INFECTION AND IMMUNITY, Apr. 1992, p. 1589–1597
0019-9567/92/041589-08$02.00/0
Copyright © 1992, American Society for Microbiology
Vol. 60, No. 4

Improved Rat Model of *Pneumocystis carinii* Pneumonia: Induced Laboratory Infections in *Pneumocystis*-Free Animals

CAROLE J. BOYLAN* AND WILLIAM L. CURRENT

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana 46285

Received 23 October 1991/Accepted 14 January 1992

An immunosuppressed rat model of *Pneumocystis carinii* pneumonia is described that utilizes simple, noninvasive intratracheal (i.t.) inoculation of cryopreserved parasites and results in development of severe *P. carinii* pneumonia within 5 weeks. This is an improvement over the most commonly used models of *P. carinii* pneumonia that rely on immune suppression to activate latent *P. carinii* infections and that often require 8 to 12 weeks to produce heavy infections of *P. carinii*. It is also less labor intensive than more recent models requiring surgical instillation of parasites. Our report describes a series of preliminary studies to select an appropriate strain of rat; to determine suitable methods for inducing uniform immunosuppression, *P. carinii* inoculation, and laboratory maintenance of *P. carinii*; and to determine effective animal husbandry methods for maintaining animals free from serious secondary infections. Results of our more detailed studies demonstrate that animals receiving two or three i.t. inoculations of approximately 10^6 cryopreserved *P. carinii* organisms have a predictable course of disease progression which includes moderate *P. carinii* infections within 3 weeks, severe *P. carinii* pneumonia in 5 weeks, and a high percentage of mortality due to *P. carinii* pneumonia in 6 weeks. Parasites were distributed evenly between the right and left lungs, regardless of the number of *P. carinii* inoculations administered. Non-*P. carinii*-inoculated immunosuppressed control rats maintained in microisolator cages remained free of *P. carinii*, thus providing an important control that is missing from many *P. carinii* pneumonia models. Most non-*P. carinii*-inoculated control animals and *P. carinii*-inoculated rats treated with trimethoprim-sulfamethoxazole that were housed in open caging in the same room containing heavily infected animals had no detectable infections after 5 to 6 weeks of immunosuppression; however, some had a small number of *P. carinii* in their lungs. Because heavy, reproducible infections are achieved 5 weeks after i.t. inoculation, because few animals are lost to secondary infections, and because animals can be maintained as noninfected contemporaneous controls, this animal model is useful for the maintenance of *P. carinii* strains, for studies of the transmission and natural history of *P. carinii*, for the production of large numbers of organisms for laboratory studies, and for the evaluation of potential anti-*P. carinii* drugs.

Pneumocystis carinii pneumonia is a leading cause of morbidity and mortality in persons with AIDS. Historically, it has been the initial disease manifestation in more than 60% and ultimately occurred in at least 85% of AIDS patients in North America (4, 13). *P. carinii* pneumonia is fatal in 5 to 10% of initial episodes and has been the cause of death in approximately 25% of AIDS patients reported in autopsy series (13). Although recent, widespread implementation of prophylaxis for *P. carinii* pneumonia has apparently been successful in lowering its incidence, it remains an important disease; more deaths presently occur in the United States from *P. carinii* pneumonia than from all reportable diseases (8). Thus, a major goal for biomedical research is increased success in diagnosis, prevention, and treatment of *P. carinii* pneumonia. To meet these goals, researchers must have access to reliable animal models of *P. carinii* pneumonia that can be used as a source of large numbers of organisms for laboratory studies and as a tool for evaluating the efficacy of new drugs.

Historically, most animal models used to study *P. carinii* relied on steroid-induced immunosuppression to activate latent *P. carinii* infections (1, 3, 6, 9–11, 14, 17, 18). The presence of latent *P. carinii* infections in many breeding colonies of Sprague-Dawley rats resulted in this rodent becoming the animal of choice for most investigators (1, 3, 9–11, 17). The utility of this animal model is often limited by unknown baselines of *P. carinii* infection, the long time required for development of heavy infections (8 to 12 weeks), the lack of control over parasite strains, and often the presence of secondary infections that render many of the animals unusable. This animal model is becoming more difficult to maintain in most laboratories because of the reduced availability of rodents with latent *P. carinii*. Most suppliers have eliminated or are in the process of eliminating primary and opportunistic pathogens, including *P. carinii*, from commercial production colonies.

To circumvent many of the problems associated with the conventional (dirty rat) model of *P. carinii* pneumonia, investigators have developed surgical procedures to introduce *P. carinii* into the lungs of virus-free, *P. carinii*-free rats (2). Although the surgical procedures are labor intensive, they provide a means of obtaining consistent, heavy *P. carinii* infection in immunosuppressed rats. In this communication, we describe the development of an improved animal model of laboratory-induced *P. carinii* infections that results in severe *P. carinii* pneumonia within 5 weeks after noninvasive intratracheal (i.t.) inoculation of parasites. This model produces consistent, heavy infections of *P. carinii* with minimal secondary bacterial infections and has been used extensively in our laboratory to evaluate candidate drugs for anti-*P. carinii* pneumonia activity and for propagation of a strain of *P. carinii* for laboratory studies.

(Presented in part at the International Workshop on *Pneumocystis*, *Cryptosporidium*, and Microsporidia, Bozeman, Mont., 30 June to 2 July 1991 [abstract WS-65].)

* Corresponding author.

MATERIALS AND METHODS

Preliminary studies. A series of preliminary studies were conducted to select an appropriate strain of rat; to determine suitable methods of immunosuppression, *P. carinii* inoculation, and maintenance of *P. carinii*; and to determine effective animal husbandry methods for maintaining animals free from serious secondary bacterial infections. These studies are summarized below before a presentation of the materials and methods used in the more-detailed studies to develop the animal model.

(i) Selection of rat strain. Three strains of rats, Sprague-Dawley, Fisher 344, and Lewis, were evaluated and compared to determine which was best suited for steroid-induced immunosuppression and maintenance of *P. carinii* pneumonia. All 20 rats in each of the three strains developed heavy *P. carinii* infections 6 weeks after immunosuppression and i.t. inoculation of parasites; however, the inbred, virus-free Lewis rats developed fewer secondary bacterial infections and were more docile. Female Lewis rats (Harlan Sprague-Dawley, Inc., Indianapolis, Ind.) weighing 120 to 140 g were utilized in all subsequent studies to develop the model.

