INFECTION AND IMMUNITY, June 1979, p. 939-947
0019-9567/79/06-0939/09$02.00/0

Vol. 24, No. 3

Experimental *Pneumocystis carinii* Pneumonia in Different Strains of Cortisonized Mice

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Received for publication 20 November 1979

Pneumocystis carinii pneumonia was produced in eight different strains of mice by the administration of corticosteroids, low (8%)-protein diet, and tetracycline in the drinking water. Heavier degrees of *P. carinii* infection were most consistently found in C3H/HeN mice; intermediate levels occurred in BALB/c AnN, C57BL/6N, B10.A(2R), AKR/J, and Swiss Webster mice; lighter degrees were found in DBA/2N and DBA/IJ mice. Histopathologically, *P. carinii* organisms were morphologically indistinguishable from human and rat *P. carinii*, and elicited a predominantly mononuclear response that was similar among the various mouse strains. The optimal cortisone acetate regimen was 1 mg injected subcutaneously twice weekly. Higher doses shortened the life span of the mice, presumably by inducing overwhelming bacterial infection. This problem occurred not only in different strains of mice, but also in the same strain of mice obtained from different breeders. Thus, cortisonized mice should be useful in the study of experimental *P. carinii* infection. Success of this model depends on the corticosteroid dose, as well as the strain, source, general health, and preexisting microbial flora of the mice chosen for study.

Pneumocystis carinii is an important cause of pneumonia in immunosuppressed patients (20). Although *P. carinii* is widely distributed as a saprophyte in nature, only a few animal species have been studied as experimental models for the human disease. Pneumocystis pneumonia develops in 8 to 12 weeks in rats by the administration of corticosteroids and antibiotics; the mechanism appears to be reactivation of latent infection (5). The rat model has contributed significantly to our understanding of the pathogenesis of *P. carinii* infection and has served as the basis for epidemiological, immunological, and therapeutic studies (8, 9, 11-13, 24). Pneumocystis pneumonia has been produced in cortisonized rabbits (17), but this system has not been further explored.

New animal models would not only enhance our knowledge of *P. carinii* but also provide an additional source of organisms for laboratory studies. The mouse is of particular interest because its well-defined genetic system might reveal important differences in host susceptibility to *P. carinii* infection.

The present study was undertaken to produce *P. carinii* pneumonia in different strains of mice by the administration of corticosteroids.

MATERIALS AND METHODS

Sources of mice. Groups of mice were initially obtained through an interagency agreement between the National Cancer Institue (NCI) and Veterans Administration. The NCI contracts with different commercial breeding laboratories to maintain colonies of selected strains of mice. These mice are usually raised apart from the breeder's own mice. The NCI mice of a particular strain are histocompatible regardless of breeder location; however, their nutrition, microbiological flora, and general state of health will vary from breeder to breeder. To minimize this variation, some strains of mice were purchased commercially from the same breeder. Other strains of mice not available through the NCI were also purchased commercially.

The following strains of mice were used: C3H/HeN from the National Institutes of Health (NIH) (Charles River Lab, Cambridge, Mass.) and Charles River Lab own stock; C57BL/6N from NIH (Simonsen Labs, Simonsen, Calif.) and Charles River Lab own stock; BALB/cAnN from NIH (Charles River Lab and Laboratory Supply, Indianapolis, Ind.) and from Charles River Lab own stock; DBA/2N from NIH (Charles River Lab); B10.A(2R) from NIH (ARS Sprague Dawley, Madison, Wis.); DBA/1J from Jackson Labs (Bar Harbor, Me.); AKR/J from Jackson Labs; outbred Swiss Webster from NIH (Charles River Lab) and Harland Industries (Indianapolis, Ind.).

Experimental design. Female mice of all strains were studied and weighed about 18 to 20 g when obtained. Male C3H/HeN and BALB/cAnN mice weighing 18 to 20 g and male Swiss Webster retired breeder mice weighing 40 g were also studied. The mice were housed in standard cages with no filter tops in a room with mice used by other investigators. The

mice were allowed to acclimate on a diet of regular food and tap water ad libitum for 2 weeks before any studies were begun.

The mice of a particular strain to be studied were randomly divided into groups of three to six per cage and individually labeled. The mice were usually weighed weekly throughout the study. Food was provided either as regular or low (8%)-protein diet; intake was not measured. Tap water with or without tetracycline (1 mg/ml) was provided ad libitum in a single bottle for each cage, and was changed twice weekly; in selected instances the consumption of water for different cages was measured.

Corticosteroids were administered either as twice-weekly subcutaneous injections of cortisone acetate or as dexamethasone added to the drinking water, according to the regimens outlined in Table 1. The initial cortisone acetate dosage regimens A to D were chosen on the basis of the work of Frenkel and Havenhill (6) to provide a spectrum of from mild weight loss to severe wasting and shortening of life span. Later, other dose schedules were studied. The dexamethasone regimens were adapted from studies by Hendley and Weller in rats (8) and from our own previous experience.

