Studies of Morphology and Immunofluorescence of *Pneumocystis carinii* (36764)

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Pneumocystis carinii is the causative agent of a specific pneumonitis of man. In European countries this disease occurs most frequently as endemics in nurseries and institutions for infants. In the United States the disease occurs sporadically in patients of various ages who are undergoing immunosuppressive therapy for malignancy or organ transplantation, or who suffer from congenital immunoglobulin deficiencies. *P. carinii* is ubiquitous in a variety of animals (1). It infests the intraalveolar space of the lung as an opportunist and rarely penetrates tissue. Pneumonitis may be induced in animals by the administration of large doses of corticosteroid or cytotoxic drugs (2, 3). There are many similarities between human and animal *P. carinii* pneumonitis with respect to the pathogenicity, histology of the diseased tissue and morphology of the organism. However, the possibility of species specificity and antigenic differences between animal hosts exists and has not heretofore been investigated. Knowledge of species difference or similarity of *P. carinii* in animals and man would be useful in the further understanding of epidemiology, in the development of serological tests and in interpolation of experimental data from the animal model to disease of man. The objective of this study is to determine whether *P. carinii* from the rat and human share common antigenic determinants and to examine further the morphology of the organism.

Materials and Methods. Propagation of P. carinii in rats. Sprague-Dawley rats weighing approximately 200 g were given 25 mg of cortisone acetate subcutaneously twice weekly. They were fed Purina Lab Chow. For the first 4 wk 50 mg of chlortetracycline was added to 100 ml of drinking water and increased thereafter to 100 mg/100 ml to prevent bacterial infection. When the animals became ill or died, usually after 8 to 11 wk, the lungs were removed immediately with trachea and bronchi intact. A small plastic catheter was inserted aseptically into the left main stem bronchus. A syringe was attached to the catheter and the lung was gently irrigated with physiologic saline to harvest *P. carinii* from the alveolar spaces. The right lung was used for impression smears, histologic study and stored at −30° for the fluorescent antibody study.

Study of morphology. The lung irrigate was centrifuged and cells suspended in sterile physiologic saline. To prevent the clumping of the organisms by mucus a few drops of irrigate suspension was mixed into 1 ml of 5% acetylcysteine (Mucomyst, Mead Johnson Co.) and after 10 min, the specimen was impinged onto a microscope slide by cytocentrifuge (Shandon Scientific Co.). Impression and suspension smears were examined by improved polychrome methylene blue (Hema-Tek, Ames), Giemsa and methenamine silver stains. *P. carinii* organisms from human host were obtained by percutaneous needle aspiration of the lung, a procedure used for the diagnosis of *P. carinii* pneumonitis and also from impression smears of postmortem lung tissue. Human specimens were stained in the same manner as those from the rat. According to the morphologic difference observed, the terms "cyst," "sporozoite" and "trophozoite" are used in the following text because of convenience and the inclination to classify *P. carinii* as protozoan.

Immunization. Lung irrigates contained large numbers of *P. carinii* organisms along

with macrophages. By differential centrifugation, a fairly pure preparation of *P. carinii* organisms was obtained and the organisms were washed several times with physiologic saline. The organisms were counted after staining a smear of 0.001 ml of appropriately diluted suspension with methenamine silver. No attempt was made to separate cyst forms from trophozoites. Approximately 3 to 3.5 $\times$ 10^6 cyst forms per ml of the suspension in saline was used for the immunization. A 0.1 ml suspension of *P. carinii* with 0.1 ml complete Freund adjuvant was injected into rats subcutaneously. One booster injection of 0.1 ml was given 1 mo later and a second booster 10 days after the first. Ten days after each booster injection, blood was collected and tested for the presence of antibody by indirect fluorescein antibody method. Homologous *P. carinii* organisms showed brilliant fluorescence. Serum collected from each rat 10 days after the second booster injection was pooled and used for the study described herein.