(ii) Immunosuppression regimen. Two immunosuppressive therapies were compared. Depo-Medrol (methlyprednisolone acetate; The Upjohn Co., Kalamazoo, Mich.; 40 and 30 μg/g for weeks 1 and 2, continuing weekly at 20 μg/g) administered subcutaneously resulted in a more controlled, uniform dose of immunosuppression than did dexamethasone (Schering Corp., Kenilworth, N.J.) administered in the drinking water. Animals immunosuppressed with methylprednisolone acetate developed heavy, consistent *P. carinii* infections within 6 weeks following i.t. inoculation of parasites.

(iii) Preparation and maintenance of *P. carinii* inoculum. Parasite inoculum was prepared from the lungs of heavily infected donor rats housed in microisolator cages (Lab Products Inc., Maywood, N.J.; cage bottom no. 18727; stainless steel wire lid no. 10428; and filter top no. 18704). Rats were anesthetized with approximately 135 μg of ketamine (ketamine hydrochloride injection USP, 100 mg/ml; Aveco Co., Inc., Fort Dodge, Iowa) per g and 4 μg of xylazine (20 mg/ml; Mobay Corp., Shawnee, Kans.) per g, and the thoracic and abdominal cavities were aseptically opened. After the abdominal aorta and vena cava were severed, the pulmonary circulation was aseptically perfused by using a 10-cm^3 syringe and 23-gauge needle to inject approximately 7 ml of 4°C Alsever's solution (GIBCO Laboratories, Grand Island, N.Y.) into the pulmonary artery. Perfused lungs were collected, suspended in 1:40 (wt/vol) Dulbecco modified Eagle medium supplemented with 10% fetal calf serum, glutamine (2 mM), pyruvate (1 mM), penicillin-streptomycin (100 U/100 μg/ml), and amphotericin B (Fungizone; 0.1 μg/ml) (S-DMEM), and homogenized (Brinkman Polytron homogenizer, speed 5 for 10 to 15 s). Trophozoites, precysts, and cysts of *P. carinii* were separated from most of the host cell debris by two repetitions of centrifugation at 40 × *g* and 1,000 × *g* for 10 min. The supernatant was saved from the 40 × *g* centrifugation, whereas the pellet was saved from the 1,000 × *g* centrifugation. The final *P. carinii*-enriched pellet was resuspended in S-DMEM, counted with a hemacytometer, resuspended at approximately 2×10^7 *P. carinii* organisms per ml in S-DMEM containing 7.5% (vol/vol) dimethyl sulfoxide, cryopreserved by using a 1°C/min cooling cycle (model 700 automated cell freezer; Cryo-Med, Division of Forma Scientific Inc., New Baltimore, Mich.), and stored as 1-ml aliquots in liquid nitrogen for up to 1 year. Prior to use, each batch of inoculum was cultured for bacterial and fungal contaminants on brain heart infusion and Sabouraud dextrose agar plates. Only batches of culture-negative inocula were used. Cryopreserved inoculum was thawed at 37°C and resuspended in an equal volume of S-DMEM, and 0.1 ml containing approximately 10^6 *P. carinii* organisms was inoculated i.t. into the lungs of each immunosuppressed rat.

(iv) Method of *P. carinii* inoculation. Two inoculation techniques for inducing *P. carinii* infections were evaluated. One week following initial immunosuppression, *P. carinii* was introduced deep into the trachea either by i.t. intubation with a 3-in. (7.6-cm), 20-gauge curved stainless steel animal feeding tube (Popper and Sons, Inc., New Hyde Park, N.Y.) or by surgical instillation into the trachea as described by Bartlett et al. (2). Inoculation i.t. following light halothane anesthesia proved to be the most satisfactory method because it was rapid and noninvasive and reduced both the anesthesia requirements and the physical stress associated with surgical exposure of the trachea. Lightly anesthetized rats were suspended by their upper incisors on a wire loop at the top of a Plexiglas slant board (8 by 4 in. [ca. 20 by 10 cm], 60-degree angle). The animal's tongue was grasped and pulled to one side of the lower incisors, and a stainless steel feeding tube attached to a 1-cm^3 syringe containing 0.1 ml of inoculum and then 0.4 ml of air was directed with slight pressure along the back of the tongue into the larynx. Tracheal rings were palpated against the feeding tube to confirm correct placement before depositing the inoculum in the trachea approximately 0.5 cm above the primary bronchi.

(v) Prevention of secondary infections. In most immunosuppressed animal models of *P. carinii* pneumonia, tetracycline is routinely added to the drinking water to control secondary bacterial infections (1–3, 6, 9–11, 17). In our preliminary studies, hyperchlorinated drinking water (approximately 8-mg/ml available chlorine) was compared with water containing tetracycline (0.5 mg/ml of water). In our animal facilities, tetracycline in the drinking water provided no apparent advantage over hyperchlorinated drinking water with respect to the occurrence of secondary bacterial infections in immunosuppressed rats housed in both microisolator and wire-bottom cages. This observation is consistent with recent reports indicating that tetracycline fails to produce effective blood levels when administered in the drinking water (15). Hyperchlorinated drinking water administered without tetracycline significantly reduced labor requirements since animals were maintained five per cage in wire-bottom caging with an automatic watering system, thus reducing the potential bacterial contamination from animal caretakers who manually change water bottles. Also important in preventing serious secondary bacterial infections was handling rats infrequently (once weekly for weighing and for injection of methylprednisolone) and the use of sterilized latex gloves. In the remaining studies reported herein, tetracycline was not included in the drinking water and the rats were maintained on hyperchlorinated water and autoclaved, standard (not low protein) rodent chow (Ralston Purina Co. no. 5001).