Histopathology. The mice were sacrificed by an overdose of halothane usually after 8 to 12 weeks, or when they appeared ill. Attempts were made to salvage specimens from mice already dead, but cannibalism sometimes occurred. At autopsy, histopathological sections were made of the lungs and stained with hematoxylin and eosin and methenamine silver. Lung imprint smears were stained with Giemsa and cresyl echt violet (2). In selected instances, cultures were performed on standard bacteriological and fungal media.

A standardized procedure was established for grading the intensity of *P. carinii* infection. At least three pieces of lung were removed for histopathological examination: one each from the upper and lower portions of the right lung, and one from the midportion of the left lung. The lung sections were stained with methenamine silver, coded, and read blindly. The following scoring system for infection was used: 0 = no *P. carinii* found; 0.5+ = minimal infection, <1% alveoli involved; 1+ = light, 1 to 25% alveoli involved; 2+ = moderate, 25 to 50% alveoli involved; 3+ = heavy, 50 to 75% alveoli involved; 4+ = very heavy infection, ≥75% alveoli involved. For comparison, the mean score of the group was calculated only from those members where *P. carinii* was demonstrated (i.e., with a score ≥ 0.5+).

TABLE 1. *Experimental corticosteroid regimens for mice*

Regimen	Cortisone acetate[a] (mg)	Diet	Tetracycline[b] in H_2O?
A	None	Regular	Yes
B	1.0	Low protein	Yes
C	2.5	Low protein	Yes
D	5.0	Low protein	Yes
E	1.5	Low protein	Yes
F	1.0	Regular	Yes
G	2.5	Regular	Yes
H	None	Low protein	Yes
I	None	Regular	No

Regimen	Dexamethasone[c] (mg/1,000 ml)	Diet	Tetracycline prepn in H_2O[b]
J-1	0.75	Low protein	Sumycin
J-2	1.00	Low protein	Sumycin
J-3	1.50	Low protein	Sumycin
J-4	2.25	Low protein	Sumycin
J-5	1.00	Low protein	Polyotic

[a] Dose injected subcutaneously twice weekly.
[b] Concentration of tetracycline preparations in drinking water was always 1 mg/ml.
[c] Concentration in drinking water.

RESULTS

Cortisone acetate regimens. Initial studies were performed on DBA/2N, BALB/cAnN, and Swiss Webster mice (Fig. 1A). Mice treated with cortisone acetate regimens B (1 mg), C (2.5 mg), and D (5 mg) experienced considerable weight loss as compared with mice on regimen A (controls). Regimens B and C permitted survival times of at least 6 weeks, whereas regimen D shortened life span considerably. Since the heaviest degree of *P. carinii* infection occurred in regimen C (Table 2), this appeared to be the most promising regimen.

The same studies were then performed in C3H/HeN and C57BL/6N mice (Fig. 1B). Regimens C and D resulted in a profound shortening of life span. At the same time, about 70 BALB/cAnN mice had been obtained, through the NCI interagency agreement, from a different breeder than the first group of BALB/c mice. These 70 BALB/c mice were started on regimen C of cortisone acetate and were going to be sacrificed at weekly intervals to study the sequential development of *P. carinii* infection. However, the mice started dying in great numbers within 2 weeks after starting cortisone. Regimen C was discontinued in the surviving mice, and regimen B was instituted. The remaining mice survived a mean duration of 41 days (Table 2).

Regimen B was thus selected as the standard cortisone acetate regimen. In other studies of sequential *P. carinii* infections not reported here, regimen B was administered to large numbers of C3H/HeN and BALB/cAnN mice with good survival.

Dexamethasone regimens. Dexamethasone was administered to selected groups of mice in their drinking water in doses of 0.75 to 2.25 mg/liter (regimens J1 to J5) (Table 1). The number of mice in different treatment groups was small. Although dexamethasone is considerably more potent than cortisone acetate, there was no correlation between dexamethasone dose and length of survival of the mice (Table 3). It

