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Comparison of Histological and Immunological Techniques for Detection of *Pneumocystis carinii* in Rat Bronchial Lavage Fluid

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We compared histological and immunological techniques in the early diagnosis of *Pneumocystis carinii* pneumonia in bronchial lavage fluid of steroid-treated rats. The rats were sacrificed weekly and lavage fluids were: (i) examined with cresyl echt violet and Giemsa stains; (ii) examined for *P. carinii* antigens by indirect fluorescent-antibody, counterimmunoelectrophoresis, and double-diffusion techniques, using high-titer specific antisera to *P. carinii* raised in rabbits. *P. carinii* was detected in lavage fluid by cresyl echt violet at 2 weeks of steroids and persisted even with steroid tapering; the intensity of the infection in lavage fluid closely paralleled that in the lungs. *P. carinii* was not detected in lavage by Giemsa stain until 4 weeks and disappeared from the fluids with steroid tapering. *P. carinii* was detected by indirect fluorescent antibody as early as 1 week of steroids, and the results correlated well with those of cresyl echt violet. *P. carinii* antigens were not detected in lavage fluids or serum by counterimmunoelectrophoresis or double-diffusion techniques. Although precipitin lines sometimes occurred, they were nonspecific. In this model, cresyl echt violet and indirect fluorescent antibody were the preferred techniques for the early diagnosis of *P. carinii* infection in bronchial lavage fluid.

Pneumocystis carinii is the most common cause of diffuse pneumonia in immunocompromised patients (12, 34). Effective therapeutic agents are available for this otherwise fatal infection, but the only reliable means of establishing the diagnosis has been by the use of invasive procedures to demonstrate the organism in lung tissue (37). *P. carinii* has been detected by selective histological stains in sputum (9), pharyngeal smears (8), transtracheal or bronchial aspirates (20, 32), and gastric aspirates (4), but the overall yield from these procedures has been low. There have been several reports of successful histological diagnosis of human *Pneumocystis* pneumonia by bronchopulmonary lavage in small numbers of patients (3, 7, 16). The ready availability and low morbidity of flexible fiberoptic bronchoscopy and the ability to sample large volumes of alveolar effluent with lavage through the bronchoscope suggest this is an attractive diagnostic procedure, if sensitive techniques can be developed to detect *P. carinii* in lavage fluid.

The purpose of this study was to compare histological and immunological techniques for early detection of *P. carinii* in the bronchial lavage fluid of experimentally infected rats. The results indicate that the selective cell wall stain, cresyl echt violet (CEV), and immunofluorescence are the most sensitive methods for the detection of *P. carinii* in this model.

MATERIALS AND METHODS

Techniques for infection and bronchopulmonary lavage. Adult male Sprague-Dawley rats weighing about 250 g were used. These rats were part of a larger group of rats which had been used to study the growth characteristics and pathogenesis of experimental *P. carinii* infection (40). The rats were divided into three groups. Group A (controls) was composed of 10 rats (6 rats raised in a conventional colony and 4 germfree rats raised in isolators), which ate a regular diet and drank tap water with or without tetracycline (1 mg/ml). These rats were sacrificed at varying intervals throughout the study. Group B consisted of 20 rats on the standard treatment regimen of cortisone acetate (25 mg) injected subcutaneously twice weekly, low (8%) protein diet, and tetracycline in the drinking water for 8 to 9 weeks to produce *P. carinii* infection; two or three of these rats were sacrificed at weekly intervals. Group C consisted of 14 rats on the standard treatment regimen for 4 weeks; then a regular diet was instituted and steroids were tapered to zero over the next 3 weeks. These animals were followed for a total of 11 weeks. Two or three group C rats were sacrificed each week during and after steroid tapering. Since

Pneumocystis pneumonia developed in all 34 group B and C rats, the results of these groups have been pooled in some parts of the study.

The rats were sacrificed by exposure to halothane in a closed container. The chest was opened aseptically, heart blood was aspirated, and tissue specimens were cut from both lungs. The trachea was then exposed and aseptically cannulated with a polyethylene catheter. Lungs were lavaged with 20 ml of sterile saline in 5-ml amounts.