Evaluation of *P. carinii* infections. The severity of *P. carinii* infections was determined by monitoring percent survival and body weight loss and by evaluating parasite burdens in the lungs collected at necropsy. In our initial studies, Giemsa- and methenamine silver-stained lung impression smears were used to determine the severity of *P. carinii* infections. Giemsa-stained impression smears were used to

evaluate all lung stages of the organism (trophozoites, precysts, and cysts), whereas methenamine silver stain was specific for only the cyst stage. Each impression smear was assigned an infection score, a logarithmic representation of the actual number of parasites seen: 0, no parasites found in 30 microscopic fields; 1, 1 to 5 parasites per 10 microscopic fields; 2, approximately 1 parasite per field; 3, 2 to 10 parasites per field; 4, >10 but <100 parasites per field; 5, >100 but <1,000 parasites per field. Impression smears containing >1,000 parasites per field were assigned a value of 6. Giemsa- and methenamine silver-stained slides were examined by using a final magnification of ×1,000 and ×400, respectively. All slides were randomized and then examined microscopically by two persons using a blinded protocol. In rare cases in which infection scores of the two observers differed by more than 0.5, the slides were reevaluated and the actual infection score was determined.

During the study to investigate the kinetics of *P. carinii* infection following i.t. inoculation (see below), another procedure was evaluated to determine the severity of *P. carinii* infections in the lung. Infection scores obtained from microscopic examination of Giemsa- and methenamine silver-stained impression smears were compared with infection scores obtained from methenamine silver-stained lung homogenates, the new evaluation procedure. Stained slides of lung homogenates were prepared at the time of necropsy and were scored by the same criteria described above for methenamine silver-stained impression smears. Rats were anesthetized with ketamine-xylazine (as described above), exsanguinated via the right atrium to remove excess blood from the pulmonary circulation, and then necropsied. The lungs were removed from each rat, weighed, and then homogenized in a 40× (wt/vol) volume of sterile water for approximately 15 s (Brinkman Polytron homogenizer, setting 5). Large particulates comprising host cell debris were allowed to settle for approximately 10 min, and then 4-μl samples of the supernatant containing *P. carinii* cysts were distributed evenly onto the wells of Teflon-coated, 12-well microscope slides (catalog no. 99910090; 6-mm well diameter; Shandon Inc., Pittsburgh, Pa.) and stained with methenamine silver. Infection scores from lung homogenates diluted 1:40 in water (wt/vol) were slightly lower but comparable to those of Giemsa- and methenamine silver-stained lung impression smears (see Tables 2 and 3).

Optimizing i.t. inoculation of *P. carinii*. Immunosuppressed rats were given one, two, or three i.t. inoculations of parasites (0.1 ml of S-DMEM containing 10^6 *P. carinii* parasites administered 48 h apart) to determine the optimum number of inoculations required to produce consistent, heavy *P. carinii* infections. To reduce the number of animals used, these rats also served as *P. carinii*-infected controls for therapy studies to evaluate candidate drugs for anti-*P. carinii* pneumonia activity. Noninfected control rats and trimethoprim-sulfamethoxazole (TMP-SMX, trimethoprim [0.2 mg/ml] and sulfamethoxazole [1 mg/ml] in hyperchlorinated water ad lib)-treated rats were also included in the therapy studies to confirm the *P. carinii*-free status of the rats and to demonstrate the efficacy of TMP-SMX as a positive control drug. In each of four studies, 10 rats were assigned to the following treatment groups: rats receiving one, two, or three inoculations of *P. carinii*; *P. carinii*-infected rats (two *P. carinii* inoculations) treated with TMP-SMX; and nontreated, noninfected control animals. Six weeks after the initial *P. carinii* inoculation, rats were necropsied, and impression smears collected from the left lung were stained and evaluated for severity of *P. carinii* infections. Infection scores were then compared with the number of *P. carinii* inoculations each group received.

***P. carinii* distribution in the lungs following i.t. inoculation.** Immunosuppressed rats were given one, two, or three i.t. inoculations of *P. carinii*, and the distribution of parasites in the left and right lungs was determined. Ten rats were assigned to each of four treatment groups: rats receiving one, two, or three inoculations of *P. carinii* (0.1 ml of S-DMEM containing 10^6 *P. carinii* organisms) and noninfected controls. Six weeks after *P. carinii* inoculation, rats were necropsied, and Giemsa- and methenamine silver-stained impression smears were prepared from the left lung of each animal. The remaining left and right lungs from each rat were separated, homogenized, and prepared for methenamine silver-stained lung homogenates as described above. Infection scores from lung impression smears and lung homogenates were evaluated and compared to determine whether the two procedures yield comparable infection scores. Lung homogenates prepared from the left and right lungs of each rat were then evaluated to determine the distribution of organisms within the lungs.

Kinetics of *P. carinii* infection. Immunosuppressed rats given one, two, or three i.t. inoculations of *P. carinii* were evaluated to determine the severity of *P. carinii* infections during weeks 3 to 6 postinoculation (p.i.). Thirty-two rats were assigned to each of four treatment groups: rats receiving one, two, or three *P. carinii* inoculations and noninoculated controls. Eight rats from each treatment group were necropsied at 3, 4, 5, and 6 weeks after i.t. inoculation to determine the severity of *P. carinii* infections. After impression smears were collected from the left lung, the remainder of the lung tissue was weighed and prepared for methenamine silver-stained lung homogenates. Stained impression smears of the lungs and stained lung homogenates were evaluated and compared to determine the severity of *P. carinii* infection at 3, 4, 5, and 6 weeks p.i.