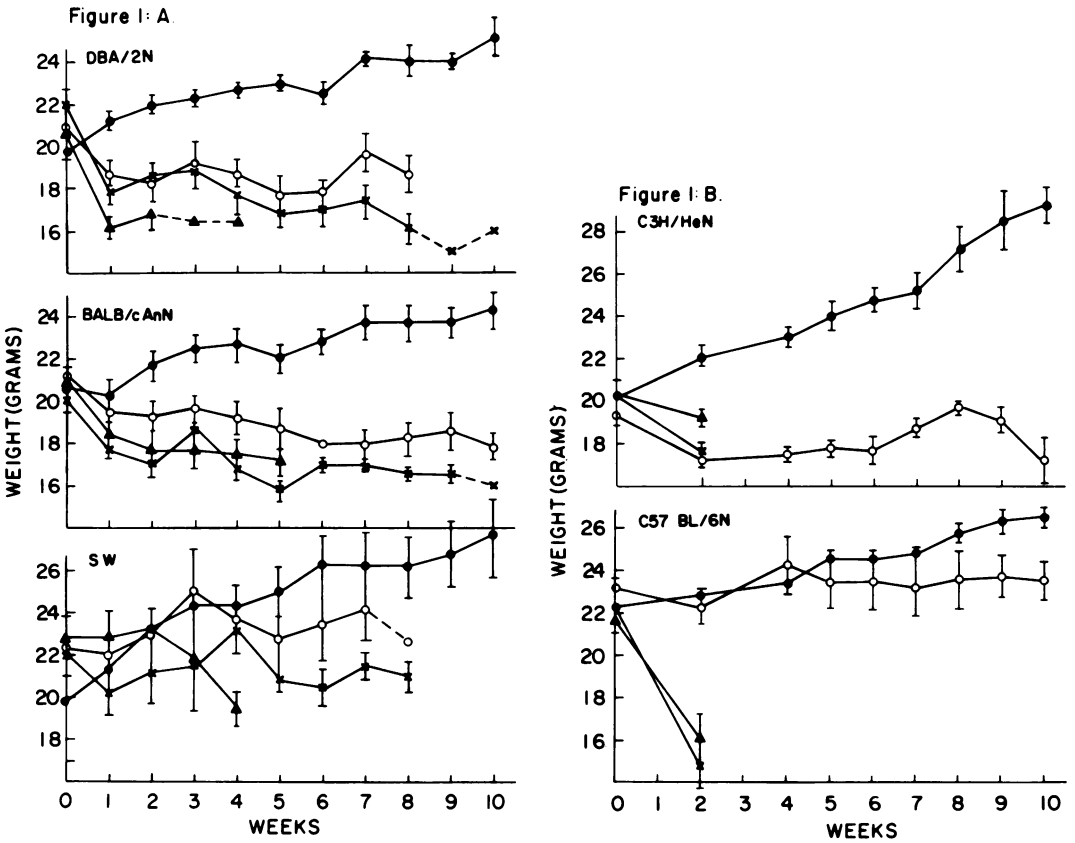

FIG. 1. *Sequential weight changes in mice in different corticosteroid regimens. Each point represents the mean ± 1 standard error of the mean weight of at least three mice. Dotted line indicates less than three mice in group.* (●) *Regimen A, controls.* (○) *Regimen B, 1 mg;* (×) *regimen C, 2.5 mg; and* (▲) *regimen D, 5 mg of cortisone acetate injected subcutaneously twice weekly.*

was possible that other factors (e.g., palatability of drinking water, antibiotic preparation, etc.) might have influenced the amount of dexamethasone consumed. Thus, studies were designed to measure the amount of drinking water consumed on different dexamethasone regimens. The total amount of water consumed by the animals of a cage each week was measured, and the result was expressed as the mean volume of water consumed per mouse per week.

Water consumption was compared for two different groups of C57BL/6N mice. The mean (± standard error of the mean) volumes of water consumed were 57 ± 4 ml per mouse per week for regimen J2 and 64 ± 7 ml per mouse per week for regimen J5. The survival time and intensity of *P. carinii* infection were similar for both groups (Table 3). Among AKR/J mice the mean volumes (± standard error of the mean) consumed were 55 ± 4 ml per mouse per week for regimen J2, 62 ± 3 ml per mouse per week for regimen J3, and 104 ± 9 ml per mouse per week for regimen J5. The survival time and intensity of *P. carinii* infection were virtually identical for regimens J2 and J5, whereas mice on regimen J3 lived considerably longer and were more heavily infected (Table 3). The reasons for the differences in water consumption are unclear. Sumycin, the tetracycline preparation in regimens J2 and J3, is a sweet-tasting suspension intended for human pediatric use. Sumycin tended to settle out in the water bottles; it is possible that the high concentration of Sumycin sometimes attained in the sipper tubes was unpalatable to the mice. Polyotic, the tetracycline used in regimen J5, is a bitter-tasting powdered veterinary preparation which dissolved well in the drinking water.

***P. carinii* infection on cortisone acetate.** *P. carinii* was found in all strains of female mice receiving cortisone acetate injections (Table 2). The intensity of the infection could be grouped into three broad categories. C3H/HeN mice consistently had the heaviest degree of infection.

