Development and Evaluation of a Direct Fluorescent Antibody Method for the Diagnosis of Pneumocystis carinii Infections in Experimental Animals

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Received for publication 4 May 1973

Because no fully satisfactory diagnostic method has been available for use in pneumocystis infection, an attempt was made to apply the fluorescent antibody technique in the identification of Pneumocystis carinii. Hyperimmune sera were prepared in rabbits against P. carinii from human and rat sources. After proper adsorption, these antisera were conjugated with fluorescein isothiocyanate and used as reagents in a direct fluorescent antibody procedure. Each of the two reagents was found to stain trypsin-treated P. carinii organisms from either human or rat sources, indicating the presence of common antigens. Stained organisms were demonstrated in the hypopharyngeal material from rats in which pneumocystis infection had been activated by the administration of corticosteroid. From the results reported here, the procedure outlined is considered sufficiently sensitive and specific to justify tests on pneumocystis infections in man. The findings in a series of specimens from human subjects will be reported separately. The method also provides an extended approach to related research problems. The need for controls of the procedure at all points is emphasized.

Up to the present time, the diagnosis of Pneumocystis carinii pneumonitis (PP) has been dependent on lung biopsy. The value of conventional staining methods for tracheal aspirates or sputa is controversial (1, 12, 14). Although there is great need for a reliable diagnostic method for P. carinii infection, there is no report on the use of fluorescent antibody method as a diagnostic procedure in PP. The application of the fluorescent antibody method has been emphasized in the rapid identification of various other microorganisms in clinical laboratories (4, 5).

Using an indirect fluorescent antibody procedure (IFA), Lim et al. (10) showed that rat P. carinii bind specifically with homologous antisera obtained after spontaneous recovery of the animals from corticosteroid-activated pneumocystosis. For a direct fluorescent antibody method (DFA) for clinical use, antigens from animals would be desirable. Preliminary results indicated that reagents prepared from an animal source could be used in tests of both human and animal preparations.

In the present study (taken from a dissertation submitted in partial fulfillment of the requirement of the Dr. P. H. degree), a practical DFA procedure and the testing of its dependability have been shown. In actual tests, specimens were taken from rats with PP which had been activated by corticosteroid treatment. The use of this procedure in testing specimens from humans with suspected PP will be the subject of a separate paper.

MATERIALS AND METHODS
Preparation of P. carinii antigens. P. carinii organisms were obtained from lung tissue of 200-g naturally infected rats of either sex, injected with cortisone acetate (Merck, Sharp and Dohme) (25 mg) subcutaneously twice weekly (6) for 8 weeks or until fatal pneumocystis developed. Chlorotetacycline (0.05%) and amphotericin B (0.05%) were given in the drinking water throughout the experimental period to prevent secondary infection during corticosteroid treatment. P. carinii organisms of human source were taken from lung tissue obtained at necropsy from histologically proven PP patients-four from the Center for Disease Control, Atlanta, Ga., and one from St.

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Jude Children's Hospital, Memphis, Tenn. All specimens were re-examined for degree of infection by May-Grunwald-Giemsa (MGG) staining.

The infected lung tissue was homogenized, using mortar and pestle, in Eagle minimal essential medium with Hank solution (MEM) containing 100 U of penicillin per ml and 1 mg of streptomycin per ml, and clarified by low-speed centrifugation at 400 rpm for 3 min, discarding the sediment. To the supernatant fluid a sample of warmed 0.25% trypsin (General Biochemicals, Chagrin Falls, Ohio) was added, and the mixture was incubated at 37 C for 10 min and centrifuged at 900 rpm for 10 min. After the sediment had been discarded, the supernatant fluid was centrifuged at 300 rpm for 30 min. This final sediment was reconstituted to make a 10 or 20% suspension of organisms by volume in MEM. The suspension was fractionated by sucrose density gradient centrifugation in cellulose nitrate tubes with the following sucrose concentrations: 12, 24, 36, 48, and 60%, respectively. A 20-ml fraction of a 5% P. carinii sample was placed on top of the gradient and centrifuged at 2500 rpm for 60 min at 4 C. A 21-gauge hypodermic needle was used to puncture the bottom of the tube, and drops of the visible zones were collected in separate tubes. The fractions were washed and centrifuged as above. These organisms were quantitated and used in the immunization of the rabbits and in slide preparations for DFA or IFA staining. In calculating the number of organisms, the standard method for the examination of dairy products was followed (15).