Processing and evaluation of lavage fluids by histological techniques. *Pneumocystis* organisms in the lavage fluids were concentrated and separated from rat alveolar macrophages, using the differential centrifugation technique described by Masur and Jones (23). The lavage fluid was centrifuged at 30 g for 5 min, and the supernatant was respun at 1,300 × g for 30 min. The sediment from the high-speed centrifugation was suspended in 1 to 2 ml of sterile saline. Drops (20 μl) of this suspension were air dried on glass slides, heat fixed, and stained by the following techniques: (i) Giemsa, which stains *P. carinii* cysts and trophozoites; (ii) CEV which selectively stains the cell wall of cysts (1).

Stained slides of the high-speed sediment were coded for evaluation. Each slide was scanned completely; a semiquantitative grading system ranging from 0 to 4+ was established to assess the intensity of *P. carinii* infection. With CEV stain, the grade assigned was based on the number of cysts per slide (Table 1). A similar approach was attempted for Giemsa-stained slides but was not satisfactory because of difficulty in distinguishing individual *Pneumocystis* organisms from host cells and debris.

As part of the larger study (40), tissue blocks were prepared from lungs of rats obtained at autopsy and stained with methenamine silver. These specimens were coded and read by an observer (P.D.W.) who did not participate in the histological evaluation of the lavage fluids. A semiquantitative system from 0 to 4+ was established to grade the intensity of *P. carinii* infection in the lungs. This system, which has also been used in mice, has been published in detail previously (38). Nonparametric statistical methods (Spearman's rho, Kendall's tau), (5, 33) were used to study the correlation of histological assessment of *P. carinii* infection in lungs and bronchial lavage fluids.

Cultures of lavage fluid. Unspun lavage fluids were streaked onto agar plates (e.g., tryptic soy with 5% sheep blood, Columbia CNA, MacConkey, and Sabouraud dextrose containing gentamicin) for the detection of bacterial and fungal contaminants. Bacterial colonies were processed for identification by the clinical microbiology laboratory of the Veterans Administration Hospital. Fungal cultures were read at 1, 2, and 3 weeks, and all yeasts and molds were identified by the clinical mycology laboratory of the University of Kentucky Hospital.

Preparation of antigens and antisera. The immunological studies on lavage fluids were performed, using antisera to *Pneumocystis* organisms raised in rabbits. A total of 13 rabbits were immunized with *Pneumocystis* organisms obtained from either autopsy lung tissue or bronchial lavage fluid. Heavily infected rat lungs were homogenized with a Teflon pestle and fine wire mesh screen, digested with collagenase and hyaluronidase, and centrifuged on a discontinuous Ficoll-Hypaque density gradient. This method, as reported previously (39), separates *Pneumocystis* organisms from rat lung tissue, and also fractionates *P. carinii* into layers preferentially rich in cysts or trophozoites. Quantitation of *Pneumocystis* organisms by this method was based on cyst counts. Layer 1 from the Ficoll-Hypaque gradient (FHL_1) contained predominantly trophozoites, FHL_3 and FHL_4 contained predominantly cysts and were pooled in all procedures, FHL_2 was intermediate in terms of relative proportions of cysts and trophozoites. A hydrochloric acid extract (13) and a sonicated specimen of FHL_3 and FHL_4 were also employed as immunogens in one rabbit each. Three rabbits were immunized with lyophilized lavage fluids from heavily infected rats. A negative control antiserum was raised in one rabbit with an homogenate of germfree rat lungs.

TABLE 1. *Semiquantitative grading of the intensity of P. carinii infection in rat bronchial lavage fluid*

Grade	*P. carinii* cysts per slide
0	0
+0.5	1 to 12
+1	12 to 24
+2	24 to 48
+3	>48, but not all fields
+4	Cysts in all fields

Most rabbits were immunized with a series of subcutaneous injections of *P. carinii* in normal saline mixed with complete or incomplete Freund adjuvant. The primary series consisted of one to three weekly injections followed by a series of booster injections weeks to months later. Some rabbits received a series of intravenous injections of *P. carinii* in saline, either after the subcutaneous injections, or as the sole method of immunization. The rabbits were bled weekly for 1 to 3 weeks after the last injection, and serum specimens for each rabbit were pooled and stored at −20°C.