TABLE 2. *P. carinii infection in female mice*

Group	Regimen	Survival (days)[a]	No. of mice with PC[b]/total	Mean PC[b] score
C3H/HeN	A	74 ± 2	1/6	0.5
	B	64 ± 3	8/9	2.4
	C	24 ± 5	2/3	2.0
	D	15 ± 3	4/5	1.4
	F	66 ± 0	4/4	1.5
	G	50 ± 1	4/4	1.0
BALB/cAnN	A	101 ± 9	2/9	0.5
	B	87 ± 7	1/5	2.0
	C	69 ± 2	6/6	2.0
	D	47 ± 5	2/3	1.8
	C → B[c]	41 ± 2	17/19	1.0
	H	72 ± 1	0/4	0.0
	I	74 ± 0	0/4	0.0
C57BL/6N	A	79 ± 13	1/6	0.5
	B	72 ± 5	8/9	1.1
	C	14 ± 2	0/3	0.0
	D	14 ± 0	0/1	0.0
B10.A(2R)	A	64 ± 1	1/6	0.5
	B	45 ± 8	3/4	1.8
	E	18 ± 1	4/6	0.8
	G	26 ± 2	3/4	0.8
AKR/J	A	89 ± 0	2/6	0.5
	B	25 ± 3	6/6	1.1
Swiss Webster	A	97 ± 3	0/6	0.0
	B	57 ± 4	2/3	0.5
	C	47 ± 8	3/4	1.0
	D	29 ± 11	2/3	0.8
DBA/2N	A	96 ± 2	0/6	0.0
	B	59 ± 0	0/3	0.0
	C	59 ± 10	3/6	1.0
	D	24 ± 3	2/5	0.5
DBA/1J	A	49 ± 0	0/3	0.0
	B	13 ± 2	1/5	0.5
	E	19 ± 3	0/4	0.0
	F	21 ± 2	1/5	1.0
	G	28 ± 0	2/3	0.8
	H	46 ± 0	2/4	0.5

[a] Mean ± standard error of the mean.
[b] PC, *P. carinii* infection.
[c] Started on regimen C and switched to regimen B.

TABLE 3. *P. carinii infection in male and female mice*

Group	Regimen	Sex	Survival (days)[a]	No. of mice with PC[b]/total	Mean PC[b] score
C3H/HeN	A	M	59 ± 0	1/6	0.5
	B	M	54 ± 3	5/5	2.6
BALB/cAnN	A	M	59 ± 0	2/6	0.5
	B	M	54 ± 3	5/5	2.4
Swiss Webster	A	M	92 ± 13	1/3	0.5
	J1	M	104 ± 0	1/1	1.0
	J3	M	105 ± 0	3/3	1.7
	J4	M	80 ± 8	2/2	1.2
	J(total)	M	96 ± 8	6/6	1.4
C57BL/6N	J2	F	61 ± 6	4/4	1.2
	J5	F	67 ± 0	3/3	1.3
	J(total)	F	64 ± 3	7/7	1.3
AKR/J	J2	F	35 ± 6	4/4	1.1
	J5	F	34 ± 5	4/4	1.1
	J3	F	82 ± 0	3/3	2.3
	J(total)	F	47 ± 7	11/11	1.4

[a] Mean ± SEM.
[b] PC, *P. carinii* infection.

The results of two different groups of C3H/HeN mice were very similar and thus have been pooled. *P. carinii* infection was higher in C3H/HeN mice treated with cortisone acetate and low-protein diet (regimens B and C) than in mice treated with the same doses of cortisone acetate and regular diet (regimens F and G).

Intermediate levels of infection were found in BALB/cAnN, C57BL/6N, B10.A(2R), AKR/J, and outbred Swiss Webster mice. All groups of mice on regimen B survived at least 6 weeks, except AKR/J mice, which survived an average of 25 days. The results of two different groups of C57BL/6N mice were similar and have been pooled.

DBA/2N and DBA/1J mice tended to have both a lower frequency and lighter intensity of *P. carinii* infection. The survival of DBA/2N mice was similar to that of other mice, whereas the survival of DBA/1J mice was considerably shorter on a variety of cortisone acetate regimens.

Male mice were studied to compare the possible effect of sex on susceptibility to *P. carinii* infection. Regimen B administered to groups of male C3H/HeN and BALB/cAnN mice produced a considerable degree of weight loss (Fig. 2). The intensity of *P. carinii* infection in both groups was similar (Table 3); this resembled the intensity of *P. carinii* infection in female C3H/HeN mice but was considerably heavier than that of female BALB/cAnN mice.

***P. carinii* infection on dexamethasone.** Female C57BL/6N and AKR/J mice on oral dexamethasone had about the same degree of *P. carinii* infection as did their counterparts on regimen B cortisone acetate (Tables 2 and 3). The only exception was the larger surviving group of AKR/J mice on regimen J3.

Small groups of outbred retired breeder male Swiss Wester mice were administered different dose regimens of dexamethasone. The pooled results indicated that these mice lost weight and survived a long period of time (Fig. 2) and had an intermediate level of *P. carinii* infection (Table 3).