Preparation of antiserum and its titration. Rabbits weighing 1.5 to 2.5 kg were used. A primary series of immunizations consisted of four consecutive injections once a week; 0.1 ml of 6 x 10^9/ml, or approximately 600,000 organisms, was thoroughly mixed with complete Freund adjuvant and injected into the footpads. In some cases the organisms were sonically disrupted in a 10-kc Raytheon sonic oscillator, run at 60% maximal output for 10 min; in others, whole organisms were injected. After an interval of 6 weeks, a series of four booster injections were given intravenously at 3 to 4 days intervals twice a week, gradually increasing the number of organisms 10^4, 2 x 10^4, 5 x 10^4, and finally 10^6.

Serum samples on the day after the last injection were titrated for antibody by immunodiffusion and IFA tests in comparison with preimmunization sera. All tests were made both before and after absorption of the antiserum with mouse powder or normal rat serum. Ouchterlony immunodiffusion (ID) plates were prepared using 1% Noble agar method in Barbitone buffer (pH 8.0) with Merthiolate in a 1:1000 final concentration. Serum samples in serial dilutions were placed in outer wells, and either sonically treated antigens or extracted antigens were placed in the center well. The plates were incubated at room temperature and read at 24, 48, and 72 h. The absorbed antisera were also titrated by IFA with labeled goat antirabbit immunoglobulin (IgG).

Conjugation of antiserum with FITC. To 10 ml of antiserum, an equal volume of saturated ammonium sulfate solution (pH 7.0) was added, and the mixture was incubated in the cold (4 C) overnight. The material was then centrifuged at 9,000 rpm at 4 C, after which the supernatant fluid was discarded and the precipitate was dissolved in 5 ml of 0.85% NaCl (11). This solution, containing the globulin fraction, was dialyzed against pH 7.6 buffered saline (PBS) overnight to eliminate ammonium ions. Protein content of the globulin solution was determined by the biuret method with a Beckman spectrophotometer at a wave length of 540 nm. The sample was diluted with 0.85% NaCl and carbonate-bicarbonate buffer (pH 9.0) so that the final solution contained 10 mg of protein per ml and 10% carbonate-bicarbonate buffer, and then cooled to 4 C. To this was added 0.025 mg of fluorescein isothiocyanate (FITC) per mg of protein, and the mixture was stirred overnight at 4 C. The conjugate was transferred to a cellulose membrane and dialyzed against pH 6.3 0.175 M phosphate buffer (PB) overnight, with one change of the buffer solution. The conjugate, freed from unbound dye, was put on a diethylaminoethyl-cellulose anion exchange column (1.5 by 30 cm) and equilibrated with PB, pH 6.3 (13). For separation of labeled antibody, elution was made with 0.125 M salt in PB (pH 6.3), and the fractions were collected, put into dialyzing bags separately, and dialyzed against pH 7.6 PBS overnight at 4 C. The molar fluorescein protein ratio was determined from the respective values of optical density OD 520 and OD 660 on each fraction and of the purified antiserum, using the nomogram for molar fluorescein/protein ratio (16).

DFA staining procedure. Smears from purified P. carinii or trypsined impression smears of infected tissue were fixed with acetone at room temperature for 10 min. Tissue smears were incubated with 0.25% trypsin at 37 C for 10 min and washed in PBS (pH 7.6). The working dilution of the labeled antiserum, previously evaluated, was placed on the fixed smears on the slides, incubated at room temperature for 45 min, and washed twice with pH 7.6 PBS for 10 min each. The slides were then counterstained with a 1:20 dilution of eriochrome black (7) for 5 s and mounted in buffered glycerin.

Comparison of sensitivity of DFA and MGG staining. For evaluation of sensitivity in identification of the various forms of the organisms, the DFA test was compared with conventional staining. Two sets of smears were made from infected lung tissue of the same animal; one set was stained by DFA and the other with MGG. Using a fixed counting time for both methods, the number of parasites counted within that time limit was used to determine whether the DFA procedure was superior in terms of rapidity of identification of the parasites.

