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Comparison of Histologic and Quantitative Techniques in Evaluation of Therapy for Experimental *Pneumocystis carinii* Pneumonia

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***Pneumocystis carinii* pneumonia was induced in rats by the administration of corticosteroids, and histologic and quantitative techniques were compared in the evaluation of the severity of the disease and response to therapy. A highly significant correlation was found between the histologic score of the extent of alveolar involvement (the standard method of assessment) and the number of *P. carinii* cysts and nuclei in lung homogenates, lung weight, and lung weight/body weight ratio. Clear differences were noted between rats which responded well and rats which responded poorly to therapy by all techniques. Quantitation of *P. carinii* cysts and nuclei revealed a 10^4-fold reduction in organism burden with successful treatment. Thus, these techniques should be helpful in the development of testing of new antimicrobial agents in the rat model of pneumocystosis.**

Pneumocystis carinii, an organism of low virulence, is an important cause of pneumonia in the immunocompromised host, particularly malnourished infants, children with immunodeficiency disorders, and patients of all ages receiving immunosuppressive therapy (20). With the emergence of the acquired immunodeficiency syndrome, the number of cases of pneumocystosis has risen dramatically (1). The problems in the treatment of *P. carinii* pneumonia in patients with acquired immunodeficiency syndrome (i.e., slow response, frequent relapse, high rate of adverse drug reactions) (8, 16) have stimulated efforts to develop new forms of therapy (3, 7, 13).

Drug development for pneumocystosis has mainly been conducted in animal models in which rats administered corticosteroids for about 8 weeks spontaneously develop the disease (5, 10–13, 15, 22). Assessment of drug efficacy has been based on histopathologic examination of the extent of *P. carinii* pneumonia in the lungs. While this process has been useful, it is largely subjective and has not permitted the development of uniform or quantitative standards. Thus, it has been difficult to compare the results of one investigator with those of another even if the same treatment regimen was used.

We have previously found a high degree of correlation between a semiquantitative histologic scoring system of the intensity of *P. carinii* pneumonia and the number of *P. carinii* cysts in lung homogenates of infected animals (21). More recently, we have developed the ability to quantitate *P. carinii* nuclei in lung homogenates and in tissue culture (2–4); this technique has allowed evaluation of cysts, trophozoites, and intermediate stages in the life cycle of *P. carinii* and has been a sensitive indicator of organism replication in vitro.

In the present study, we applied these methods to the evaluation of drug treatment of experimental pneumocystosis by comparing the histologic score with *P. carinii* cyst and nuclei counts, lung weight, and lung weight/body weight ratio.

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MATERIALS AND METHODS

Experimental protocol. The animals evaluated here were part of a larger study designed to compare the efficacy of different inhibitors of folic acid synthesis in the treatment of pneumocystosis. Adult male Sprague-Dawley rats obtained from Harlan Industries, Madison, Wis., weighing about 250 g were housed in a conventional colony room, ate a regular diet, and drank plain tap water for 1 to 2 weeks before the study. They were then placed on the standard immunosuppressive regimen of 25 mg of cortisone acetate injected subcutaneously twice weekly, a low (8%) protein diet, and tetracycline (1 mg/ml) in the drinking water (21). The animals were weighed at regular intervals; sentinel rats were sacrificed regularly to monitor the development of pneumonia. After 5 to 6.5 weeks, when the infection reached moderate intensity, the rats were randomly divided into different treatment groups. Drugs for the treatment of *P. carinii* pneumonia were administered for 3 weeks, during which time the rats remained on the immunosuppressive regimen. Control rats received no therapy. At the end of treatment, the rats were sacrificed by an overdose of halothane anesthesia.

Evaluation of the therapy of pneumocystosis was complicated by the fact that these heavily immunosuppressed rats were very susceptible to infections with other microorganisms and to the toxic effects of drugs. As a result, animals frequently died before completing the full course of *P. carinii* therapy. We decided to base assessment of treatment efficacy on the extent of disease in the lungs rather than on animal survival. Since our preliminary data suggested that 10 days were sufficient to observe a therapeutic response, rats which received drugs for this period of time were included in the data analysis.