***P. carinii* infection without corticosteroids.** Most mice on regimen A (controls) were not infected with *P. carinii* on lung sections. Small numbers of C3H/HeN, BALB/cAnN, C57BL/6N, B10.A(2R), AKR/J, and male Swiss Webster mice had minimal infection. Female Swiss Webster, DBA/2N, and DBA/1J mice showed no infection.

Histopathology. Histopathologically, *P. carinii* infection in mice was similar to that in rats, except that the rats were more heavily infected. Detailed studies will be published elsewhere. Mouse *P. carinii* on methenamine silver-stained

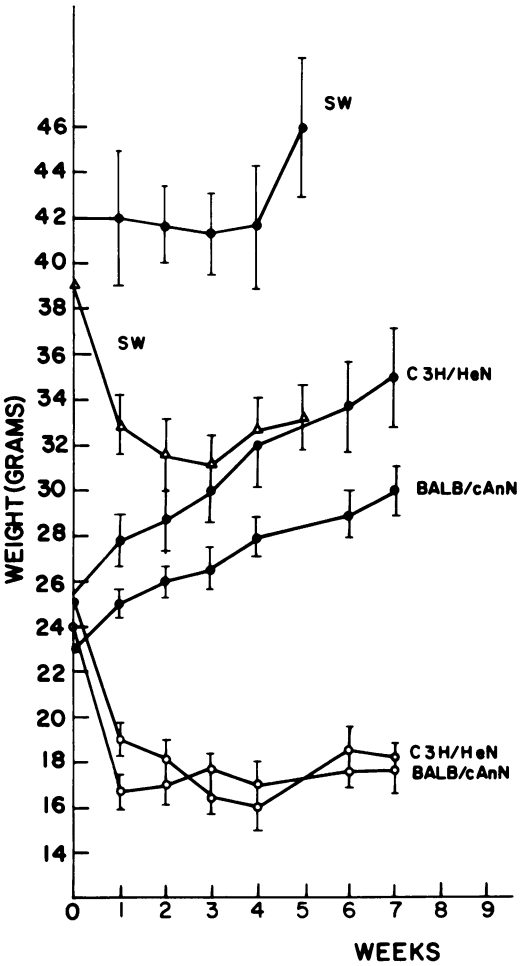

FIG. 2. *Same as Fig. 1. Regimen J represents pooled results of different oral dexamethasone doses added to drinking water.* (●) *Regimen A,* (○) *regimen B, and* (△) *regimen J.*

lung sections (Fig. 3) and on Giemsa-stained smears (Fig. 4) was morphologically indistinguishable from rat and human *P. carinii.* The organisms were found along the walls of alveoli and also in small clusters (Fig. 5). On hematoxylin and eosin sections there was primarily a mononuclear cell response. The appearance and distribution of *P. carinii,* as well as host inflammatory response, were similar for the various strains of mice.

Fungal and bacterial infection. Fungal cultures were performed on the lungs of cortisonized and control mice. Initial culturing effects were directed mainly toward mice on higher cortisone dosage regimens; later cultures were taken at random from different groups of mice. Of 34 cortisonized mice cultured, 12 (38%) grew out fungi. The predominant organisms were *Candida* sp., *Trichosporon cutaneum,* and *Penicillium* sp. Four of the 12 mice were cage mates on regimen C, and 2 others were cage mates on regimen D. Fungi were seen in histological lung sections in 5 of the 12 mice. *P. carinii* was present in 8 of the 12 mice and in 16 of the 22 culture-negative mice. *P. carinii* could be easily distinguished from fungi on the basis of morphology, size, and distribution.

Fungi grew out of only one of seven lungs of control mice that were cultured; *P. carinii* was present in three of the seven control mice.

Bacterial cultures were performed on different body sites of several mice dying shortly after beginning corticosteroid treatment. There was no predominant pathogen found. The most frequent organisms were *Escherichia coli, Klebsiella pneumoniae,* and group D streptococci; *Pseudomonas aeruginosa* was uncommon. The bacteria were isolated from multiple organs (e.g., lung, liver, kidney) and were usually resistant to tetracycline.

DISCUSSION

There has been little apparent interest in studying *P. carinii* infection in mice. During a study of the effects of corticosteroids on different animal species, Frenkel et al. found mild *Pneumocystis* pneumonia in one of four autopsied mice (5). However, this work has not been further pursued. Recently we have transmitted human and rat *P. carinii* to athymic (nude) mice without the use of corticosteroids (21). Ueda et al. (18) found naturally occurring pneumocystis pneumonia in a nude mouse colony, and also produced the infection in a few heterozygous littermates by administration of cortisone acetate and cyclophosphamide.