Evaluation of antisera. Antisera were absorbed twice at room temperature with either rat liver powder (150 mg per ml of antiserum) or with uninfected rat lungs. Some antisera were further absorbed with serum from uninfected rats.

The sera were tested for antibodies to *P. carinii* with an indirect fluorescent antibody (IFA) technique. Clean Teflon-coated glass slides, each containing eight wells, were used. Five microliters of FHL_2 or FHL_3 and FHL_4 were placed in the wells and heat fixed. A 25-μl amount of the serially diluted test serum was added to the wells; the slide was incubated at 37°C in a moist chamber for 45 min and washed twice with phosphate-buffered saline (pH 7.2, 7.4). A 25-μl amount of fluorescein-conjugated goat anti-rabbit immunoglobulin G (Cappel Laboratories) at an appropriate dilution was then added to each well; the slide was incubated and washed as above. The slide was then mounted with glycerin-phosphate-buffered saline and read with Leitz Orthoplan fluorescence microscope (Leitz/Opto-Metric Div. of E. Leitz Inc.). The intensity of fluorescence was graded on a scale from 0

(negative) to 4+ (maximum). The highest dilution with a 1+ intensity of fluorescence was considered to be the peak antibody titer.

Evaluation of lavage fluids by immunological techniques. The same lavage fluid specimens used in the histological studies were used in immunological studies. Two types of immunological tecnhiques were employed: (i) IFA; (ii) immunodiffusion, including double diffusion (DD) and counterimmunoelectrophoresis (CIE).

The IFA technique was the same as that used in testing antisera except that lavage fluid replaced Ficoll-Hypaque gradient layers as the antigen. The lavage fluids were coded for reading. Up to eight specimens could be read on a single slide.

Immunodiffusion studies were performed on glass slides coated with 14 ml of 1.0% agarose in barbital buffer (pH 8.2; ionic strength, 0.05). Wells 3 mm in diameter and 2 to 4 mm apart were cut in the agarose. DD experiments were performed in moisture boxes at room temperature, and results were read at 12 and 14 h. CIE studies were carried out for 60 min at 390 V, 30 mA, at room temperature by methods described previously (6). Known concentrations of purified pneumococcal capsular polysaccharide antigen were electrophoresed against type-specific pneumococcal antiserum (Statens Seruminstitut) as a positive control in each experiment.

RESULTS

Evaluation of lavage fluids by histological stains. Examples of lavage fluids positive for *P. carinii* by CEV and Giemsa stain are shown in Fig. 1a. *P. carinii* cysts can easily be distinguished from background with CEV stain. The clump of trophozoites in Fig. 1b illustrates the difficulty in distinguishing individual organisms by Giemsa stain.

In Fig. 2, the results of semiquantitative assessment of the intensity of *P. carinii* infection by examination of bronchial lavage fluid stained with CEV are compared with the results of examination of methenamine silver-stained sections of lung in the same animals. There was a highly significant correlation ($r_s = 0.832$; $P < 0.001$) between these two techniques, suggesting that examination of bronchial lavage fluid provides a reliable indication of the extent of the pathological process in the lungs.

The sequential changes in the intensity of *P. carinii* infection in bronchial lavage fluid by CEV stain are shown in Fig. 3. The infection steadily increased over time in group B rats on the standard steroid treatment regimen and reached peak intensity by 7 weeks. Although the intensity of the infection diminished with steroid tapering (group C rats), the infection persisted even after steroids were discontinued.

In Table 2, group B and C rats have been combined. The sequential changes of *P. carinii* infection in the lungs and bronchial lavage fluid

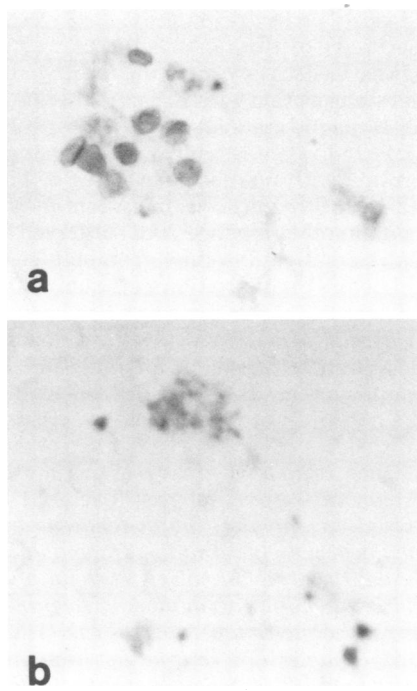

FIG. 1. *P. carinii in rat bronchial lavage fluid identified by histological stains. (a) A group of P. carinii cysts (CEV stain, ×1,250). (b) A cluster of P. carinii trophozoites (Giemsa, ×1,250).*