Histopathologic assessment of *P. carinii* pneumonia. At death or time of sacrifice, the left lung from each animal was removed, infused with 4% buffered formaldehyde solution through the bronchus until fully expanded, and fixed further in formaldehyde for several hours to several days until processed for histologic preparation. The lungs were inflated

TABLE 1. Duration of treatment of *P. carinii* pneumonia and survival of different rat groups

Group	Regimen	No. of rats	Survival (days)		Treatment (days)		Histologic score (median)
			Median	Range	Median	Range	
A	Corticosteroid control	31	58	55–63	20	13–21	4+
B	Poor response	12	57	57–58	21	19–21	4+
C	Intermediate response	12	58	58[a]	20	20[a]	2+
D	Good response	24	60	57–63	21	21[a]	0
E	Normal control	18	67	55–71	21	21[a]	0

[a] All test animals were sacrificed at the end of the study period.

because the collapsed alveoli in uninflated lungs hampered histopathologic examination. Three horizontal sections were taken from the upper, middle, and lower portions of the lung and stained with hematoxylin-eosin, which provided a general view of pulmonary architecture, and Grocott methenamine silver stain, which selectively stained the cyst wall of *P. carinii*. The lung sections were coded and read blindly. The following scoring system, as described previously (21), was used to evaluate the severity of *P. carinii* pneumonia: 0, no infection; 0.5+, minimal; 1+, light; 2+, moderate; 3+, severe; and 4+, very severe.

Quantitation of *P. carinii* cysts and nuclei. The right lung was used for the quantitation of *P. carinii* cysts and nuclei. Procedures for obtaining and quantitating *P. carinii* in fresh air-dried specimens of lung homogenate, as well as criteria for standardization and reproducibility, have been described in detail previously (2–4). Stains used to identify *P. carinii* included cresyl echt violet, which selectively stains the cell wall of the cyst, and Diff-Quik, which stains the nuclei of cysts, trophozoites, and intermediate forms. The lower limit of detection by this evaluation method is 1.47×10^5 organisms per lung. Specimens to be studied were coded and read in a blinded manner.

The total number of *P. carinii* in the lung was also divided by the weight of the lung and expressed as the number per gram of lung. However, since the cyst and nuclei counts per lung and per gram of lung were very similar and also showed a very high correlation in linear regression analyses ($r = 0.99$, $P < 0.001$), all cyst and nuclei counts reported here are presented on a per lung basis.

Lung weight and lung weight/body weight ratio. The right lung wet weight and body weight of each animal were recorded at the time of sacrifice.

Application of histologic and quantitative techniques to treatment of *P. carinii* pneumonia. Histologic examination was used initially as the standard method of drug evaluation. Rat groups were selected for analysis in this study on the basis whether they showed a poor, intermediate, or good response to therapy of pneumocystosis by the semiquantitative histologic scoring system. Data obtained by this method were then compared with *P. carinii* cyst and nuclei counts, lung weights, and lung weight/body weight ratios. No attempt was made to interpret the efficacy of a particular drug regimen per se. This information will be reported in detail separately (P. D. Walzer, manuscript in preparation).

The following rat groups were used (Table 1). Group A, the corticosteroid control group, remained on the immunosuppressive regimen throughout the study and had a median histologic score of 4+. Group B, the poor-response group, received either 100 mg of trimethoprim per kg per day orally or 100 mg of diaveridine per kg per day orally and had a median score of 4+. Group C, the intermediate-response group, received the oral combination of 50 mg of trimethoprim per kg per day or 50 mg of diaveridine per kg per day plus 15 mg of dapsone per kg per day and had a median score of 2+. Group D, the good-response group, received the oral combination of 50 to 100 mg of trimethoprim per kg per day or 50 to 100 mg of diaveridine per kg per day plus 250 to 500 mg of sulfamethoxazole per kg per day and had a median score of 0. Group E, the normal control group, ate a regular diet and drank plain tap water, received no immunosuppressive treatment, and had a median score of 0. Twenty group E rats were sacrificed at the beginning of the study, and 18 were sacrificed at the end of the study.

Statistical analysis. Linear regression analysis and the Wilcoxon rank sum test in the Clinfo Data Management and Analysis System were used when appropriate.