Antigenicity study. The indirect fluorescent antibody (IFA) method was applied using rat *P. carinii* antiserum and goat anti-rat γ-globulin antiserum conjugated with fluorescein isothiocyanate (Hyland Lab.). To remove parenchymal and nonspecific fluorescence absorption of rat antiserum was performed as follows. Lungs of three normal rats were homogenized together, washed four times with phosphate-buffered saline (PBS) and divided into three portions of approximately 2 g each. One milliliter of rat antiserum was sequentially passed through each of the three aliquots of lung tissue homogenate allowing 30 min with constant agitation for absorption with each aliquot at room temperature. One other aliquot of antiserum was absorbed with a mixture of the rat lung homogenate and *Candida albicans*. The same procedure was used for absorption with *P. carinii*-infested rat lung and human lung homogenate. The rat antiserum was tested against eight strains of rat and five strains of human *P. carinii* organisms. Smears of *P. carinii* suspension and lung tissue impression smears were air dried and fixed in acetone for 10 min. Three small drops of rat antiserum diluted to 1:128 with PBS was overlaid on the smears and incubated in moist chamber at 37° for 45 min. These smears were then washed with three changes of PBS at pH 7.2 for 30 min. This procedure was then repeated with goat anti-rat γ-globulin antiserum conjugated with fluorescein isothiocyanate. These slides were mounted with 35% glycerine in PBS and examined with a Zeiss microscope with mercury lamp and BG-12 exciter filter.

Five randomly selected human lung specimens known to be infested heavily by *P. carinii* and seven rat lung tissues embedded in paraffin were also tested. Lung tissue sections were deparaffinized and the fluorescent antibody method applied as described above. As controls and tests for immunofluorescence specificity, normal rat serum or saline in place of rat antiserum, normal rat and human lung tissue, and rat antiserum absorbed with rat lung tissue infested with *P. carinii* and human lung tissue with human *P. carinii* organisms were used.

Results. As shown in Fig. 1, there were generally two forms: "cyst" and "trophozoite." The cyst wall did not stain by Giemsa or polychrome methylene blue but stained prominently by methenamine silver stain (Fig. 3). "Sporozoites" inside the cyst and free trophozoites were well-stained by Giemsa and polychome methylene blue stains. Trophozoites varied greatly in size and shape. Seemingly young forms were smaller with darker cytoplasm and older forms were much larger in size with lightly staining and vacuolated appearance. Most of the trophozoite forms in various stages of development have U-shaped or paired nuclei, but there was no clear discernible evidence of binary fission in the trophozoite stage. Some of the large trophozoite forms with larger cytoplasm demonstrated some capsular material which could be visualized by methenamine silver stain and the eccentric nuclear material showed appearance of nuclear division (Fig. 1, D-F). This form was thought to be an early transforming stage from trophozoite to cyst form. The sporozoite