Testing the DFA procedure in H-P materials from rats. Hypopharyngeal (H-P) material was taken from the rats twice a week, two swabs being taken each time, using sterile nasopharyngeal swabs (Calgiswab, Colab) under light ether anesthesia. The swabs were either treated in N-acetyl-l-cysteine (NALC) solution or directly smeared on microscope slides. To homogenize the mucous H-P material, the swab specimens to be treated were immersed in 1 ml of 2.5% NALC (pH 8.0) in siliconized tubes for 15 to 30
min, followed by centrifugation at 2,500 rpm for 20 min. The supernatant fluid was discarded, the sediment was washed with Hanks balanced salt solution, and microscope smears were prepared from the sediment.

Each smear was made with 0.01 ml of NALC-treated specimen or with the material from one direct swab. The enumeration of parasites was performed by counting all organisms in a smear.

The number of stained cysts in each smear was expressed by a score. Because a smaller number appeared in H-P material than in lung tissue the scores are necessarily different (Tables 1 and 2).

To express the relative number of cysts in the lung tissue stained by DFA, an impression smear measuring approximately 1 cm² was prepared, and the entire smear was examined under high, dry power. Tissue samples were taken from the most consolidated area under gross examination, with at least 10 smears being made from each specimen.

Control animals. H-P materials of untreated animals in the control group were also taken once a week in the same manner as the treated animals, from the first day of the experiment through 5 weeks. After the animals were sacrificed, re-examination was made by DFA of impression smears of lung tissue as well as tissue of the ascending respiratory tract for presence of P. carinii organisms. The intact bronchi and trachea were dissected by sterile technique, and saline-lavage was made with a 2-ml syringe fitted with a 23-gauge needle. The material was centrifuged, and smears were prepared in the same manner as for the H-P material.

RESULTS

Fractionation and quantitation of P. carinii organisms. The fractions of P. carinii organisms obtained by centrifugation of the sucrose gradients at 2,500 rpm for 60 min at 4°C were as follows. Organisms believed to be trophozoites of small size, obtained at 36% aqueous sucrose solution, specific gravity (SG) 1.1562; large trophozoites and some cyst forms, obtained at 48% aqueous sucrose solution, SG 1.2186; and some precystic forms and cysts in the sediment fraction, 60% aqueous solution, SG 1.2865. The fractions obtained at 48 and 60% sucrose were combined, centrifuged, and washed, and the organisms were suspended in physiological saline to make a 20% concentration (approximately 7.5 x 10⁶ to 10⁷/ml, standard method of direct microscope counting) (15).

Antibody assay. The rabbit hyperimmune sera prepared against P. carinii organisms of rat origin reacted with the sonically disrupted soluble portion of antigen to give several precipitin lines on Ouchterlony ID plates. After absorption of the serum with mouse powder and normal rat serum, there remained a single precipitin line which was considered to be the P. carinii specific reaction. The titers of antisera from three rabbits (numbers 2, 4, and 5) before conjugation were 1:160, 1:320, and 1:128, respectively, by IFA. Hyperimmune sera prepared with P. carinii organisms of human origin exhibited multiple precipitin lines in reaction to sonically treated P. carinii organisms from human sources. This antigen reacted with anti-human IgG on ID, suggesting that the human P. carinii had surface-bound globulins. After absorption of the immune serum with normal human serum, a single precipitin line remained. This line was eliminated with a sample of antisera which was incubated with human P. carinii antigen overnight at 4°C.

The pooled fractions of labeled antibody were used for determination of molar fluorescein/protein ratio and protein concentrations. The mole F/P ratios ranging from 1.0 to 5.0 and protein concentrations from 1 to 5 mg/ml were chosen for titration.

DFA staining. The purified P. carinii from rats, stained with appropriately diluted labeled antiserum, exhibited brilliantly delineated organisms of various sizes and shapes. The cystic wall stained intensely in all organisms, but

| Table 2. Pneumocystis score in hypopharyngeal smears and lung tissue of rats by direct fluorescent antibody technique |
|---|---|---|
| Rat no. | Hypopharyngeal smear* | Lung tissue* |
|---|---|---|
| 28 | 3 | 2 |
| 31 | 4 | 3 |
| 32 | 4 | 2 |
| 33 | 1 | 1 |
| 37 | 2 | 1 |
| 42 | 3 | 1 |
| 51 | 2 | 3 |
| 54 | 4 | 4 |
| 55 | 2 | 2 |

* Hypopharyngeal smears taken at death or during week preceding death.
* Lung tissue impression smears made at death.
* Died after corticosteroid was withheld.