RESULTS

Comparison of histologic and quantitative techniques in assessment of *P. carinii* pneumonia. The histologic scoring system was first compared with quantitation of *P. carinii* cysts and nuclei (Fig. 1A and B). A highly significant correlation was found between the histologic score and the number of *P. carinii* cysts ($r = 0.933$, $P < 0.001$) and nuclei ($r = 0.927$, $P < 0.001$). Some overlap in organism counts at different histologic scores occurred. The number of *P. carinii* cysts and nuclei at the lower histologic scores (i.e., ≤ 2) were usually higher than the predicted regression line, suggesting that these quantitative techniques were more sensitive than histology in detecting light infection. This question is addressed more directly in the assessment of treatment.

A highly significant correlation was found between the number of *P. carinii* cysts and number of nuclei ($r = 0.947$, $P < 0.001$) (Fig. 1C). Nuclei counts were usually 5- to 10-fold higher than cyst counts in the same specimens.

The histologic score showed a strong correlation with the lung weight ($r = 0.812$, $P < 0.01$) and the lung weight/body weight ratio ($r = 0.842$, $P < 0.001$).

Assessment of treatment. The rat groups exhibited clear differences in response to treatment of pneumocystosis which could not be related to simple differences in survival or duration of treatment (Table 1). Histologic scoring revealed these differences to be highly significant among all rat groups (Fig. 2A; Table 2). The median score of 4 in the untreated or poor-response rat groups fell to 0 in the good-response group. Some overlap in the range of values occurred, particularly among the poor and intermediate responders.

A similar pattern of response to treatment was found with quantitation of *P. carinii* cysts (Fig. 2B) and nuclei (Fig. 2C). Median cyst and nuclei counts fell from 9.11×10^8 and 4.11×10^9 in untreated rats to 1.47×10^5 and 1.54×10^6, respectively, in the good responders.

The sensitivity of all three techniques in detecting minimal or residual infection was compared directly in group D rats which had a good response to treatment (Fig. 2). Of these 24 rats, *P. carinii* was demonstrated by nuclei quantitation in 12, cyst quantitation in 7, and histology in 7. In seven rats, nuclei quantitation was the only method able to detect the organism. Thus, nuclei quantitation appeared to be the most sensitive technique in detecting minimal or residual infection.

The response to treatment could also be distinguished

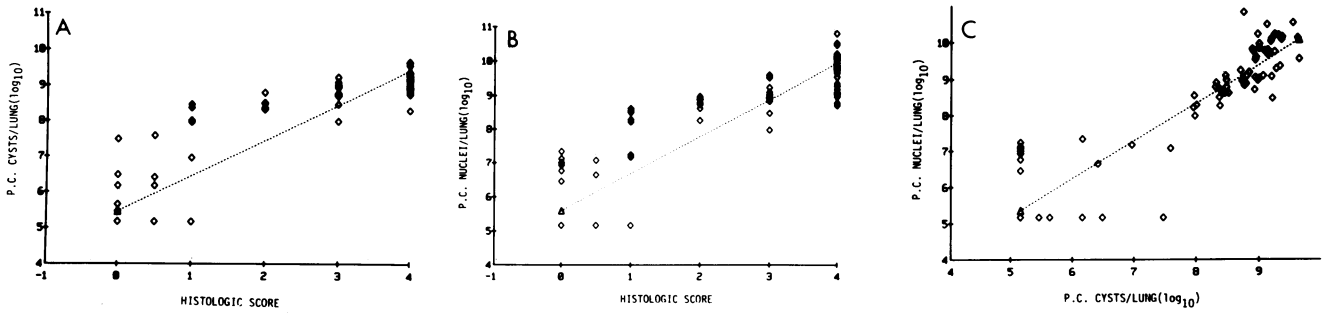

FIG. 1. Correlation of histologic score with the number of *P. carinii* cysts (A) and nuclei (B) in rat lung homogenates in 117 rats. Correlation of the number of *P. carinii* cysts with the number of *P. carinii* nuclei in rat lung homogenates (C). The data were generated by a computer program of the Clinfo Data Management and Analysis System. Darkened areas represent the same or very closely related data points, so not all animals were individually portrayed. Animals at the lower limit of detection are plotted as a single point regardless of the number of subjects; this gives the regression lines an artificially low appearance.

among rat groups on the basis of lung weight (Fig. 3A) and lung weight/body weight ratio (Fig. 3B). The median lung weight and lung weight/body weight ratio fell from 2.0 g and 0.014, respectively, in untreated rats to 1.1 g and 0.006, respectively, in the good responders.