The 5-week *P. carinii* pneumonia model for drug screening. Near the end of our studies to characterize the immunosuppressed rat model of *P. carinii* pneumonia, it was apparent that 5 weeks after i.t. inoculation of *P. carinii* was the optimal time to necropsy the animals and to evaluate the extent of *P. carinii* infections. This 5-week model was used in our laboratory in studies to evaluate the potential anti-*P. carinii* pneumonia activity of experimental compounds. These studies routinely had 8 to 10 immunosuppressed rats assigned to each of the following treatment groups: *P. carinii*-infected, nontreated rats (infected controls); *P. carinii*-infected animals given TMP-SMX in the water (positive drug treatment controls); non-*P. carinii*-infected control animals; and *P. carinii*-infected animals receiving experimental compounds. One week after initiation of immunosuppression, rats received two i.t. inoculations of *P. carinii* 48 h apart and were assigned to control groups or to therapy or prophylaxis treatment groups. Animals used to screen drugs for prophylactic activity against *P. carinii* pneumonia started receiving therapy 24 h after initial *P. carinii* inoculation, and animals harboring a 2-week-old *P. carinii* infection were used to screen drugs for therapeutic activity. Both therapeutic and prophylactic treatments were continued until week 5 of *P. carinii* infection. In addition to monitoring percent survival and percent body weight loss, we determined the efficacy of candidate drugs against *P. carinii* pneumonia by evaluating the lungs for severity of *P. carinii* infections at necropsy. Impression smears were collected from the left lung, and the remainder of lung tissue was weighed and homogenized for cyst quantitation as described above.

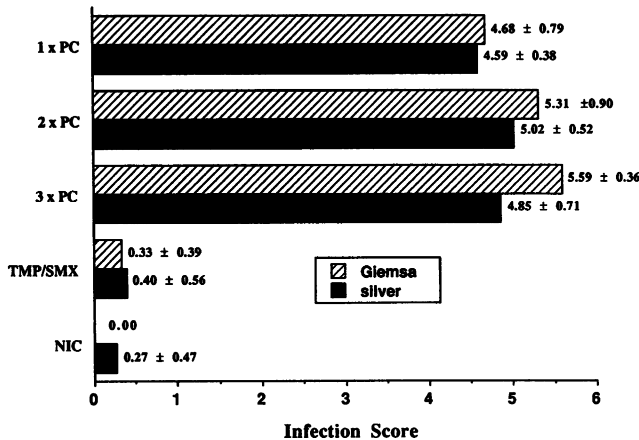

FIG. 1. Mean infection scores (± standard deviation) 6 weeks after one, two, or three i.t. inoculations of *P. carinii* (1 × PC, 2 × PC, 3 × PC, respectively). Each infection score represents the mean of four studies, each with 8 to 10 rats per group. Impression smears were stained with Giemsa or methenamine silver. All life cycle stages (trophozoites, precysts, cysts) were scored when reading Giemsa-stained smears (×1,000 magnification), whereas only cysts were scored when reading silver-stained smears (×400 magnification). *P. carinii*-inoculated rats treated with TMP-SMX served as positive drug treatment controls. Noninoculated control (NIC) rats were immunosuppressed and housed in open cages in the same room as heavily infected animals. Distributions of the infection scores among rats from the four studies are shown in Table 1.

Stained lung homogenates were evaluated to determine the severity of *P. carinii* infection at 5 weeks. Treatment groups showing signs of anti-*P. carinii* pneumonia activity were further evaluated by using stained lung impression smears. Finally, TMP-SMX-treated animals and *P. carinii*-infected and non-*P. carinii*-infected animals were evaluated and compared to confirm the validity of the model.

RESULTS

Optimizing i.t. inoculation of *P. carinii*. Animals receiving two or three i.t. inoculations of *P. carinii* developed consistent, heavy *P. carinii* infections (mean infection scores of 5.31 to 5.59) within 6 weeks p.i. (Fig. 1; Table 1). Most rats receiving only one inoculation also demonstrated heavy *P. carinii* infections within 6 weeks; however, these animals showed an increased incidence of lower infection scores compared with animals receiving two to three *P. carinii* inoculations (Table 1). Mean percent mortality and mean percent body weight loss (data not shown) were higher in animals receiving two or three inoculations and were attributed to heavy parasite burdens. TMP-SMX administered in the drinking water (weeks 2 to 6 p.i.) was highly effective in eradicating parasites from the lungs of infected rats, and little or no evidence of *P. carinii* was found in the lungs 6 weeks after parasite inoculation (Fig. 1; Table 1). Although some (approximately 15%) non-*P. carinii*-inoculated, immu-

TABLE 1. Distribution of infection scores 6 weeks following one, two, or three i.t. inoculations of *P. carinii* (1 × PC, 2 × PC, or 3 × PC, respectively)

Study no.	No. of rats in each group/no. of rats necropsied	Reported cause of death[a]	No. of methenamine silver-stained impression smears[b] with the following infection score:						
			<1.0	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	≥6.0
1 × PC									
1	10/10		0	0	0	1	4	5	0
2	10/10		0	0	0	1	2	7	0
3	9/9		0	0	1	1	4	3	0
4	10/7	3 PCP	0	0	0	0	1	6	0
2 × PC									
1	10/10		0	0	0	0	5	4	1
2	8/6	2 PCP	0	0	0	0	0	5	1
3	8/6	2 PCP	0	0	0	1	2	1	2
4	10/5	5 PCP	0	0	0	0	0	5	0
3 × PC									
1	10/10		0	0	0	2	3	4	1
2	9/6	3 PCP	0	0	0	0	3	3	0
3	10/10		0	0	0	0	3	5	2
4	10/7	3 PCP	0	0	0	1	1	4	1
Non-*P. carinii* inoculated[c]									
1	10/10		7	3	0	0	0	0	0
2	10/10		9	1	0	0	0	0	0
3	9/9		8	1	0	1	0	0	0
4	10/10		10	0	0	0	0	0	0
TMP-SMX (positive treatment control)									
1	10/10		4	6	0	0	0	0	0
2	9/9		5	2	2	0	0	0	0
3	10/10		8	2	0	0	0	0	0
4	10/9	1 KF	8	1	0	0	0	0	0

[a] PCP, *P. carinii* pneumonia: infection scores ranged from 5.0 to 6.0 (Giemsa impression smears); KF, kidney failure resulting from immune suppression-induced purulent streptococcal nephritis.
[b] Impression smears collected from the center of the left lung.
[c] Noninoculated, immunosuppressed rats housed for 6 weeks in open cages in the same room as heavily infected animals. Non-*P. carinii*-inoculated rats in study 3 were housed in the same cage rack as the 1, 2, and 3 × PC groups, whereas those in studies 1, 2, and 4 were housed in separate cage racks.

