Direct Fluorescent-Antibody Method for the Diagnosis of
Pneumocystis carinii Pneumonitis from Sputa or Tracheal
Aspirates from Humans

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A direct fluorescent antibody (DFA) method was applied to sputum or tracheal aspirate from 68 patients with clinical or radiological evidence suggesting Pneumocystis carinii pneumonitis, and to 50 control patients. P. carinii was detected by DFA in specimens from 33 of the 69 clinical cases and 3 of the 50 controls. Specimens of lung from 11 of 33 DFA-positive cases were examined histologically, and 9 were positive. Four of 35 DFA-negative cases were examined histologically, and all were negative. Sputa or tracheal aspirates from 6 patients who were positive by both DFA and histological examination were examined also by methenamine silver staining; none could be diagnosed conclusively by this method. The results indicate that the DFA method is a sensitive and dependable procedure for the laboratory diagnosis of P. carinii pneumonitis in man.

In a previous study (10) a direct fluorescent antibody (DFA) procedure was developed and shown to be satisfactory for detecting Pneumocystis carinii in hypopharyngeal smears from rats whose infection had been activated by corticosteroid administration. In the present study this DFA was evaluated on clinical specimens from human sources.

The need for a rapid and reliable laboratory diagnostic procedure has been emphasized because of the increased frequency of P. carinii pneumonitis associated with the wide use of immunosuppressive chemotherapy.

Currently, the diagnosis of P. carinii pneumonitis depends primarily on biopsy of lung with examination of the tissue by the conventional silver impregnation technique (1, 14). A method using sputa or tracheal aspirates would be simpler and safer; however, with current staining procedures the examination of such specimens has been successful less often than biopsy in the detection of P. carinii (2, 8, 9, 17, 18).

The DFA procedure, as described in a preceding paper (10) provided greater sensitivity than conventional staining techniques in the examination of hypopharyngeal smears of rats. In this study the reliability of the procedure was tested as applied to specimens of sputum and tracheal aspirate of human origin.

MATERIALS AND METHODS

Description of clinical specimens. The chief sources of clinical specimens were The University of Michigan Medical Center in Ann Arbor, Mich., and Children's Memorial Hospital in Chicago, Ill. The specimens were obtained from patients in respiratory distress who developed varying degrees of pulmonary infiltration as demonstrated radiologically. Most of these patients had immunological deficiency diseases of various types or were receiving immunosuppressive chemotherapy or adrenocorticosteroids for cancer.

A total of 108 specimens of sputum or tracheal aspirate were studied from 68 patients with clinical indications of P. carinii pneumonitis (PP). Thirty-five of the patients had strong evidence of PP; in 33 others the clinical findings merely suggested P. carinii infection. Ten patients were known to have congenital immunological disorders with histories of recurrent infection of the respiratory system, 48 patients were receiving immunosuppressive therapy for lymphoreticular neoplasms or other types of malignancy, and the remaining 10 patients were in other categories.

Control studies were carried out on 70 sputa or tracheal aspirates from 50 patients who had acute infectious disease of the lower respiratory tract but no clinical basis for suspicion of PP. (These specimens were collected primarily for bacteriological culture.) Single specimens were obtained from most patients, but in some cases additional specimens were examined. When a patient was found to have a positive DFA test for P. carinii, paired sera, when available, were tested by the indirect fluorescent antibody (IFA) method for rises in antibody titers. Histological corroboration was sought whenever possible. The histological examinations were performed in the Department of Pathology in The University of Michigan and included the methenamine silver staining technique.

Processing of specimens with mucolytic reagents. Because of their viscosity, most of the
specimens were treated by a mucolytic process. Such specimens were tested both before and after treatment. For homogenization of tracheal aspirates or sputa, a freshly prepared N-acetyl-L-cysteine (NALC) solution (19) was added to the specimen in the original container to make a final concentration of 0.25 to 0.3%. The material was mixed thoroughly until visible mucolysis occurred, and then incubated at room temperature for 10 to 20 min. For the purpose of concentration, 1/N NaOH-trisodium acetate mixture was added to make a final concentration of 1%, using the procedure described by Dye and Kubica for Mycobacterium tuberculosis (3). After thorough mixing, the specimen was transferred to a siliconized centrifuge tube with at least 4 volumes of 0.85% saline solution. The mixture was then either allowed to stand at room temperature for 10 to 20 min or centrifuged at 400 rpm for 1 min to eliminate coarse particles. The supernatant fluid was transferred to another tube and centrifuged at 3,000 rpm for 20 to 30 min. The sediment was used as a concentrated specimen. Hypopharyngeal swabs were directly smeared on microscope slides or treated in the NALC (pH 8.0) for 15 to 20 min, followed by centrifugation at 2,500 rpm for 20 min. Smears for microscopy were prepared from the saline-washed sediment.