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cytoplasmic staining varied somewhat from one organism to another. Some apparently immature organisms showed strong staining of both internal structures and the external membrane. In the large cysts that were considered to be mature forms or degenerating organisms, the FA staining was found mainly in the external membrane, whereas the internal portion of the cysts showed mostly the counterstaining. With the labeled anti-rat *P. carinii* antiserum, *P. carinii* from both rat and human sources showed brilliant staining. The staining characteristics of various forms of human *P. carinii* did not differ noticeably from those of rats. The labeled anti-human *P. carinii* antibody reacted with *P. carinii* from rats as strongly as with human *P. carinii* (Fig. 1). With this antiserum, the *P. carinii* in the tissue smears of both rats and humans showed the characteristic, intense membrane staining with less brilliance of the internal cystic portion of the parasites. With trypsin-treated tissue preparations, the intensity of the staining was enhanced, so that aggregated masses of parasites and single organisms on the slide were clearly delineated. In IFA tests *P. carinii* from man and rats showed the same cross-reactions seen in the DFA procedure.

**Control procedure.** The following control procedures demonstrated the specificity of the reaction. (i) Labeled normal serum, whether from rabbits or man, did not stain either animal or human *P. carinii*. (ii) After absorption of the labeled antibody with concentrated *P. carinii* the fluorescence was eliminated. (iii) In the two-step process of staining, as well as in the usual one-step staining, the fluorescence was markedly reduced by unconjugated antiserum. (iv) Uninfected tissue from animal and human lungs showed no fluorescence. (v) Staining of other species of microorganisms obtained from appropriate cultures with labeled anti-*P. carinii* antibody showed no cross-reactions. Organisms tested were: *Candida albicans*, *C. tropicalis*, Blastomyces dermatitidis, *Cryptococcus neoformans*, *Rhodotorula sp.*, *Histoplasma capsulatum*, and microorganisms usually present in the respiratory tract, such as group A streptococci, *Staphylococcus*, *Diplococcus pneumoniae*, *Haemophilus influenzae*, *Escherichia coli*, and *Pseudomonas* sp.

**Detection of *P. carinii* in H-P materials.** Various forms of *P. carinii* were detected in H-P materials of rats treated with corticosteroids (Fig. 2). *P. carinii* were detected in rats as early as 2 weeks after corticosteroid treatment was started. Nine animals were positive by 5 weeks, 14 by 7 weeks, all 16 by 9 weeks. The average number of organisms detected in the H-P smears increased with time, reaching a peak at 9 weeks. *P. carinii* were demonstrated in the H-P material for a prolonged period after the corticosteroid treatment had been terminated. The *P. carinii* score in the H-P remained at the peak for 2 to 3 weeks, thereafter declining to lower levels until 8 to 9 weeks (Fig. 3). Of 3 animals that survived beyond 9 weeks, 2 were negative from the 10th week continuously until the 13th week, whereas one showed intermittent excretion of organisms up to the 13th week. The numbers of *P. carinii* in the lung tissue were relatively well correlated with the numbers in H-P smears.

*P. carinii* were demonstrated in the H-P material of convalescent animals after discontinuation of corticosteroids. They were detected in one animal as long as 8 weeks, in the upper respiratory tract as well as lung tissue. In another animal, which died 6 weeks after the discontinuation of cortisone acetate, there were many cystic forms that were in regression, but the parasites were not detected in the upper respiratory tract.
MGG staining demonstrated pre-cystic forms and mature cysts; however, the free forms and small trophozoites were difficult to differentiate from the cellular background by this method (Table 3).

