Fluorescent Antibody Studies on Experimental Pneumocystosis (35338)

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Pneumocystis carinii is now well known as an endogenous, pneumotropic organism which causes an atypical interstitial plasma cell pneumonia in man. In the Western countries, the affected hosts are those who suffer from innate immunological deprivation (1–3) (congenital hypogamma-globulinemia), hemopoietic malignancies (4, 5), or are recipients of immunosuppressive therapy (3, 6, 7). In Europe and some of the Eastern countries, however, the disease mainly involves infants under 1 year (2–6 months), precisely the premature and debilitated babies who are cared for in institutions (8–12). The latter form occurs as a primary disease with high morbidity and mortality rates (8, 9, 11).

P. carinii is widely distributed among rodents and certain other animals, usually without causing disease. Not infrequently, it has been recovered in humans on random autopsy specimens which are not associated with pneumonitis (2, 4, 8). Many investigators (13–17) have produced pneumonitis in laboratory animals from latent pneumocystis by long-term immunosuppressive therapy and massive doses of corticosteroids.

The taxonomic position of *P. carinii* is accepted as a protozoan of uncertain relationships. The mode of transmission is not clearly understood and the organism has not been cultured *in vitro* (4, 13, 16). Accordingly, one critical problem is how to make an early diagnosis to support successful treatment. The diagnosis of pneumocystic pneumonia has been based on the histopathology of postmortem materials during the past few decades. Recently, Gram-Weighert's staining procedures and silver impregnation techniques have been recommended (2, 6, 18) for biopsy specimens, but these are applicable only in advanced stages of the disease. Immunohistochemical study has been carried out with postmortem materials in human pneumocystosis and reported to be useful in epidemiological studies (14, 19, 20).

The purpose of this study was to develop a rapid diagnostic method using fluorescent antibody (FA) techniques. Using rat pneumocystosis as a model, an indirect fluorescent antibody (IFA) technique has been successfully applied on fresh impression smears of lung tissue infected with *P. carinii*.

Methods. One-year-old male rats (Lewis strain), over 200 g each were obtained from Microbiological Associates (Rockville, Maryland). Four rats were placed into two equal test groups; Group 1 rats were used for antigenic material and Group 2 rats for antiserum. Rats in Groups 1 and 2 were injected with cortisone acetate (Merck, Sharp and Dohme, Columbus, Ohio), 1 ml (25 mg/ml) subcutaneously twice weekly for 8 weeks. Chlortetracycline (Lederle Laboratories, Pearl River, New York) was given in the drinking water (0.05%, v/v, final) throughout the experimental period to prevent secondary infection while receiving corticosteroid. Control rats from the same source were kept in a separate cage without cortisone conditioning.

Lungs from Group 1 rats were harvested after 8 weeks of cortisone injections. Group 2 animals were kept for another 8 weeks after withdrawal of corticosteroid conditioning to allow for antibody titer rise. Beginning from the time of withdrawal, blood samples were drawn weekly by cardiac puncture for antibody detection.

Light pressure impression smears of lungs from Group 1 and control rats were made on clean slides and acetone fixed for 10 min at room temperature. After air drying for 30 min the slides were incubated with 2 drops of antiserum (from Group 2 rats) for 45 min in a moist chamber at room temperature.

They were then washed twice in buffered saline (pH 7.6) for 10 min each; incubated at room temperature with 2 drops of fluorescein isothiocyanate (FITC) labeled rabbit anti-rat gamma globulin (Nutritional Biochemical Corporation, Cleveland, Ohio) for another 45 min; carefully washed in two changes of buffered saline for 10 min each; counterstained with 1:50 dilution of Eriochrome black B for 5 sec and mounted in buffered glycerin (pH 7.6).

Control procedures for the specificity of the IFA staining were constituted as follows: antiglobulin conjugate staining of smears of normal and infected tissue; the infected tissue smears staining against pre-bled normal serum and heterologous unlabeled serum (antiserum from rats infected with *Mycoplasma pneumonia*) in the place of homologous unlabeled antiserum.

Preparations were observed for fluorescence with a Zeiss GFL microscope using the BG I heat barrier, BG 12 exciter, and OG4/GG4 ocular barrier filters. Black and white photomicrographs were taken with Polaroid 3000 Type 37 Land film at ×1000 magnification with exposure times ranging 15–24 sec.

Results and Discussion. The corticosteroid conditioned rats showed 10–45 g of weight loss (mean 32 g) while control rats gained 108 g by the end of the eighth week. Group 1 rats for impression smears were sacrificed 8 weeks after corticosteroid conditioning; one animal possessed low grade focal lesions in both lungs except in peripheral areas, while the other rat revealed irregular, confluent lesions extending to the periphery, indicating a moderate degree of pneumocystic infection.