by different methods of examination are compared without regard to the intensity infection. Even before steroids were administered, a few rats had scattered foci of *Pneumocystis* organisms in their lungs, consistent with the subclinical carrier state in nature. *P. carinii* was first detected in bronchial lavage fluid by CEV stain at 2 weeks of steroids; by 3 weeks all lavage fluids were positive. By contrast, *P. carinii* was not detected in lavage fluid by Giemsa stain until 4 weeks of steroids and disappeared from the fluid with steroid tapering. *P. carinii* trophozoites were much more numerous than mature cysts.

Evaluation of antisera. Antisera were studied by the IFA technique. The appearance of *P. carinii* in Ficoll-Hypaque gradient layers and in lavage fluids on immunofluorescent staining was identical (Fig. 4a and b). The organisms exhibited a typical rim pattern around their periphery. Considerable variation in antibody titers was achieved (Table 3). Whole organisms derived from Ficoll-Hypaque gradients were more immunogenic than soluble fractions of the organisms or lyophilized bronchial lavage fluid contents. Highest antibody titers were obtained when these organisms were injected both sub-

cutaneously and intravenously.

Specificity of the rabbit antisera was evaluated in several ways. Antiserum titers were abolished by absorption with *P. carinii* derived from Ficoll-Hypaque gradient layers and with *P. carinii*-infected rat lungs, but were unchanged by absorption with uninfected rat lung or lung infected with *Streptococcus pneumoniae* type 25. No immunofluorescence was observed when phosphate-buffered saline or rabbit sera obtained before immunization were substituted for anti-*Pneumocystis* antisera or when lavage fluid from germfree rats was substituted for lavage fluid from infected rats or for *P. carinii* Ficoll-Hypaque gradient layers on the slides.

The antisera were also tested for cross-reacting antibodies to a variety of other organisms. Organisms tested for cross-reacting antibodies to *P. carinii* were as follows: (bacteria) *Streptococcus pneumoniae, Staphylococcus aureus, Staphylococcus epidermidis, Haemophilus influenzae. Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, F. meningosepticum, Mycobacterium tuberculosis* (H37 Ra), and *Mycobacterium smegmatis*; (fungi) *Candida albicans, Torulopsis glabrata, Cryptococcus neoformans, Histoplasma capsulatum, Blastomyces dermatitidis, Aspergillus* sp., *Penicillium* sp., *Paecilomyces* sp., *Scopulariopsis* sp., and *Trichosporon cuneatum*; (mycoplasmas) *Mycoplasma pulmonis*. The antisera were either absorbed with washed suspensions of heat-killed or Formalin-inactivated organisms, or these organisms replaced *P. carinii* on the slides. In no instance could cross-reacting antibodies be demonstrated.

Evaluation of lavage fluids by IFA. The results of IFA correlated very well with those of CEV (Table 2). *P. carinii* was detected in one of three rat lavage fluids by 1 week of steroids and was found in all lavage fluids by 3 weeks. Since *Pneumocystis* organisms were easily distinguished by IFA and a small volume (5 μl) of fluid was used, the slide could be scanned rapidly.