DISCUSSION

The corticosteroid-treated rat model has been very helpful in the development of new antimicrobial agents for the treatment of *P. carinii* pneumonia. The present study was undertaken to determine whether quantitative techniques we have previously developed for *P. carinii* (2–4, 21) could be applied to evaluate therapy in this animal model. The histologic scoring system served as the standard for comparison, and special procedures were followed (e.g., lung inflation, sampling the same areas of each lung, coding the specimens) to ensure that this method accurately reflected the extent of *P. carinii* pneumonia in the lung. A highly significant correlation was found between the histologic score and the *P. carinii* cyst count, nuclei count, lung weight, and lung weight/body weight ratio. By all these parameters, clear differences were noted between rats which responded well to treatment and rats which responded poorly or received no treatment. Rats in the intermediate-response group also showed statistically significant differences from the good and poor responders, but some overlap in the range of values with these groups was present.

Quantitation of *P. carinii* cysts proved to be a reliable and sensitive indicator of response to therapy. Not only was this technique as sensitive as histology in detecting *P. carinii*, but also the graphic portrayal of the data on semi-log paper enabled the reader to obtain a more accurate interpretation of the full range of values in each rat treatment group. Other authors have found examination of lung homogenates for *P. carinii* cysts to be more sensitive than histology in detecting light infection (6, 19). Selective cell wall stains, such as