**Preparation of smears for microscopy and DFA staining.** As a first step, smears were made routinely with the unprocessed sputa or tracheal aspirates. Portions of raw specimens were spread thinly with a 3-mm bacteriological loop on clean microscope slides. The homogenized as well as the concentrated specimens were spread in the same way. All smears were air-dried at room temperature. Usually the smears from the raw and homogenized specimens were stained while the concentrated smears were being processed. (If *P. carinii* was identified in sufficient number from raw and homogenized smears, the concentration process was omitted.)

The method of preparing the DFA reagents as well as the staining procedures were outlined in a previous paper (10). In brief, the staining procedures were as follows. The labeled anti-*P. carinii* antibody, prepared from either human or animal sources, was placed on the smear and incubated at room temperature for 45 min, followed by two washings with pH 7.6 phosphate-buffered saline (PBS) for 10 min each. The slides were then counterstained with a 1:20 dilution of Eriochrome black for 5 s (7) and mounted in buffered glycerin. In most instances six to eight smears were examined, and the mean number of *P. carinii* per smear was recorded. Control specimens were handled in the same way as the test specimens, including the concentration steps.

**Serum antibody titration by IFA technique.** The smears of *P. carinii* from rats were first incubated with serial twofold dilutions of the test sera, followed by two washings in PBS, pH 7.6, for 10 min each. The smears were then covered with either anti-human immunoglobulin (Ig) G at 1:5 or anti-human whole gamma globulin at 1:30 dilution for another 45 min at room temperature. The smears were counterstained and mounted as usual.

**Methenamine silver staining.** The methenamine silver nitrate procedure was carried out by the method of Gomori as described by Grocott (6).

Preparation of *P. carinii* antigens and specific antiserum for DFA staining have been cited in detail in a preceding paper (10). In brief, rabbits were injected with relatively purified *P. carinii* organisms from either human or animal sources. Hyperimmune serum thus obtained was conjugated with fluorescein isothiocyanate, after proper absorption and evaluation of its specificity.

**RESULTS**

In preliminary tests it was shown that *P. carinii* from both man and animals were not damaged by the mucolytic process employed in this experiment. After at least 30 min in 1/N NaOH (pH 14) the parasites showed intense staining with specific labeled antiserum; after 60 min some organisms had begun to disintegrate, showing odd shapes. Treating the organisms for 60 min with the reducing agent 3% NALC (pH 8.0) alone did not change either the morphological appearance or the surface antigen.

**Effect of mucolytic treatment on the clinical specimens.** The viscosity of sputa or tracheal aspirates varied from one specimen to another. The mucolytic activity of a 0.3% final concentration of NALC solution at pH 8.0 was efficient in homogenizing the specimen within 1 to 5 min. For the tracheal material this homogenization process alone, without further concentration, was sufficient for the detection of *P. carinii*. However, the concentration step was necessary for specimens which showed few or no organisms in unconcentrated material. Nasopharyngeal swabs, treated in 2.5% NALC solution, were also suitable for detecting *P. carinii* after one washing with saline; the sediment obtained by centrifugation contained well-preserved cellular background and parasites.

**Identification of *P. carinii* from clinical specimens by DFA.** A total of 108 specimens from 68 patients with diffuse interstitial pneumonitis were examined by DFA technique. *P. carinii* were identified in 33 patients (Fig. 1). Clinical and radiological evidence for PP was strong in 25 of these, and suggestive in 8 as shown in Table 1. The 50 patients with negative evidence and not suspected of having PP were considered as controls. The results with 70 specimens from the 50 control patients are included in Table 1. *P. carinii* were detected in three of these individuals on one or more examinations.

In Table 2 the results of histological examination (methenamine silver) on the 15 patients from whom tissues were obtained by biopsy or necropsy are recorded. Of 11 patients positive by DFA, 9 were also positive histologically. Of the four negative by DFA, all were negative by histological findings.
titers. The acute sera usually showed lower titers, whereas the titers of sera taken 2 to 4 weeks after pentamidine therapy reached high levels (1:64 to 1:256). Two patients who were known to have congenital agammaglobulinemia did not show antibody reaction beyond a 1:4 dilution of sera. In addition to the data in Table 3, the IFA titers of four individuals whose sputa were negative by DFA were found to be relatively low, ranging from 1:8 to 1:32.