The present study demonstrates that the cortisonized mouse can serve as an experimental model for pneumocystis pneumonia. *P. carinii* was found in all strains of mice tested. Differences in *P. carinii* infection among individual mouse strains must be interpreted with caution, in some cases, because of the small numbers of mice involved. Nevertheless, the intensity of *P. carinii* infection in the mouse strains could be divided into three broad categories.

There are several possible explanations for the strain differences in *P. carinii* infection found. One is that they represent genetic differences in susceptibility to the organism. The mouse strains used here are similar to those used in studies of genetic susceptibility to a variety of organisms, including *Toxoplasma gondii,* which shares cartain similarities with *P. carinii.* In a study of *T. gondii,* DBA/2 mice remained sus-

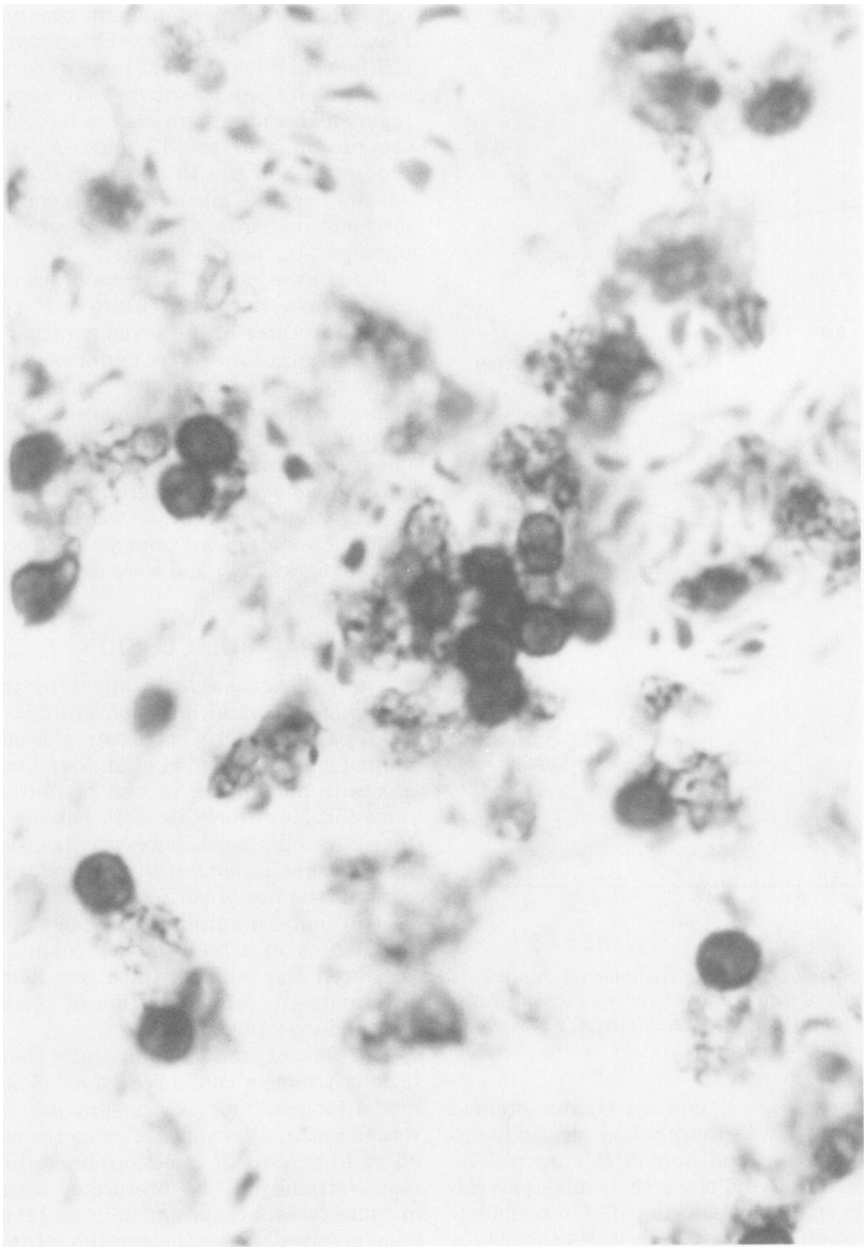

FIG. 3. *P. carinii cysts in mouse lung section; methenamine silver stain, ×1,250.*

ceptible and DBA/1 mice remained resistant to both high and low inoculum challenges, whereas the susceptibility of BALB/c mice varied markedly with inoculum size (1). The authors have recently postulated that susceptibility to *T. gondii* is under multigeneic control (25).

A major problem in studying *P. carinii* is that the pneumonia produced by corticosteroids in mice represents reactivation of latent infection rather than exogenous organism challenge. Evaluation of the inflammatory response of the mice to *P. carinii* was also impeded by corticosteroids. Recent studies of Sendai virus infection have shown that sensitive mouse strains exhibited more extensive histological evidence of pulmonary damage to exogenous challenge than did more resistant mouse strains (15).