TABLE 2. Distribution of *P. carinii* in the left versus right lung and comparison of infection score evaluation procedures

No. of *P. carinii* inoculations[a]	No. of rats in each group/no. of rats necropsied[b]	Reported cause of death[c]	Mean infection scores ± SD			
			Impression smears[d]		Silver-stained homogenate	
			Giemsa	Silver	Left lung	Right lung
0	10/10		0.30 ± 0.33	0.40 ± 0.43	0.23 ± 0.18	0.10 ± 0.09
1	10/9	1 PCP	5.42 ± 0.67	5.33 ± 0.53	4.77 ± 0.23	4.72 ± 0.31
2	10/5	5 PCP	5.30 ± 0.48	5.10 ± 0.46	4.50 ± 0.23	4.50 ± 0.23
3	10/6	4 PCP	5.50 ± 0.35	5.42 ± 0.34	4.58 ± 0.12	4.63 ± 0.18

[a] 0, noninoculated, immunosuppressed rats housed for 6 weeks in open cages in the same room as heavily infected animals.
[b] Animals necropsied 6 weeks p.i.
[c] PCP, *P. carinii* pneumonia: infection scores ranged from 4.5 to 6.0 (Giemsa-stained impression smears).
[d] Impression smears collected from the center of the left lung.

nosuppressed rats housed in standard wire-bottom cages in the same room as heavily infected animals had small numbers of *P. carinii* in their lungs after 6 weeks (Fig. 1; Table 1), the remaining animals (approximately 85%) maintained in microisolator cages remained free of detectable *P. carinii*. Housing non-*P. carinii*-inoculated, immunosuppressed rats in the same cage rack as heavily infected animals resulted in a higher incidence of moderate *P. carinii* infection compared with noninoculated controls housed in a separate cage rack (Table 1, study 3). Mean *P. carinii* infection scores determined by evaluation of stained lung impression smears were comparable for both Giemsa and methenamine silver stains for each of the animals evaluated (Fig. 1; Table 1).

Distribution of *P. carinii* in lungs following i.t. inoculation. Parasites were distributed evenly between the left and right lungs regardless of the number of *P. carinii* inoculations administered (Table 2). Animals receiving two or three *P. carinii* inoculations had higher mortality rates and more consistent, heavy mean *P. carinii* infections than rats receiving one *P. carinii* inoculation. All mortalities were attributed to heavy *P. carinii* burdens. In addition, little or no evidence of *P. carinii* pneumonia was found in the lungs of non-*P. carinii*-infected animals housed in wire-bottom cages in the same room when these animals were placed in a cage rack without *P. carinii*-infected animals.

Kinetics of the *P. carinii* infection. Mean *P. carinii* infection scores and infection score incidence at different times after i.t. inoculation of *P. carinii* are presented in Tables 3 and 4, respectively. *P. carinii* infections were well established in all groups by 3 weeks, regardless of the number of inoculations received, and mean infection scores at this time ranged from 3.87 to 4.04 (Table 3). *P. carinii* pneumonia progressed consistently over time for each of the inoculation groups, and by week 5, mean infection scores of all animals receiving two or three *P. carinii* inoculations were 4.97 or greater. The severity of *P. carinii* pneumonia at 6 weeks was directly related to the number of inoculations received; however, high mortality rates resulting from heavy *P. carinii* pneumonia during weeks 5 and 6 p.i. reduced the number of animals available for evaluation.

The 5-week *P. carinii* pneumonia model for drug screening. Infection scores obtained from *P. carinii*-infected control animals, non-*P. carinii*-infected control animals, and *P. carinii*-infected TMP-SMX-treated animals from 10 studies

TABLE 3. Mean infection scores 3, 4, 5, and 6 weeks following i.t. inoculation of *P. carinii*

Week p.i.[a]	No. of rats in each group/no. of rats necropsied	Reported cause of death[b]	Mean infection scores ± SD		
			Impression smears[c]		Lung homogenate (silver)
			Giemsa	Silver	
1 × PC					
3	8/7	1 KF	3.08 ± 0.72	3.86 ± 0.34	3.87 ± 0.37
4	8/8		4.84 ± 0.45	4.74 ± 0.29	4.70 ± 0.29
5	8/5	3 PCP	4.77 ± 0.44	4.75 ± 0.28	5.32 ± 0.10
6	8/3	5 PCP	5.50 ± 0.71	5.17 ± 0.47	5.23 ± 0.05
2 × PC					
3	8/8		3.87 ± 0.68	4.21 ± 0.50	4.04 ± 0.14
4	8/8		5.04 ± 0.54	4.63 ± 0.31	4.84 ± 0.39
5	8/6	1 PCP; 1 MAL	5.37 ± 0.67	4.97 ± 0.45	5.28 ± 0.18
6	8/4	4 PCP	5.40 ± 0.53	5.01 ± 0.37	4.82 ± 0.43
3 × PC					
3	8/8		4.43 ± 0.77	4.54 ± 0.48	3.96 ± 0.26
4	8/8		5.20 ± 0.51	5.08 ± 0.37	5.02 ± 0.23
5	8/7	1 KF	5.35 ± 0.52	5.14 ± 0.45	5.24 ± 0.16
6	8/3	5 PCP	6.00 ± 0.00	5.37 ± 0.45	5.13 ± 0.19

[a] 1 × PC, 2 × PC, 3 × PC, one, two, or three *P. carinii* inoculations, respectively.
[b] PCP, *P. carinii* pneumonia; KF, kidney failure resulting from immune suppression-induced purulent streptococcal nephritis; MAL, malocclusion of the front incisors.
[c] Impression smears collected from the center of the left lung.