Evaluation of lavage fluids by immunodiffusion. Examination of rat lavage fluids by DD or CIE with any of the rabbit antisera failed to reveal precipitin bands specific for *P. carinii* (Table 2). Specific precipitin bands could also not be detected by immunodiffusion under the following conditions: (i) with intact, sonicated, or HCl extracts of Ficoll-Hypaque gradient layers; (ii) with serum from infected rats; (iii) with varying dilutions of antigens or antisera; (iv)

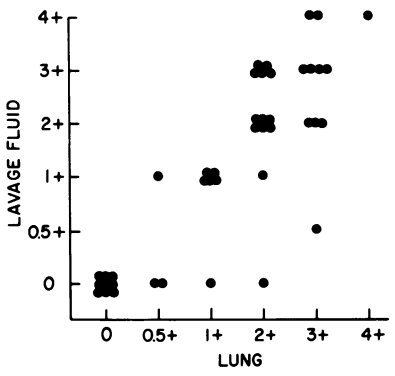

FIG. 2. *Semiquantitative assessment of the intensity of P. carinii infection in rat bronchial lavage fluid (vertical axis) stained with CEV and lung parenchyma (horizontal axis) stained with methenamine silver. Each point represents a single rat.*

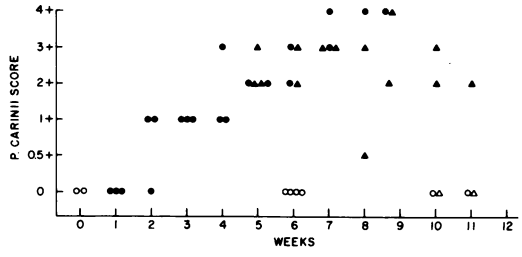

FIG. 3. *Sequential changes in the intensity of P. carinii infection in rat bronchial lavage fluids.* ●, *Group B rats on the standard steroid treatment regimen;* ▲, *group C rats on tapering doses of steroids;* ○ *and* △, *group A (control) rats which drank plain tap water* (○) *or water with tetracycline* (△).

TABLE 2. *P. carinii infection changes*

Technique	No. of positive specimens/no. of specimens examined at weeks:											
	0	1	2	3	4	5	6	7	8	9	10	11
Lungs												
Histology	1/3	2/3	3/3	3/3	4/4	4/4	4/4	4/4	3/3	3/3	2/2	1/1
Bronchial lavage fluid												
Cresyl violet	0/3	0/3	2/3	3/3	4/4	4/4	4/4	4/4	3/3	3/3	2/2	1/1
Giemsa	0/3	0/3	0/3	0/3	3/4	4/4	3/4	3/4	2/3	2/3	0/2	0/1
IFA	0/3	1/3	2/3	3/3	4/4	4/4	4/4	4/4	3/3	3/3	2/2	1/1
DD and CIE	0/3	0/3	0/3	0/3	0/4	0/4	0/4	0/4	0/3	0/3	0/2	0/1