A second hypothesis is that the strain differ-

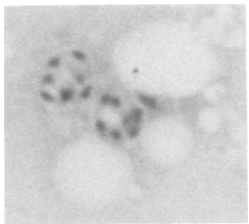

FIG. 4. *P. carinii cysts with up to eight daughter forms in mouse lung imprint smear; Giemsa stain, ×1250.*

ences represent genetic differences in susceptibility to corticosteroids. Corticosteroids have two types of effects on lymphoid cells: rats, mice, hamsters, and rabbits are steroid "sensitive," whereas ferrets, guinea pigs, monkeys, and humans are steroid "resistant" (3). We are unaware of published reports of differences in the effects of long-term corticosteroids administration among various strains of mice. In the present study, most of the mice lost weight on corticosteroid treatment and exhibited a nonspecific mononuclear inflammatory response. Studies comparing the effects of corticosteroids on C3H/HeN and C3H/HeJ mice would be of interest. C3H/HeJ mice exhibit some unique characteristics: their B lymphocytes are unresponsive to the stimulating effects of bacterial endotoxin, and their macrophages have a selective defect in tumoricidal capacity (16).

A third hypothesis is that the strain differences in *P. carinii* infection merely reflect the microbial flora of the environment where the mice were raised. The fact that similar degrees of *P. carinii* occurred in the same strains of mice (e.g. C57BL/6N) obtained from different breeders suggests this hypothesis is less likely. Five mouse strains were supplied, at least in part, by the same breeder (Charles River Lab) and thus were probably subjected to similar environmental conditions. Yet, different degrees of *P. carinii* infection were found.

The corticosteroid regimens for experimental pneumocystis pneumonia have been most extensively studied in rats. Corticosteroids were first used by Weller (22, 23). Frenkel et al. studied different corticosteroid dosages and introduced the use of tetracyclines to prevent *Corynebacterium kutscheri* infection (5). The regimen of 25 mg of cortisone acetate injected subcutaneously twice weekly and a tetracycline (0.5 to 1.0 mg/ml) in the drinking water has become the most widely used method of producing *P. carinii* pneumonia. Hendley and Weller found that dexamethasone added to the drinking water can also be used (8). Hughes et al. demonstrated that protein calorie malnutrition enhances the development of *P. carinii* pneumonia (10). We routinely employ low (8%)-protein diet with the corticosteroid regimens in rats because it consistently produces heavier degrees of *P. carinii* infection.

In the present study a variety of corticosteroid regimens were employed, usually in combination with low-protein diet. The optimal dose of cortisone acetate in mice was 1 mg injected subcutaneously twice weekly; this is equivalent to slightly less than half the 25-mg rat dose (14 mg/kg per day versus 30 to 35 mg/kg per day) (6) and may help explain why the mice had lighter degrees of *P. carinii* infection. The lack of changes in survival or in degree of *P. carinii* infection among the mice to different doses of dexamethasone is unexplained and requires further study. With the most widely used dexamethasone regimen (J2 or J5, 1 mg/1,000 ml) a 20-g mouse drank about 55 to 65 ml of water per week or 8 to 9 ml per day. This mouse thus consumed 0.4 to 0.5 mg of dexamethasone per kg per day, which approximates the amount of dexamethasone consumed by rats found by Hendley and Weller using a similar regimen (8). In terms of glucocorticoid potency, this is about equivalent to a regimen of 1 mg of cortisone acetate (7).

The major problem in mice treated with immunosuppressive agents has been the risk of overwhelming bacterial infection, particularly *P. aeruginosa* (4). In the present study, overwhelming bacterial infection was the presumptive cause of early death in mice treated with higher doses of corticosteroids. Limited culturing revealed no predominant pathogen. Of particular interest were the differences in survival of the same strain of mice (e.g. BALB/cAnN) treated with the same corticosteroid dose but obtained from different breeders. This dramatically illustrates the importance of indigenous bacterial flora in determining the value of a mouse strain in long-term studies with a slow-growing organism such as *P. carinii*.

Several measures can be taken to help prevent bacterial infection. Acidification of the drinking water has been shown to suppress *P. aeruginosa* in mouse colonies (14). Tetracycline was empirically used in the present study because it had been successful in preventing *C. kutscheri* infection in rats. We have found that tetracycline tends to select out resistant organisms (e.g. *Flavobacterium meningosepticum*) in rat lungs. Since this may also occur in mice, studies of different antibiotic regimens in mice would be helpful. In addition, careful attention should be given to the source of the animal (i.e., the breeder).