Evidence for the presence of pneumocystic organisms in the tissue was obtained first by examining smears stained with May-Grunwald-Giemsa (MGG) (see Fig. 1). The mature cysts contained eight purple-brown nuclei surrounded by pink cytoplasm and the developing sporoblasts were clearly identified among host cells in the field. Impression smears of normal lung prepared from the control rat did not show any suspicious pneumocysts using the same staining procedure.

Beginning the fourth week after the corticosteroid treatment was withdrawn, sera from the Group 2 animals were tested weekly for antibody assay. The serum samples of the eighth week postwithdrawal gave a positive FA reaction. The antisera collected after the eighth week were pooled together and used in preparation of gamma globulin by precipitating with saturated ammonium sulfate (50%,

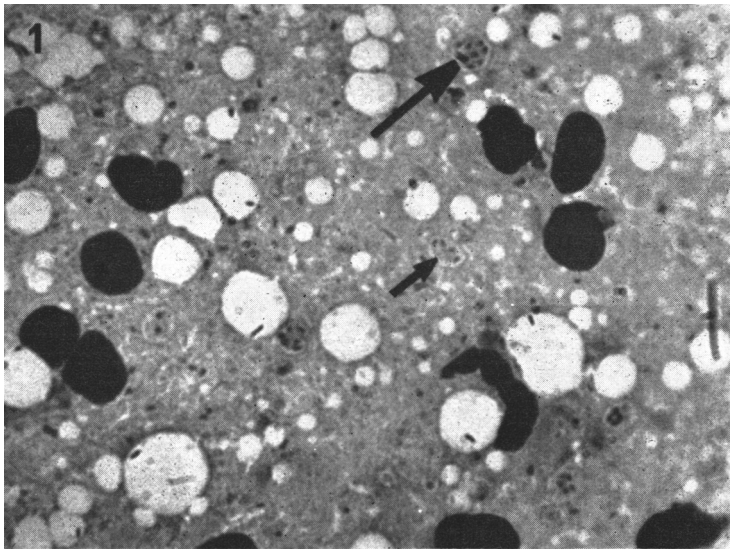

FIG. 1. May-Grunwald-Giemsa stained impression smear of lung tissue from a conditioned rat revealing pneumocysts in various stages of maturity: One cyst (large arrow) appears to contain eight spores, while another (small arrow) developing cyst possesses four; ×2652.

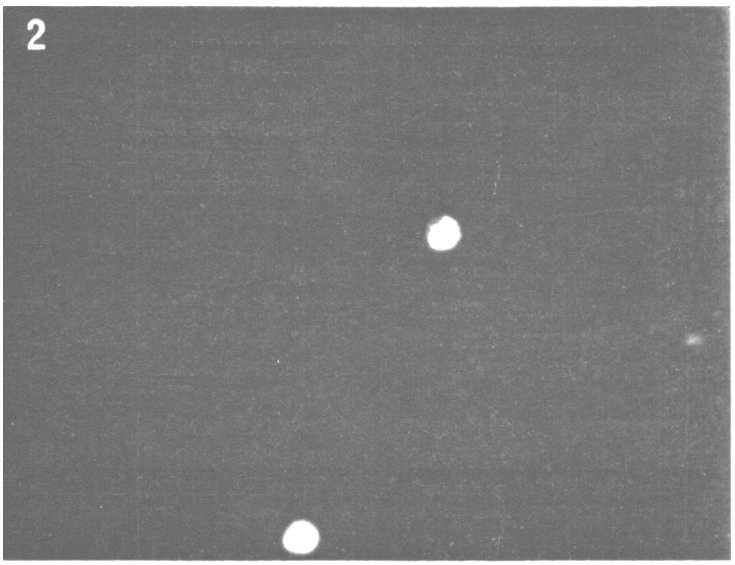

FIG. 2. Impression smear of lung tissue from a conditioned rat revealing the presence of pneumocysts by the IFA technic; ×2652.

v/v, final). After diluting the precipitated globulins 1:4 with saline, microgel precipitin tests (Ouchterlony) were performed against labeled rabbit anti-rat globulin diluted 1:8, and gave positive bands. The prepared globulin material as well as the pooled whole sera gave positive results in the IFA tests.