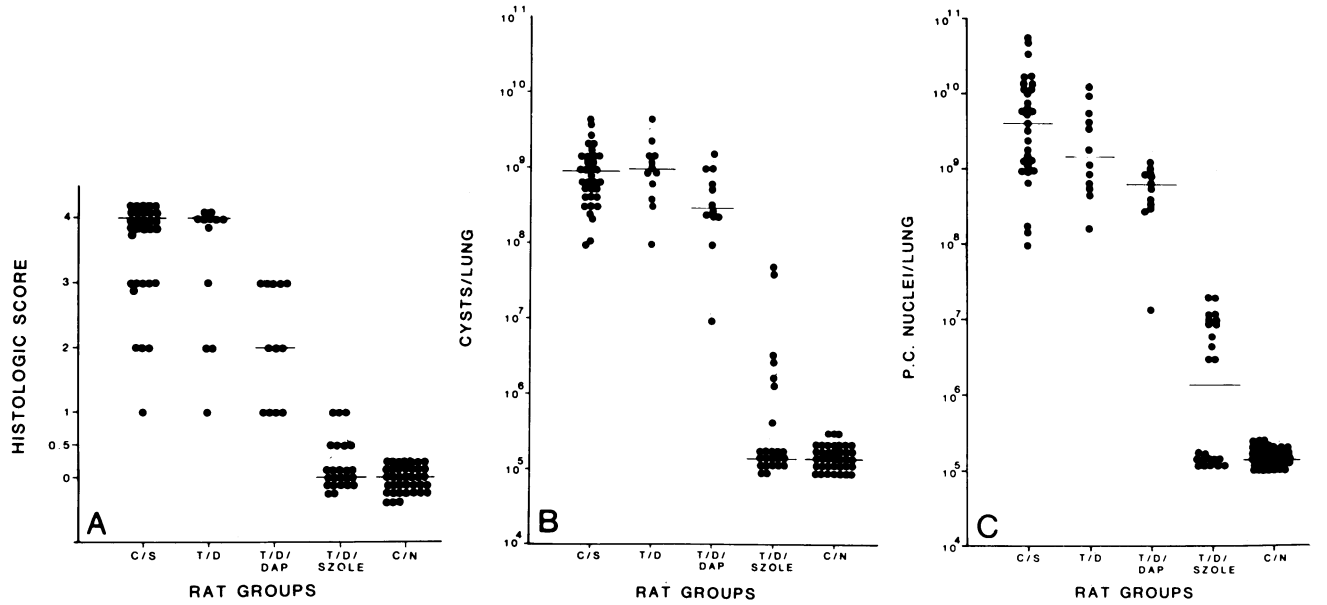

FIG. 2. Assessment of therapy of pneumocystosis in different rat groups by histologic score (A), number of *P. carinii* cysts (B), and number of *P. carinii* nuclei (C). C/S, Corticosteroid controls; T/D, trimethoprim-diaveridine treatment; T/D/DAP, trimethoprim-diaveridine-dapsone treatment; T/D/Szole, trimethoprim-diaveridine-sulfamethoxazole treatment; C/N, normal controls. Horizontal bars represent median values.

TABLE 2. Comparison of responses to treatment of *P. carinii* pneumonia among rat groups

Methods	Two-tailed *P* values of Wilcoxon rank sum tests			
	A vs B	B vs C	C vs D	D vs E
Histologic score	NS[a]	0.005	<0.001	<0.001
Cyst counts	NS	0.030	<0.001	0.015
Nuclei counts	NS	0.038	<0.001	<0.001
Lung wt	NS	<0.001	0.007	NS
Lung wt/body wt ratio	NS	0.007	<0.001	<0.001

[a] NS, Not significant statistically.

cresyl echt violet used to identify *P. carinii* cysts, are simple to use and enable even a relatively inexperienced person to scan a slide rapidly for the presence of organisms.

The major drawback to the use of cell wall stains in lung homogenates or histologic sections is that they do not permit visualization of the internal cyst contents, and thus empty cysts, dead cysts, and fully viable cysts share the same staining characteristics. Quantitation of cysts has been a less sensitive measure of *P. carinii* growth in tissue culture than has been quantitation of nuclei (2, 4). Other problems with the cell wall stains include variability in staining intensity of *P. carinii* and possible confusion with fungi (which are also stained) in very light infection.

Quantitation of nuclei consistently resulted in higher *P. carinii* counts than did cyst quantitation because Diff-Quick stains the nuclei of all forms of the organism. Nuclei quantitation was also somewhat more sensitive than cyst quantitation in detecting minimal or residual infection. The Diff-Quick stain can be performed in less than 1 min and provides excellent visualization of *P. carinii* in fresh air-dried specimens. We found good observer-to-observer reproducibility in nuclei counts among experienced personnel.

The changes in *P. carinii* counts after therapy determined by quantitation of nuclei were very similar to those obtained by cyst quantitation, suggesting that both techniques provide comparable assessment of drug efficacy. Since Diff-Quik also stains host cells, it takes longer to examine a slide than with selective cell wall stains. A greater degree of experience is required to differentiate *P. carinii* nuclei from host platelets or debris. Diff-Quik is not suitable for tissue sections, and should not be used on lung homogenates which have been frozen because variation in staining intensity might preclude accurate organism quantitation.

The question of which technique(s) should be used to evaluate *P. carinii* therapy in the rat model depends on the needs of the investigator. Histologic assessment provides important data about the status of *P. carinii* infection and host tissue changes in the lungs and can be accomplished rapidly by an experienced observer. However, tissue preparation and staining is an expensive and time-consuming process, requiring special facilities. *P. carinii* quantitative techniques provide a more precise measurement of the organism burden and its reduction after successful treatment. Cyst quantitation may be preferred for general drug screening programs, whereas quantitation of nuclei may be more effectively used in specialized studies. More definitive recommendations about these quantitative techniques can be made after drugs with different mechanisms of action are studied.

A major unresolved tissue in *P. carinii* therapy is the presence of residual organisms despite prolonged and apparently successful antimicrobial treatment. All available in vitro, rat model, and patient data suggest that currently available drugs have a static effect on *P. carinii* and are