In summary, the cortisonized mouse is a useful

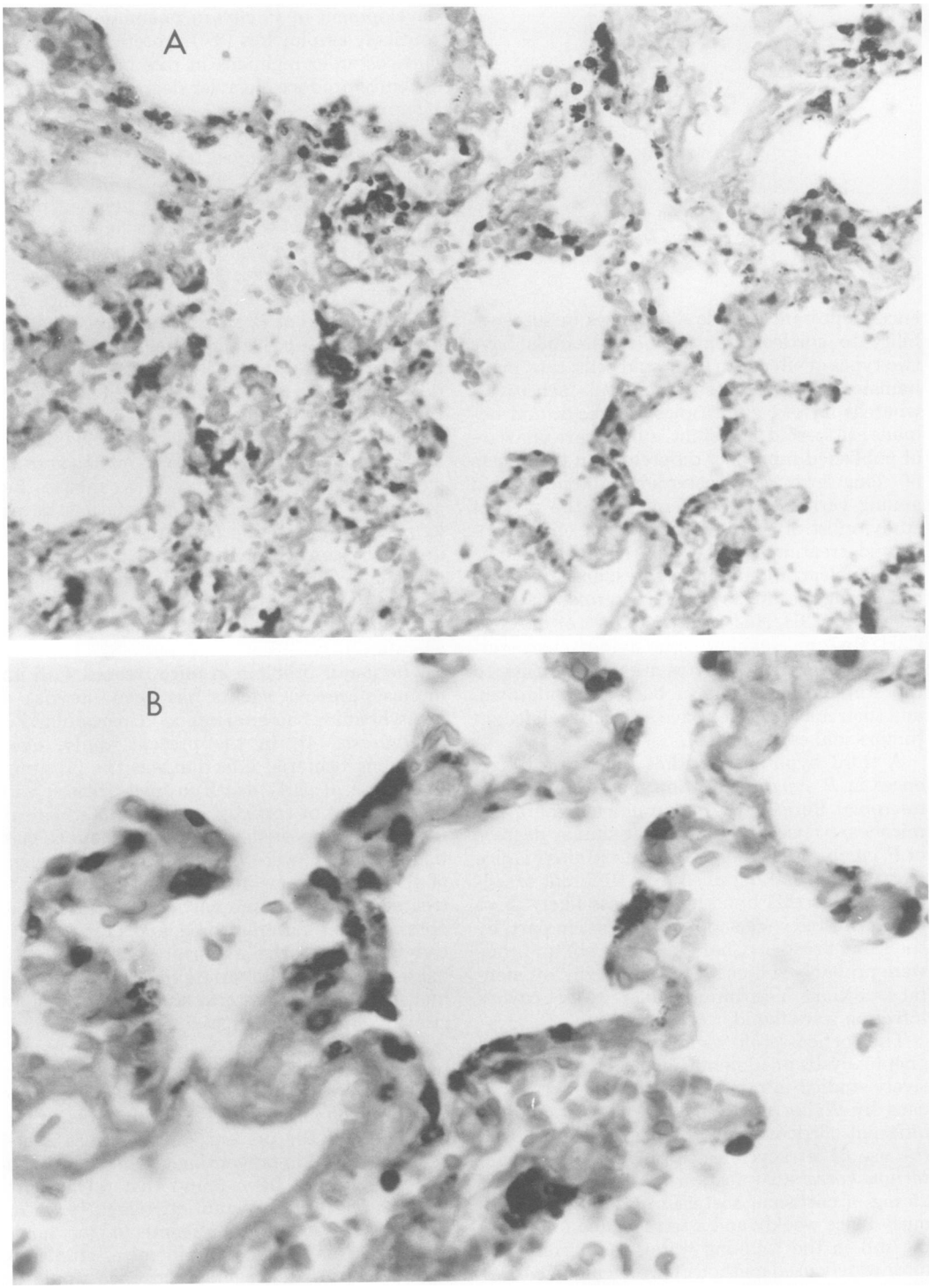

FIG. 5. *(A) P. carinii in mouse lung sections. Note arrangement of cysts along alveolar walls and in clusters. Methenamine silver stain. ×125. (B) Higher power view of (A) emphasizing alveolar wall distribution of organism. Methenamine silver stain, ×500.*

new animal model for *P. carinii* infection. However, mice are very sensitive to the effects of immunosuppression. The success of this model depends not only on the corticosteroid dose, but also on the strain, source, general health, and preexisting microbial flora of the mouse chosen for study.

ACKNOWLEDGMENTS

This work was supported in part by a grant from the Medical Research Service, Veterans Administration, by an American Cancer Society Institutional Research Grant, and by Public Health Service Biomedical Research Grant and Biomedical Research Support Grant RR-05374 from the Division of Research Facilities and Resources, National Institutes of Health.

We gratefully acknowledge the assistance of Mary Ellen Rutledge and Darrell Winsett.

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