In Table 4 the IFA results are correlated with the DFA findings on specimens from the same patients, with the exception of the two with congenital agammaglobulinemia (Table 4). Of the convalescent sera which were positive by DFA, 61.5% showed high antibody titer.

**Gomori methenamine silver (GMS) staining.** Because GMS staining of pulmonary tissue has been recommended for the diagnosis of PP, this staining method was applied to sputa and tracheal aspirates. To compare the sensitivity of the DFA with this conventional staining method, duplicate smears of tracheal aspirates from six patients confirmed by GMS staining were tested by each of the two staining methods. Conclusive identification of *P. carinii* was not possible in the material from any of the six patients because of the difficulty in discriminating between this organism and yeast cells. By the DFA method, the same material from all six patients was definitely positive.

**DISCUSSION**

*P. carinii* pneumonia in this country is almost always related to some form of immunological deficiency which, in turn, engenders the activation of latent infection or increases the susceptibility to primary infection. Identification of *P. carinii* in tracheal aspirates has been seldom successful, and diagnosis has depended on pulmonary biopsy during the advanced stage of the disease. In general, the results in this study suggest that the examination of sputa or

**FIG. 1.** *P. carinii* in smears stained by direct fluorescent antibody technique. ×640. (a) Transtracheal aspirates of patient with fatal *P. carinii* pneumonia. (b) Tracheal aspirate homogenized with N-acetyl-L-cysteine.

**TABLE 1. Correlation between direct fluorescent antibody tests of specimens and clinical and X-ray findings of the patients**

| Clinical and X-ray criteria of *P. carinii* pneumonia | No. of patients with direct fluorescent antibody test |
|-------------------------------------------------------|-----------------------------------------------------|
|                                                       | Positive* Negative* Total                            |
| Strong positive                                       | 25 8 33 10 35 35 68                                  |
| Suggestive                                            | 25 33 10 35 35 68                                  |
| Negative (controls)                                   | 3 47 50                                           |
| Total patients                                        | 36 82 118                                         |

* Agreement: 33/36 = 91.6%.
* Agreement: 47/82 = 57.3%.

**Results of IFA tests.** In Table 3 the results of IFA tests are recorded for 15 DFA-positive patients. Twofold or greater rises in serum antibody titers were observed in five paired sera by the IFA test. The titer of a single serum from each of 10 suspects in whom *P. carinii* were identified by DFA varied from 1:4 to 1:128 when titrated against rat *P. carinii*, using labeled rabbit anti-human IgG. Single serum samples from three patients taken after 4 weeks of pentamidine treatment exhibited high IFA

**TABLE 2. Correlation between direct fluorescent antibody test of sputa or tracheal aspirates and subsequent histological examination for *P. carinii***

| Histological findings (methenamine silver) | Direct fluorescent antibody test |
|--------------------------------------------|---------------------------------|
|                                            | Positive* Negative* Total       |
| Positive                                   | 9 0 9                           |
| Negative                                   | 2 4 6                           |
| Total                                      | 11 4 15                         |

* Agreement: 9/11 = 82%.
* Agreement: 4/4 = 100%.
tracheal aspirates by the DFA procedure is reliable and perhaps can be substituted for biopsy in many instances.

In the group of 68 patients with diffuse interstitial pneumonitis, 33 or 47.2% were DFA positive. It is of interest that this percentage agrees well with the results by Goodell et al. (5) that 44% of their cases of diffuse interstitial pneumonitis were identifiable as PP by histological examination.

In the control group, P. carinii were identified in three patients (6%). While Stopka et al. (21) gave no precise figures, they stated that P. carinii were observed in sputa from control infants but that the frequency was significantly lower than in the test group. In contrast to this, Robillard et al. (16) could not detect the organism in infants without symptoms but with histories of contact with known cases. The occasional finding of P. carinii in clinically negative persons probably represents inapparent infection, as described by Sheldon (20) and Esterly (4).

In patients with advanced PP, repeated sputum examinations were not necessary; the parasites were detected in the first specimen from each of nine patients with histologically confirmed diagnoses. However, in the early stages of PP, or for detection of possible carriers, concentrated material should be examined because the number of organisms may be expected to be fewer than in specimens from advanced cases. Likewise, for very viscous specimens or when a large amount of sputum must be examined, treatment with a mucolytic agent and concentration of the specimen are indicated.

