinetics of cortisone or in the degree of immunosuppression from cortisone. Alternatively, *Pneumocystis* strains in different rat populations may differ in pathogenicity. Another possibility is that rat populations may have other infections that influence susceptibility to *P. carinii*. For example, cytomegalovirus is known to suppress immune responses (12). Sendai virus is capable of suppressing immune responses in rats (5). Rat colonies may be infected with viruses, and some of these may influence immune responses.

The rat model described, which used animals matched by weight, provides a means of obtaining a more homogeneous degree of infection than was obtained with previous models. This technique should prove particularly useful for studies of chemotherapeutic agents. It also provides a more reliable source of organisms for culture and a better model for studies of pathogenesis than were previously available.

TABLE 5. Pilot studies on Sprague-Dawley rats from two suppliers

Characteristic	Charles River rats	Cox rats
No. of rats studied	5	5
Initial wt range (g)	162–217	153–180
Mean wt (g)		
Initial	190 ± 9	169 ± 5
Immunosuppressed for 8–9 wk	214 ± 9	173 ± 7
At sacrifice	220 ± 18.3[a]	194 ± 6[b]
Mean cortisone dose (mg/kg)		
Initial	132	148
Range	115–154	139–163
Final	187	206
Severity of infection score		
Giemsa impression	0.9 ± 0.8	2.0 ± 0.7
$AgNO_3$ impression	1.1 ± 0.6	2.7 ± 0.9
$AgNO_3$ section	1.4 ± 1.3	3.8 ± 0.4

[a] Sacrificed after 16 to 24 weeks of immunosuppression.
[b] Sacrificed after 16 weeks of immunosuppression.

ACKNOWLEDGMENT

This work was partially supported by Public Health Service contract N01-AI-42543 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Bartlett, M. S., P. A. Verbanac, and J. W. Smith.** 1979. Cultivation of *Pneumocystis carinii* with WI-38 cells. J. Clin. Microbiol. **10**:796–799.
2. **Brinn, N. T.** 1983. Rapid metallic histological staining. J. Histotechnol. **6**:125–129.
3. **D'Antonio, R. G., D. B. Johnson, R. E. Winn, A. F. Van Dellen, and M. E. Evans.** 1986. Effect of folinic acid on the capacity of trimethoprim-sulfamethoxazole to prevent and treat *Pneumocystis carinii* pneumonia in rats. Antimicrob. Agents Chemother. **29**:327–329.
4. **Frenkel, K. K., T. T. Good, and J. A. Schults.** 1966. Latent *Pneumocystis* infection of rats, relapse and chemotherapy. Lab. Invest. **15**:1559–1577.
5. **Garlinghause, L. E., Jr., and G. L. Van Hoosier.** 1978. Studies on adjuvent-induced arthritis, tumor transplantability, and serological response to bovine serum albumin in Sendai virus-infected rats. Am. J. Vet. Res. **39**:297–300.
6. **Hughes, W. T., S. Kuhn, S. Chaudhary, S. Feldman, M. Verzasa, J. A. Rhomes, C. Pratt, and S. L. George.** 1977. Successful chemoprophylaxis for *Pneumocystis carinii* pneumonia. N. Engl. J. Med. **297**:1419–1426.
7. **Hughes, W. T., B. L. Smith, and D. P. Jacobus.** 1986. Successful treatment and prevention of murine *Pneumocystis carinii* pneumonitis with 4,4'-sulfonylbisformanilide. Antimicrob. Agents Chemother. **29**:509–510.
8. **Hussain, Z., M. L. Carlson, I. D. Craig, and R. Lannigan.** 1985. Efficacy of tetroxoprim/sulphadiazine in the treatment of *Pneumocystis carinii* pneumonitis in rats. J. Antimicrob. Chemother. **15**:575–578.
9. **Kluge, R. M., D. M. Spaulding, and A. J. Spain.** 1978. Combination of pentamidine and trimethoprim-sulfamethoxazole in the therapy of *Pneumocystis carinii* pneumonia in rats. Antimicrob. Agents Chemother. **13**:975–978.
10. **Lanken, P. N., M. Minda, G. P. Giuseppe, and A. P. Fishman.** 1980. Alveolar response to experimental *Pneumocystis carinii* pneumonia in the rat. Am. J. Pathol. **99**:561–578.
11. **Matsumoto, Y., and Y. Yoshida.** 1984. Sporogony in *Pneumocystis carinii*: synaptonemal complexes and meiotic nuclear divisions observed in precysts. J. Protozool. **31**:420–428.
12. **Rinaldo, C. R., Jr., W. P. Carney, B. S. Richter, P. H. Black, and W. S. Hirsch.** 1980. Mechanisms of immunosuppression in cytomegalovirus mononucleosis. J. Infect. Dis. **141**:448–495.
13. **Walzer, P. D.** 1984. Experimental models of *Pneumocystis carinii* infections, p. 7–76. *In* L. S. Young (ed.), *Pneumocystis*

carinii pneumonia. Marcel Dekker, Inc., New York.

14. **Walzer, P. D., M. LaBine, T. J. Redington, and M. T. Cushion.** 1984. Lymphocyte changes during chronic administration of and withdrawal from corticosteroids: relation to *Pneumocystis carinii* pneumonia. J. Immunol. **133:**2502–2508.

15. **Yoshida, Y., Y. Matsumoto, M. Yamada, K. Okabagashi, H. Yoshikowa, and M. Nakazawa.** 1984. *Pneumocystis carinii*: electron microscopic investigation on the interaction of trophozoite and alveolar lining cells. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe A **256:**390–399.