TABLE 4. Distribution of infection scores 3, 4, 5, and 6 weeks following 1, 2, or 3 i.t. inoculations of *P. carinii* (1 × PC, 2 × PC, or 3 × PC, respectively)

Week p.i.	No. of rats in each group/ no. of rats necropsied	No. of methenamine silver-stained lung homogenates with the following infection score:						
		<1.0	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	≥6.0
1 × PC								
3	8/7	0	0	0	2	5	0	0
4	8/8	0	0	0	0	7	1	0
5	8/5	0	0	0	0	0	5	0
6	8/3	0	0	0	0	0	3	0
2 × PC								
3	8/8	0	0	0	2	6	0	0
4	8/8	0	0	0	0	5	3	0
5	8/6	0	0	0	0	0	6	0
6	8/4	0	0	0	0	2	2	0
3 × PC								
3	8/8	0	0	0	3	5	0	0
4	8/8	0	0	0	0	2	6	0
5	8/7	0	0	0	0	0	7	0
6	8/3	0	0	0	0	0	3	0

are presented in Fig. 2 and Table 5. Heavy *P. carinii* infections developed within 5 weeks in all control rats inoculated twice with *P. carinii* (mean infection score, 4.67 ± 0.39), and of these rats, approximately 20% died of heavy *P. carinii* burdens during week 4 of infection. Some non-*P. carinii*-inoculated controls as well as TMP-SMX-treated rats developed very light infections if kept in open cages in different cage racks but in the same room with *P. carinii*-infected animals, and some developed moderate infections when they were housed in cages close to heavily infected animals (Table 5, study 4). Non-*P. carinii*-inoculated, immunosuppressed rats remained *P. carinii*-free if kept in isolation (data not shown).

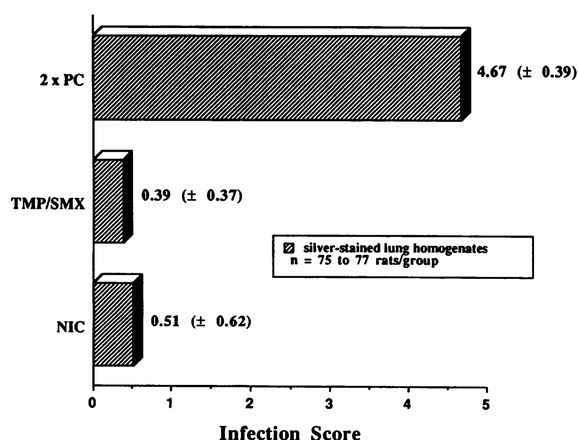

FIG. 2. Mean infection scores (± standard deviation) of control rats from the 5-week model of *P. carinii* pneumonia. Each infection score represents the mean of 10 studies, each with 8 to 10 rats per group. Only cysts were scored with methenamine silver-stained lung homogenates. *P. carinii*-inoculated rats treated with TMP-SMX served as positive drug treatment controls. Noninoculated control (NIC) rats were immunosuppressed and housed in open cages in the same room as heavily infected animals. Distributions of the infection scores among rats from the 10 studies are shown in Table 5.

DISCUSSION

The most widely used animal model of *P. carinii* pneumonia, first developed by Frenkel et al. (6), relies on activation of latent *P. carinii* infections in rats immunosuppressed by steroids. The presence of latent *P. carinii* infections in most breeding colonies of Sprague-Dawley rats resulted in this rodent becoming the animal of choice for most investigators (1, 3, 9, 10, 17). These rats have been used both as a source of organisms for laboratory studies and for most evaluations of drugs for efficacy against *P. carinii* pneumonia (1, 3, 9, 10, 12, 14, 17). Inherent to this model are the problems of inconsistent infections, the long course (8 to 12 weeks) of immunosuppression to allow development of heavy infections, a lack of control over *P. carinii* strains that may be present, and often a high incidence of secondary infections that render many of the animals unusable (1, 3, 16). These problems are thought to be primarily a result of variations among rats in the degree of latent infections, in the amount of steroids consumed or their susceptibility to steroids, and in the presence of opportunistic pathogens that may cause disease when the animals are immunosuppressed (1). Another aspect that has recently made this animal model increasingly difficult for many researchers is a marked reduction in the supply of rats with latent *P. carinii* infections. Through continuous upgrading of the pathogen-free status of most breeding stocks, animal suppliers have succeeded in reducing or eliminating inherent *P. carinii* infections in their laboratory animal colonies. Today, many of the commercial rodent colonies are free of viral and bacterial pathogens and do not have latent *P. carinii*. In the absence of substantial monetary incentives to maintain rat colonies that have latent *P. carinii*, it is likely that the traditional dirty rat model may soon be unavailable to many researchers.

A logical way to circumvent many of the problems associated with the conventional model described above is to induce controlled laboratory infections in *P. carinii*-free animals. Early attempts to induce laboratory infections met with varied success. Walzer et al. (19) successfully infected nude mice by percutaneous injection of the parasites into the lung. Although intranasal inoculation of the organism was used successfully to produce *P. carinii* pneumonia in mice in

TABLE 5. Distribution of infection scores of control rats from the 5-week model of *P. carinii* pneumonia

Study no.	No. of rats in each group/no. of rats necropsied	Reported cause of death[a]	No. of methenamine silver-stained lung homogenates with the following infection score:						
			<1.0	1.0–1.9	2.0–2.9	3.0–3.9	4.0–4.9	5.0–5.9	≥6.0
2 × PC[b]									
1	10/10		0	0	0	0	6	4	0
2	8/5	3 PCP	0	0	0	0	3	2	0
3	10/7	3 PCP	0	0	0	0	5	2	0
4	9/8	1 PCP	0	0	0	0	4	4	0
5	8/8		0	0	0	0	6	2	0
6	8/7	1 PCP	0	0	0	0	3	4	0
7	8/7	1 PCP	0	0	0	0	5	2	0
8	8/5	3 PCP	0	0	0	0	5	0	0
9	8/7	1 KF	0	0	0	0	6	1	0
10	8/7	1 PCP	0	0	0	0	3	4	0
TMP-SMX (positive treatment control)									
1	10/9	1 MAL	1	7	1	0	0	0	0
2	8/8		8	0	0	0	0	0	0
3	10/10		10	0	0	0	0	0	0
4	9/9		8	1	0	0	0	0	0
5	8/8		8	0	0	0	0	0	0
6	8/8		8	0	0	0	0	0	0
7	8/8		6	0	1	1	0	0	0
8	8/8		7	1	0	0	0	0	0
9	8/8		7	1	0	0	0	0	0
10	8/8		8	0	0	0	0	0	0
Non-*P. carinii*-inoculated[c]									
1	8/8		2	6	0	0	0	0	0
2	8/8		5	3	0	0	0	0	0
3	10/10		7	3	0	0	0	0	0
4	9/9		0	5	2	2	0	0	0
5	8/8		8	0	0	0	0	0	0
6	8/8		8	0	0	0	0	0	0
7	8/8		8	0	0	0	0	0	0
8	8/8		8	0	0	0	0	0	0
9	8/8		8	0	0	0	0	0	0
10	8/6	1 KF; 1 MAL	6	0	0	0	0	0	0

[a] PCP, *P. carinii* pneumonia; KF, kidney failure resulting from immune suppression-induced purulent streptococcal nephritis; MAL, malocclusion of the front incisors.