Using the IFA technique, the infected smears showed brilliant fluorescence up to a 1:4 dilution of the globulin preparation (Fig. 2). Staining intensities decreased markedly at higher dilutions. The cysts appeared in various sizes, round to ovoid and sickle-shaped. Figure 3 shows mature cysts with unstained nuclei. Other micrographs recording IFA reactions have revealed various forms of developing sporozoites. The intense fluorescence at the outer layers of the cysts

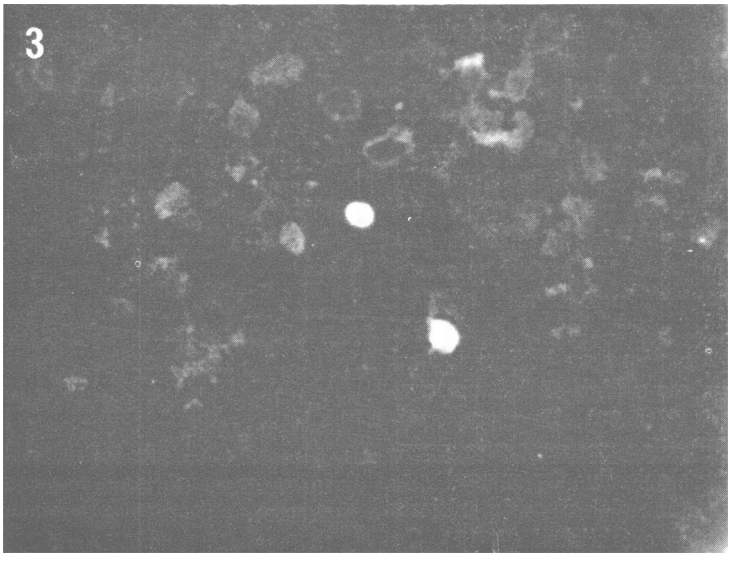

FIG. 3. Impression smear of lung tissue from a conditioned rat specifically demonstrating by IFA methods pneumocysts containing up to eight nuclei; ×2652.

exhibited distinctly walled oval or spherical bodies; the less intense, apparently soft, inner material was interpreted as homogeneous. To confirm that only cysts were fluorescing specifically in the IFA reaction, the same microscopic foci were examined before and after restaining with MGG.

Comparative findings of the restained preparations revealed various morphological pneumocystic forms that did not fluoresce by the IFA technique. This suggests that antigenic differences may exist between the various presumed developmental stages. Brzosko and Nowoslawski (19, 20) found that the PAS-positive material in the infected alveolar lumen was stained with FITC-conjugated antibody in human histological preparations. They suggested that the different intensities of fluorescence may be due to the various developmental phases of the parasites. In a report by Goetz (14), a similar observation was found; pneumocysts in the paraffin embedded lung tissue showed selective fluorochromation correlating well with the gram-positive materials observed. The antigenic properties of the pneumocysts which gave such selective, as well as varied IFA staining, definitely require further study.

The traditional diagnostic methods used in the Western countries for the sporadic cases of pneumocystic pneumonia are Gram-Weighert's stain (6), PAS and the methenamine silver impregnation techniques (18). Intensive serodiagnostic methods have been developed mainly in European countries where the disease is predominant among young infants; the complement-fixation (CF) technique was applied with 90–100% accuracy using sera from infected patients (10, 21–24) and this method has served as a useful tool in epidemiological and clinical studies.

Sera obtained from humans having natural illnesses usually show a high antipneumocystic antibody titer (21, 24). However, antibody patterns regarding pneumocystic pneumonias in immunologically suppressed patients and hypogamma-globulinemic subjects have not been clearly established (7, 16). On the other hand, there is evidence that animals having corticosteroid-induced penumocystosis demonstrate antipneumocystic globulins as shown by the CF and Ouchterlony techniques (13, 25).

Negative FA results on sera collected 4 to 7 weeks after termination of cortisone treatment may suggest either a lack of significant antigenic stimulation by a single induction of pneumocytosis in rats, or that the organisms naturally possess a low order of pathogenicity. Although positive IFA reactions were obtained with sera from infected animals, it was necessary to use concentrated globulins to obtain intense fluorescence of the pneumocysts. IFA staining is reported to be more sensitive than the direct method in human pneumocytosis (14, 19, 20).

The IFA method is relatively simple, rapid and appears as if it could be used for detecting latent or chronic pneumocytosis.

Summary. Using "induced" rat pneumocystosis as a laboratory model, experimental evidence suggests the possibility of using indirect fluorescent antibody methods for detecting latent or chronic pneumocystic disorders. Comparison of the same microscopic foci in infected tissue specimens initially stained by IFA procedures, then restained by MGG methods, revealed that antigenic differences may exist between the various developmental pneumocystic forms.

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