Inhibition of FA staining by surface-coating antibodies on the P. carinii was not a problem in general, even without pretreatment of the specimens. However, in one instance a partial blocking phenomenon was observed in sputum samples from a patient who had a combined immune deficiency disease with a history of recurrent respiratory involvement of unknown cause. The raw specimen stained by the DFA procedure revealed markedly reduced intensity of staining of P. carinii compared with the brilliant staining reaction of the treated specimens.
Therefore, at times, one may expect to find a blocking phenomenon in the DFA procedure with untreated specimens. This factor may be related to the duration of the infection or the immune state of the patient or both. It may be of significance that P. carinii were not revealed in a biopsy specimen from this patient. However, he may have been a chronic carrier of P. carinii. Except for this case, however, inhibition of DFA staining was not encountered, perhaps because almost all of the specimens tested were from patients who were receiving intensive immunosuppressive chemotherapy or had primary immunological deficiency disorders and therefore did not possess enough antibodies to bind antigens of P. carinii in vivo.

As pointed out by Rifkind et al. (13), there is no satisfactory serological method for antibody assay for PP patients in a state of secondary immunological incompetence. It is believed that these patients are unable to synthesize antibodies because of impaired cellular function during administration of the suppressants. Recently Norman and Kagan (12) reported a total of 46% of IFA-positive sera from 89 individuals who had confirmed or suspected PP as a complication of immunosuppressive therapy. On the other hand, they stated that negative IFA reactions were obtained in sera during the acute phase of fatal infections.

In the present study, the sera of 8 of 15 patients in whom P. carinii was identified by DFA showed IFA titers of 1:64 or greater; the four paired sera showed more than a fourfold rise. The acute phase sera usually showed lower titers, whereas sera taken 2 to 4 weeks after therapy showed moderate to high titers (Table 3). For the purpose of analysis, an IFA titer of 1:64 or higher was considered presumptive evidence of current or previous infection. On the other hand, 4 of the 35 clinically positive patients in whom P. carinii were not detected by DFA showed IFA titers of 1:8 to 1:32. Although detailed information on the kinds of immunosuppressants used and their doses and duration of administration is not available in all cases studied, these factors may have affected the serum antibody titers. Nevertheless, the IFA results on the paired sera demonstrated rises in titers which are considered to indicate an immune response.

Cross-reaction with other organisms, especially yeast species in the upper respiratory tract, did not create difficulty in identifying P. carinii. Freshly isolated yeast cells, primarily species of Candida, exhibit varying degrees of bluish nonspecific fluorescence around the cell walls, but after counterstaining the cells usually appear orange-red. The yeast cells as well as the P. carinii stand out clearly in the treated specimens, but there is no real difficulty in distinguishing one from the other. The nonspecific staining reaction of C. albicans cells is distinctive and, furthermore, the fungus, unlike P. carinii, may exhibit budding or pseudohyphae in the smear preparations. In case of doubt, the appropriate labeled anti-fungal reagent should be used as a control. The chances of contamination by yeast cells is greater with sputa than with carefully aspirated tracheal material. Therefore, tracheal or trans-tracheal aspirates are the specimens of choice.

P. carinii pneumonitis may be complicated by a concomitant infection with opportunistic pathogens (17, 22). DFA tests for P. carinii in tracheal aspirates should be included along with examination for complicating pathogens.

The importance of early diagnosis is well recognized (1, 11). As shown in Table 1, of 33 patients with respiratory disease only suggestive of PP, 8 were DFA positive. Although detailed follow-up was not available, these positive findings probably indicate early or inapparent infection.

Failure of current staining procedures to identify P. carinii in the sputa or tracheal aspirates of PP patients has been reported by several workers. Rifkind et al. (13) diagnosed only one of their four patients before death; Ruskin and Remington (18), Robbins et al. (15), Jacobs et al. (8), and Rosen et al. (17) reported unsuccessful results, even after careful searching of their stained preparations.

In a small series of histologically positive patients, P. carinii could not be identified with certainty by the Gomori methenamine silver technique applied to sputa, tracheal aspirates, or trans-tracheal aspirates, whereas in each case P. carinii were found by the DFA method. These limited data indicate that, at least for some patients, the DFA method is superior to the widely accepted methenamine silver staining procedure for examination of sputa or tracheal aspirates.

The results of the present study suggest that the DFA procedure can give reliable laboratory assistance in the diagnosis of PP and in the recognition of inapparent infection with P. carinii. Also, it may fill the critical need for an easily performed laboratory test which is suitable for frequent repetition during the prolonged period of treatment. In addition, it offers promise as a research procedure. For example, it could furnish a criterion for judging the value of therapeutic measures.
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