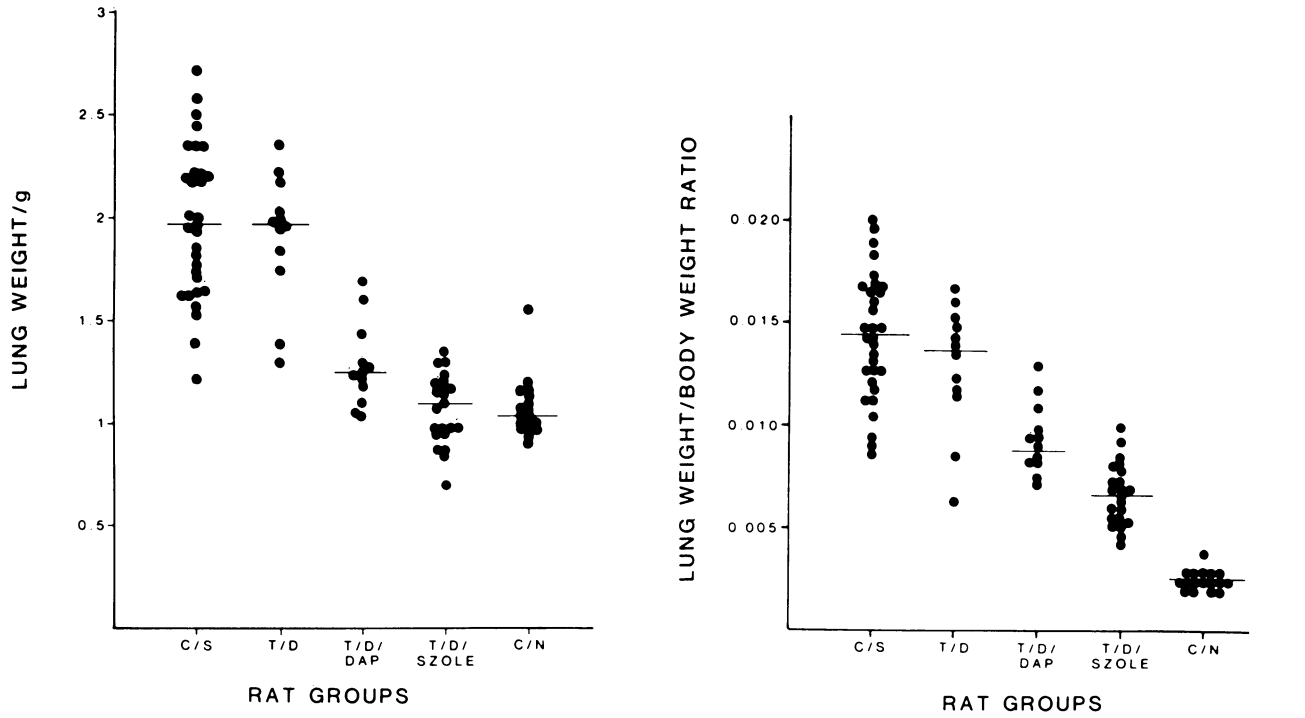

FIG. 3. Assessment of therapy of pneumocystosis in different rat groups by lung weight (A) and lung weight/body weight ratio (B). Abbreviations are described in the legend to Fig. 2. Horizontal bars represent median values.

successful in prophylaxis only as long as they are being administered (3, 10, 23). The histologic and quantitative techniques described in the present study revealed the presence of *P. carinii* in some rats which had a good response to therapy and lacked the sensitivity to rule out definitively the presence of residual organisms in animals in whom no *P. carinii* could be found. The life cycle stage of *P. carinii* responsible for this persistence is unclear, but since the cyst appears impervious to antimicrobial agents (3, 17), this form has emerged as a leading culprit. Perhaps the techniques developed for studying the life cycle stages and viability of *P. carinii* in tissue culture and fresh lung preparations (18) might be applied to studying organisms persisting after antimicrobial therapy.

Lung weight and lung weight/body weight ratio accurately reflected the extent of pneumocystosis and response to treatment in this study. These indicators have advantages over the other techniques in terms of simplicity, speed, and cost. Alterations in lung weight have been shown to reflect the severity of other experimental pulmonary infections (e.g., coccidiomycosis) (14). Since the immunosuppressed rats are prone to have bacterial and fungal infections, the lung weight and lung weight/body weight ratio appear best suited for an adjunctive role in the evaluation of *P. carinii* therapy.

In summary, this study showed that quantitative techniques accurately reflect the extent of disease and response to therapy in the rat model of *P. carinii* pneumonia. These techniques correlate well with lung histology and should be very helpful in the development and testing of new antimicrobial agents.

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