[b] Immunosuppressed rats inoculated twice with *P. carinii* (*P. carinii*-infected control rats).

[c] Non-*P. carinii*-inoculated immunosuppressed control rats housed for 5 weeks in open cages in the same room as but away from heavily infected animals. In study 4, these rats were housed in open caging near heavily infected animals.

one laboratory (7), the technique did not produce *P. carinii* pneumonia in animal models used in two other laboratories (6, 19). More recently, a rat model was developed in which consistent laboratory-induced *P. carinii* infections were achieved and which eliminated some of the undesirable variables associated with activation of latent *P. carinii* infections (2). However, labor-intensive surgical manipulation was required to inoculate the organisms into the trachea.

Our animal model of laboratory-induced *P. carinii* pneumonia represents an improvement over the existing models. The key features that make the model useful for a variety of studies include the use of relatively non-labor-intensive, controlled, uniform immunosuppression of *P. carinii*-free animals; the use of rapid, noninvasive i.t. inoculation of cryopreserved parasites; and a predictable course of disease development resulting in consistent, heavy *P. carinii* infections within 5 weeks p.i.

Incorporation of dexamethasone in the drinking water is the immunosuppressive regimen most often used in animal models of *P. carinii* pneumonia (1–3, 8–11, 14, 16–19). One major shortcoming of this method is the amount of labor required to ensure that all animals receive the same dose of dexamethasone. Because there is often considerable variability in the amount of water consumed by individual rats, they must be housed individually, daily water intake must be monitored, and drug concentrations must be adjusted to compensate for differences in individual water intake. Our preliminary studies demonstrated that weekly injections of methylprednisolone resulted in a uniform level of immunosuppression and that tetracycline was not needed in the drinking water to prevent secondary bacterial infections. This allows housing of more than one animal per cage and the use of an automatic watering system, features which greatly reduce the amount of labor for animal care because changing of individual water bottles every 1 to 3 days is eliminated. The methylprednisolone dosage originally described by Cushion et al. (5) was adjusted to accommodate for body weight loss associated with steroid therapy. Feeding standard rodent chow in combination with weekly subcutaneous injections of methylprednisolone produced a balance of immunosuppression sufficient for the maintenance of *P. carinii* pneumonia while lessening the wasting syndrome

associated with the often-used combination of steroid-induced immunosuppression and low-protein diet.

With the conventional steroid-induced *P. carinii* pneumonia model (dirty rat model), there is often a high percentage of animals which are unsuitable for use in in vitro studies because of high incidences of secondary infections, especially pulmonary bacterial infections (1, 16). *P. carinii*-free Lewis rats were selected for our model because these animals are more docile than the Sprague-Dawley or Fisher 344 rats and because they developed heavy *P. carinii* infections with minimal secondary bacterial infections. Most virus-free Lewis rats used in our studies remained free from detectable secondary bacterial and fungal infections. Deaths due to causes other than *P. carinii* pneumonia were uncommon in our 5-week model of *P. carinii* pneumonia. Of the 253 immunosuppressed animals reported in Table 5, only four deaths were attributed to causes other than *P. carinii* pneumonia; two died from steroid-induced streptococcal nephritis and two died of malnutrition attributed to malocclusion of the front incisors. Although kidney lesions due to steroid-induced streptococcal nephritis were occasionally observed at necropsy, less than 1% of the immunosuppressed animals apparently died from this cause. The virus-free status of Lewis rats used in our studies, the shorter duration of immunosuppression, and the animal housing and care procedures outlined above resulted in a lower occurrence of serious secondary bacterial infections compared with most models of *P. carinii* pneumonia; however, complete suppression of localized bacterial infections was not achieved.

Tracheal intubation with a standard 20-gauge feeding tube provides a rapid method of inoculating parasites deep into the lung without surgical manipulation, visualization of the epiglottis, or special equipment. Placement of the feeding tube i.t. is easily verified, and rats recover from light halothane anesthesia within minutes. After becoming familiar with the technique, one person can inoculate *P. carinii* into the lungs of 100 to 120 rats in 1 h.

The use of cryopreserved parasites along with i.t. inoculation of *P. carinii*-free rats provides a practical means of long-term maintenance of individual *P. carinii* isolates or strains and a consistent source of large numbers of parasites for laboratory studies. By maintaining a small stock colony of i.t.-inoculated animals in microisolator cages, one can be assured that heavily infected animals are available for laboratory use when needed. Inoculation of five rats i.t. each week should provide an uninterrupted, adequate supply of *P. carinii* for most laboratory studies.

A major shortcoming of the conventional model of *P. carinii* pneumonia that is overcome by our model is the absence of immunosuppressed, non-*P. carinii*-infected control animals. By using *P. carinii*-free rats housed in microisolators, immunosuppressed, noninfected animals can be incorporated into a variety of studies that require the presence of this type of contemporaneous control to properly interpret the data obtained.

Perhaps the most important feature of our model is the predictable course of disease progression that results in most, if not all, rats having moderate infections 2 and 3 weeks after i.t. inoculation of *P. carinii*, severe *P. carinii* pneumonia 4 and 5 weeks p.i., and approximately 50% mortality due to *P. carinii* pneumonia at 6 weeks p.i. This is preferable to the long (8 to 12 weeks), often unpredictable course of infection that occurs in conventional dirty rat models. In the early stages of our model development, rats were necropsied at 6 weeks p.i.; however, in later studies, more than 50% of *P. carinii*-infected control rats died of severe *P. carinii* pneumonia by 6 weeks p.i. Therefore, a 5-week study duration was selected to optimize percent survival among heavily infected rats used to evaluate experimental compounds for both therapeutic and prophylactic activity against *P. carinii* pneumonia. The therapeutic evaluation model allows 2 weeks for moderate *P. carinii* infections to develop and then 3 weeks to evaluate the efficacy of experimental compounds. The use of *P. carinii*-free rats and coordination of parasite inoculation and initiation of therapy within a 24-h period allows a more-controlled evaluation of prophylactic activity compared with conventional models that rely on activation of latent *P. carinii* infections.