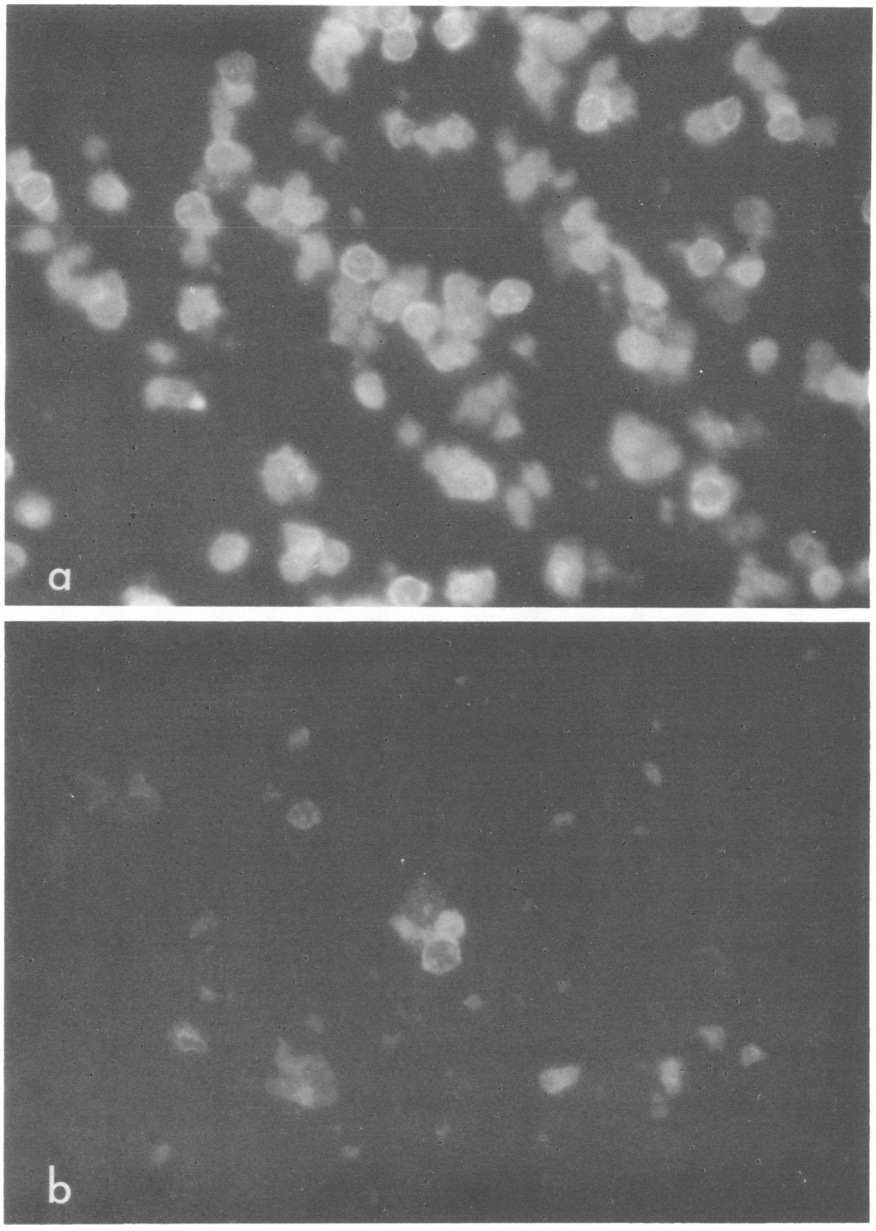

FIG. 4. *Immunofluorescent staining of P. carinii. The organisms stained brightly in a typical peripheral rim pattern. (a) P. carinii isolated from a Ficoll-Hypaque gradient (×1,250). (b) P. carinii in bronchial lavage fluid (×1,250).*

over a wide range of voltage (50 to 400 V) and current (10 to 40 mA) settings with CIE.

The problems encountered with immunodiffusion are illustrated in a typical DD experiment in Fig. 5. Well A containing lavage fluid from a rat heavily infected with *P. carinii* was reacted against rabbit antiserum in wells B to E. Multiple precipitin bands formed between lavage fluid and unabsorbed rabbit antiserum (well C); several specificities were eliminated when the antiserum was absorbed with uninfected rat lung (well B). Further absorption of the antiserum with *P. carinii* from a Ficoll-Hypaque gradient (well D) reduced but did not eliminate the remaining bands. Moreover, absorption with serum from a germfree rat (well E) gave virtually

TABLE 3. *Rabbit antisera to P. carinii*

Rabbit no.	Immunogen source	Cysts	Route	Reciprocal peak (IFA titer)
1, 2	FHL_3 and FHL_4	7.1×10^6 to 2.5×10^7	s.c.[a]	128
3, 4	FHL_3 and FHL_4	7.0×10^5 to 2.5×10^7	s.c./i.v.	512
5	FHL_3 and FHL_4	7.0×10^5 to 2.8×10^6	i.v.	256
6	FHL_3 and FHL_4	HCl extract (1.6×10^7)	s.c.	4
7	FHL_3 and FHL_4	Sonicate (3.3×10^7)	s.c.	32
8	FHL_2	2.8×10^5	s.c.	256
9	FHL_2	2.0×10^5 to 2.0×10^6	i.v.	256
10	FHL_1	1.1×10^5	s.c.	64
11, 12, 13	Bronchial lavage fluid	Lyophilized (1.2×10^5 to 6.2×10^5)	s.c.	16
14	Germfree rat	Lung homogenate (none)	s.c.	0

[a] s.c., Subcutaneous; i.v., intravenous.