**DISCUSSION**

In the use of a DFA procedure as a diagnostic test, several basic questions must be considered in relation to its specificity. In the first place the specificity of a diagnostic antiserum depends on the purity of the antigen used in its production. Also, it is recognized that staining may be inhibited by a blocking phenomenon, as indicated by the results of Brzosko et al. (2), Roberts et al. (Bacteriol. Proc., p. 60, 1965) and by Cherry et al. (3). A coating of the antigen with antibody or other substance may compete with the specific P. carinii antibody. In the antisera used in this study, special attention was given to purification of the antigens for immunization of the rabbits. It appears that little, if any, of the reactivity observed is directed to rat lung antigens, since antisera were absorbed with both normal rat serum and mouse powder, antigens from human sources gave strong reactions, and only one line of identity was seen in ID tests. As indicated by the results with trypsin-treated tissue preparations, it was possible to remove the blocking antibody which could be demonstrated as a coating of the P. carinii organisms in untreated preparations. The results indicated a high degree of specificity of the method as used.

Although antigenic relationships between P. carinii from human and animal sources were demonstrated by the results of DFA and IFA cross-reaction tests, there was a difference in antibody titers between homologous and heterologous systems. The higher titer in the homologous system observed in all tests suggested that

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**TABLE 3. Comparison of direct fluorescent antibody technique and May-Grünwald-Giemsa staining for detection of P. carinii in lung tissue (number of parasites in 10 min)**

| Rat no. | Free forms | Trophozoites | Mature cysts | Total |
|---------|------------|--------------|--------------|-------|
|         | DFA        | MGG          | DFA          | MGG   | DFA | MGG | |
| 27      | 234        | 1            | 120          | 16    | 108 | 11  | 462  | 28  |
| 31      | 211        | 5            | 169          | 20    | 169 | 28  | 490  | 53  |
| 51      | 100        | 6            | 108          | 15    | 90  | 16  | 290  | 37  |
| 54      | 190        | 8            | 131          | 22    | 169 | 10  | 490  | 59  |
| 55      | 39         | 0            | 54           | 5     | 88  | 29  | 181  | 15  |

The untreated animals showed rare organisms in H-P material. Occasional organisms (±) were detected in 2 of 10 rats on 5 weekly examinations. Rare organisms were found at autopsy in these two rats and two additional animals.

**Comparison of sensitivity of DFA and MGG staining.** For evaluation of rapidity and accuracy in identification of the various forms of the organisms the DFA was compared with MGG staining. In infected lung tissue of different animals, the numbers of organisms counted in 10 min were 8 to 16 times (mean 11 times) higher with the DFA method than with MGG staining. Free forms and trophozoites were found predominantly during the acute stage of PP, whereas most of the organisms detected by DFA during the convalescent period were cysts.
P. carinii from different species may not be entirely identical, although they may share the greater part of their antigenic specificity. The use of organisms grown in a different host may be of advantage in the identification of P. carinii by the DFA procedure or when P. carinii are used as IFA antigens, since the heterologous reagent reduces nonspecific staining of host material in the antigen, thereby insuring greater specificity of the tests.

Kim et al. (8) recently reported IFA studies showing that anti-rat P. carinii antiserum reacted only with P. carinii from rat but not with organisms from humans. However, their results cannot be compared with the results in the present study, since different procedures were used for the preparation of antigens. For example, the organisms they used for injection of animals and for IFA tests were not isolated by enzyme treatment of the tissue as in this study.

In this study no organisms other than P. carinii were stained with the conjugated anti-P. carinii sera used. However, each antiserum should be tested against other microorganisms which might be mistaken for P. carinii in the DFA test. The need is illustrated by the finding of Kendrick et al. (9) that one conjugated antipertussis serum stained both Bordetella pertussis and staphylococci; the conjugated preinjection sample also stained staphylococci but not B. pertussis.

Sensitivity of the DFA procedure has been demonstrated by comparing it with MGG, a conventional staining method, in terms of time-saving and accuracy. Whereas MGG identified only mature cysts by staining the intracytic bodies, the DFA method demonstrated all forms of the developing organisms. Comparison with methenamine silver staining was not made on rat material but has been made in a series of human cases to be published elsewhere. The DFA procedure would seem to be a convenient tool for diagnosis of P. carinii infection and also for the study of such problems as the mode of transmission, cultivation of the parasite, and the biological characteristics of P. carinii.

ACKNOWLEDGMENT

The authors are deeply indebted to Pearl L. Kendrick, who offered helpful advice in editing this manuscript.

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