In summary, we describe an immunosuppressed rat model of *P. carinii* pneumonia that results in a predictable course of disease development which includes moderate *P. carinii* infections in 2 to 3 weeks, heavy infections in 4 to 5 weeks, and a high percentage of mortality due to *P. carinii* pneumonia in 6 weeks. The model also provides uninfected, immunosuppressed contemporaneous controls, an experimental compartment that is needed to correctly interpret results obtained from many different studies. Noninvasive i.t. inoculations of cryopreserved parasites into *P. carinii*- and virus-free rats immunosuppressed by weekly injections of methylprednisolone are key features of the model that result in the development of consistent heavy *P. carinii* infections and very few secondary infections that can interfere with studies. This model is useful for maintaining over time isolates or strains of *P. carinii*, for producing large numbers of parasites for laboratory studies, and for evaluating the anti-*P. carinii* activity of experimental compounds and approved drugs.

REFERENCES

1. **Bartlett, M. S., J. A. Fishmen, M. M. Durkin, S. F. Queener, and J. W. Smith.** 1990. *Pneumocystis carinii*: improved models to study efficacy of drugs for treatment or prophylaxis of *Pneumocystis* pneumonia in the rat (Rattus sp.). Exp. Parasitol. **70:**100–106.
2. **Bartlett, M. S., J. A. Fishman, S. F. Queener, M. M. Durkin, M. A. Jay, and J. W. Smith.** 1988. New rat model of *Pneumocystis carinii* infection. J. Clin. Microbiol. **26:**1100–1102.
3. **Bartlett, M. S., S. F. Queener, M. A. Jay, M. M. Durkin, and J. W. Smith.** 1987. Improved rat model for studying *Pneumocystis carinii* pneumonia. J. Clin. Microbiol. **25:**480–484.
4. **Centers for Disease Control.** 1989. AIDS weekly surveillance report. **30 January:**1–5. Centers for Disease Control, Atlanta.
5. **Cushion, M. T., J. A. De Stefano, and P. D. Walzer.** 1988. *Pneumocystis carinii*: surface reactive carbohydrates detected by lectin probes. Exp. Parasitol. **67:**137–147.
6. **Frenkel, J. K., J. T. Good, and J. A. Schultz.** 1966. Latent *Pneumocystis* infection of rats, relapse, and chemotherapy. Lab. Invest. **15:**1559–1577.
7. **Futura, T., K. Ueda, K. Fujiwara, and K. Yamanouchi.** 1985. Cellular and humoral immune responses of mice subclinically infected with *Pneumocystis carinii*. Infect. Immun. **47:**544–548.
8. **Hughes, W. T.** 1991. Closing comments: *Pneumocystis carinii* symposium. J. Protozool. **38:**243S.
9. **Hughes, W. T., V. L. Gray, W. E. Gutteridge, V. S. Latter, and M. Pudney.** 1990. Efficacy of a hydroxynaphthoquinone, 566C80, in experimental *Pneumocystis carinii* pneumonitis. Antimicrob. Agents Chemother. **34:**225–228.
10. **Hughes, W. T., P. C. McNabb, and T. D. Makres.** 1974. Efficacy of trimethoprim and sulfamethoxazole in the prevention and treatment of *Pneumocystis carinii* pneumonitis. Antimicrob. Agents Chemother. **5:**289–293.
11. **Jones, S. K., J. E. Hall, M. A. Allen, S. D. Morrison, K. A. Reddy, V. V. Geratz, and R. R. Tidwell.** 1990. Novel pentamidine analogs in the treatment of experimental *Pneumocystis carinii* pneumonia. Antimicrob. Agents Chemother. **34:**1026–1030.

12. **Kovacs, J. A., J. L. Halpern, B. Lundgren, J. C. Swan, J. E. Parrillo, and H. Masur.** 1989. Monoclonal antibodies to *Pneumocystis carinii*: identification of specific antigens and characterization of antigenic differences between rat and human isolates. J. Infect. Dis. **159:**60–70.
13. **Kovacs, J. A., and H. Masur.** 1989. Prophylaxis of *Pneumocystis carinii* pneumonia: an update. J. Infect. Dis. **160:**882–886.
14. **Pesanti, E. L., and C. Cox.** 1981. Metabolic and synthetic activities of *Pneumocystis carinii* in vitro. Infect. Immun. **34:**908–914.
15. **Porter, W. P., Y. S. Bitar, J. D. Strandberg, and P. C. Charache.** 1985. Absence of therapeutic blood concentrations of tetracycline in rats after administration in drinking water. Lab. Anim. Sci. **35:**71–74.
16. **Walzer, P. D., C. K. Kim, and M. T. Cushion.** 1989. *Pneumocystis carinii*, p. 83–178. *In* P. D. Walzer and R. Genta (ed.), Parasitic infections in the compromised host: immunologic mechanisms and clinical applications. Marcel Dekker, Inc., New York.
17. **Walzer, P. D., C. K. Kim, H. J. Foy, M. J. Linke, and M. T. Cushion.** 1988. Cationic antitrypanosomal and other antimicrobial agents in the therapy of experimental *Pneumocystis carinii* pneumonia. Antimicrob. Agents Chemother. **32:**896–905.
18. **Walzer, P. D., M. E. Rutledge, and K. Yoneda.** 1983. Experimental *Pneumocystis carinii* pneumonia in C3H/HeJ and C3HeB/FeJ mice. J. Reticuloendothel. Soc. **33:**1–9.
19. **Walzer, P. D., V. Schnelle, D. Armstrong, and P. P. Rosen.** 1977. Nude mouse: a new experimental model for *Pneumocystis carinii* infection. Science **197:**177–179.