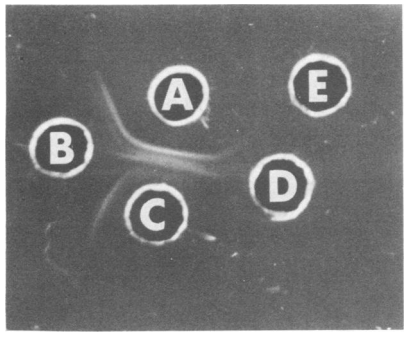

FIG. 5. *A DD study: well A, lavage fluid from a heavily infected rat; well C, unabsorbed rabbit antiserum; well B, rabbit antiserum absorbed with uninfected rat lung; well D, rabbit antiserum absorbed with uninfected rat lung and P. carinii; well E, rabbit antiserum absorbed with uninfected rat lung and serum from a germfree rat. See text for interpretation.*

the same results obtained by absorption with *P. carinii*.

With most lavage fluids, no precipitin bands of any kind were found. We attempted to demonstrate soluble *P. carinii* antigens in lavage fluids indirectly with immunofluorescence. In studies to be published elsewhere, we have found that rats whose steroids have been tapered develop high serum antibodies to *P. carinii* by an IFA technique. Sera from rats with high antibody titers were absorbed with supernatants of lavage fluids of other rats heavily infected with *P. carinii* and then tested by the IFA technique. No change in serum antibody to *P. carinii* was found after absorption, thus providing no evidence for the presence of soluble *Pneumocystis* antigens in lavage fluids.

Infection with other organisms. Cultures of lavage fluids of 27 (79%) of 34 steroid-treated rats grew out one or more species of bacteria, whereas cultures from control rats were usually sterile. *Flavobacterium meningosepticum*, which was present in 11 rats, was the predominant organism and had the following antibiotic sensitivity pattern as determined by Autobac and standard disk diffusion techniques. The organism was resistant to ampicillin, carbenicillin, cephalothin, chloramphenicol, polymyxin B, tetracycline, gentamicin, tobramycin, and amikacin and sensitive only to trimethoprim-sulfamethoxazole. Other bacteria present included coagulase-negative staphylococci, viridans streptococci, enterococci, diphtheroids, *Proteus mirabilis*, and *Yersinia enterocolitica*. Fungi were cultured from four lavage fluids (two with *Penicillium* spp. and one each with *Paecilomyces* sp. and *Geotrichum* sp.).

In contrast to lavage fluid cultures, bacteria infrequently caused parenchymal lung infection, as determined histopathologically by the presence of organisms or acute exudative inflammation.

DISCUSSION

Two basic types of stains have been used to identify *P. carinii* in tissues and body fluids. The 5- to 7-μm cyst form is classically identified by methenamine silver, which selectively stains the cyst wall. Since methenamine silver takes several hours to perform, rapid modifications of this technique or other similar stains (e.g., CEV, toluidine blue, Gram Weigert) (36) have been suggested as alternatives. The smaller (1- to 3-μm) much more numerous trophozoite form of *P. carinii* has been identified by Giemsa and, less commonly, by polychrome methylene blue stains (14). These stains do not stain the cyst wall; rather, they stain intracystic bodies (up to eight in number) termed "sporozoites", which presumably differentiate into trophozoites.

The choice of a particular stain for identification of *P. carinii* depends on the purpose for which it will be used, as well as the preference of the investigator. The selective cell wall stains

have been favored for tissue sections, but both types of stains have been used for lung aspirates and imprint smears (18). Cell wall stains have several disadvantages: (i) since they also stain fungi, caution must be used in interpreting individual forms as *P. carinii* in very light infection (31); (ii) these stains cannot distinguish viable from nonviable *P. carinii*; (iii) they provide an underestimate of the total number of *Pneumocystis* organisms in quantitation studies. On the other hand, Giemsa stains a variety of host cells, sometimes making it difficult to distinguish *P. carinii*; the small size of trophozoites and their tendency to clump have precluded accurate quantitation of this form of the organism. We and others have found that counting the number of cysts by the use of cell wall stains provides the most reliable marker of the number of *Pneumocystis* organisms (28, 39, 40).

The present study has extended the comparison of stains to bronchial lavage fluids. *P. carinii* was identified in lavage fluids earlier in the infection and was detectable longer after steroid tapering with the cell wall stain, CEV, than with Giemsa stain. Semiquantitative estimation of the intensity of *P. carinii* infection in bronchial lavage fluid by CEV stain correlated well with that in lung tissue sections by methenamine silver. Recent studies in the cortisonized rat model have suggested phase-contrast microscopy might be a sensitive method in detecting *P. carinii* in lavage fluids (23, 35). However, as with Giemsa stain, phase-contrast microscopy requires a considerable degree of sophistication. Thus, if our data can be extrapolated to humans, cell wall stains appear to be the best method for the histological identification of *P. carinii* in bronchial lavage fluids in the routine clinical laboratory.

Diagnosis of *Pneumocystis* pneumonia in immunosuppressed patients by demonstration of serum antibodies to the organism has been unrewarding (24, 27, 29). Other efforts have focused on detection of the organism or its antigenic constituents by immunofluorescence (2, 17, 21, 22, 25, 31). Lim et al. raised antisera to *P. carinii* in rabbits with organisms separated from lungs by trypsin digestion and sucrose gradient centrifugation; using a direct fluorescent antibody technique, these authors found that immunofluorescence was more sensitive than histological stains in detecting *P. carinii* in hypopharyngeal material of rats and sputum of patients (21, 22). Unfortunately, there has been little apparent interest in the application of these findings on a broader scale.

The present study demonstrates that immunofluorescence is at least as sensitive as histological stains in the early detection of *P. carinii* in bronchial lavage fluid. IFA sampled a smaller volume of bronchial lavage fluid than did CEV stain (5 μl versus 20 μl), yet detected *P. carinii* slightly earlier in the infection (1 week versus 2 weeks of corticosteroids); however, larger numbers of animals must be examined before definitive conclusions about the sensitivity of these techniques can be drawn. The early stages (i.e, weeks 1 to 3) of *P. carinii* infection in this rat model probably have the greatest clinical applicability since, in our experience, patients with *Pneumocystis* pneumonia usually have small numbers of organisms present in bronchial washings. In this setting, immunofluorescence would be very helpful in differentiating *P. carinii* from fungi.

The frequent presence of other organisms in bronchial lavage fluids of our rats emphasizes the need to demonstrate specificity of antisera to *P. carinii* (11). No cross-reacting antibodies to bacteria or fungi were found by IFA. Whereas these organisms primarily represented colonization rather then lung parenchymal infection, the situation is analagous to the seriously ill patient who often becomes colonized with hospital microbial flora. The predominant organism in our rats was *F. meningosepticum*, which was resistant to all antibiotics except trimethoprim-sulfamethoxazole.

A recent study has shown a high frequency of circulating *P. carinii* antigen by CIE in children with *Pneumocystis* pneumonia (29). By contrast, we were unable to raise precipitating antibodies to *P. carinii*, or to find soluble *P. carinii* antigens in the lavage fluid or serum of any of our rats. The reasons for these differences are unclear. It is possible they represent technical factors (e.g., different methods of preparing *P. carinii* antigens or antisera). Whereas recent interest has focused on tissue culture as a source of purified preparations of *P. carinii* (19, 28–30), our method has produced no detectable morphological alterations in the organism (39) and has been highly satisfactory for IFA work. It is also possible that there are antibodies in the rat which are either complexed with or block the detection of *P. carinii* antigens. Whereas this subject needs further study, rat *P. carinii* pneumonia in many other respects closely resembles the human disease (10).

A major unresolved question in studies of the detection of soluble *P. carinii* antigens is that of specificity. This has been illustrated by the presence of circulating *P. carinii* antigens in immunosuppressed patients without histological evidence of the organism (24a) and the problems we encountered with DD. In the most detailed

published immunodiffusion study of *P. carinii* to date, Kagan and Norman could not find specific precipitins in rabbit or monkey antisera to *P. carinii* (15). With certain parasites (e.g., schistosomes), it was only after the antigen was coupled to a specific agent (methylated bovine serum albumin) that precipitating antibodies could be produced (26).

Further studies are needed to improve the immunological diagnosis of *P. carinii* infection. Such studies should include new methods of producing antisera to *P. carinii*, and the evaluation of other sensitive techniques (e.g., coagglutination, radioimmunoassay, enzyme-linked immunosorbent assay) for the detection of *P. carinii* antigens in bronchial lavage fluid and in serum. These studies can initially be performed in rats, but ultimately need evaluation in humans.

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