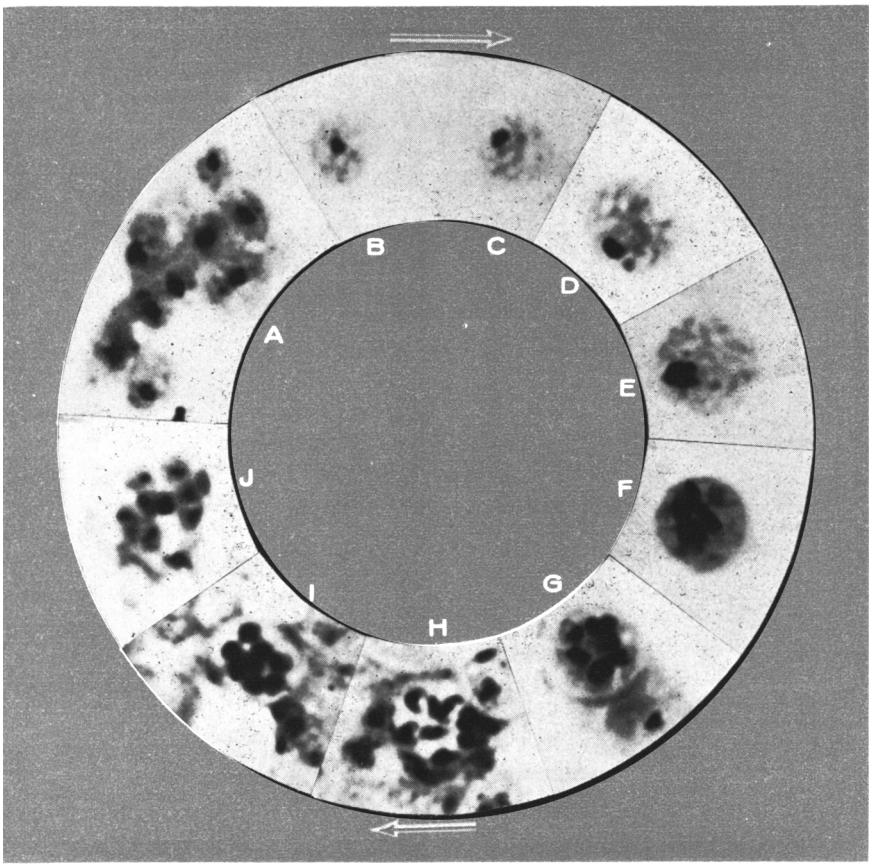

FIG. 1. Proposed life cycle of *P. carinii*. (A–C) Trophozoites; (D–G) transitional stage with thick cell wall and nuclear division (Thick cell wall can be visualized by methenamine silver stain.); (H) early cyst with crescent-shaped sporozoites; (I) cyst with maturing sporozoites; (J) cyst with eight mature sporozoites with paired nuclei.

so formed in the cyst seemed to be crescent shaped in the early stage (Fig. 1, H) and to become roundish or ovoid with an eccentric nucleus (Fig. 1, I).

Pooled sera from immunized rats contained a high antibody titer against homologous *P. carinii* as determined by the indirect fluorescent antibody method. Rat *P. carinii* organisms treated with antiserum diluted to 1:2048 emitted bright fluorescence. However, the most intense fluorescence was observed at dilutions between 1:128 and 1:512. Absorption with the normal pooled rat lung tissue or normal rat lung tissue containing *C. albicans* did not decrease the degree of specific fluorescence of *P. carinii* and none of the control stains showed appreciable fluorescence. The background parenchymal tissue fluorescence was minimal but there was appreciable cytoplasmic fluorescence of some of the macrophages. The cell walls of the cyst forms and the trophozoites fluoresced with clarity and could be compared after Giemsa stain of the same organism as illustrated in Fig. 2. Impression smears and material obtained by alveolar washings of seven rat lungs showed brightly fluorescing *P. carinii* cyst walls and trophozoites. *P. carinii* from smears of five human lung specimens, however, did not show fluorescence after tagging with rat *P. carinii* antisera. When the rat antiserum was absorbed with rat lung homogenate containing large numbers of *P. carinii* organisms, the fluorescence activity was com-

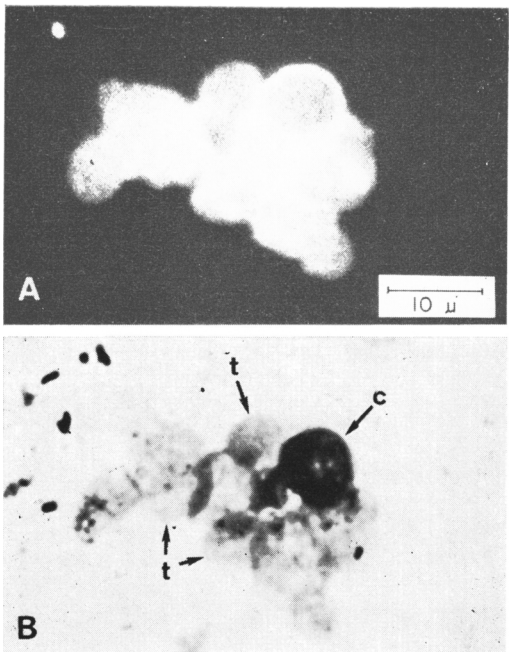

FIG. 2. Cluster of *P. carinii* seen by IFA method (A) and the same cluster stained with Giemsa stain (B), cyst form (c) and trophozoites (t).

pletely abolished, while the absorption with the human *P. carinii*-infested lung tissue did not alter the degree of fluorescence of rat *P. carinii* organisms. This suggested the specificity of the antiserum against the rat *P. carinii* strain and the antigenic difference between rat and human *P. carinii* strains. Study of the paraffin-embedded lung tissue sections also revealed antigenic dissimilarity between rat and human *P. carinii* strains by the IFA method. All of the seven rats' lung sections revealed fluorescing intraalveolar organisms as a "honeycomb" appearance and none of the five selected human lung tissue sections with heavy *P. carinii* infestation showed fluorescence.

Discussion. Inability to cultivate *P. carinii* has hindered more extensive study of this organism, its habitat and life cycle, pathogenesis of human infestation and epidemiology. By irrigation and aspiration of *P. carinii* from the pulmonary alveoli of rats, we were able to obtain fairly pure preparations of organisms and to study the morphology of individual forms in different developmental stages. Electron microscopic observations of *P. carinii* in our laboratory as well as by other investigators reveal thick-walled cyst forms and thin-walled trophozoite forms (4, 5). We have observed these two distinct forms clearly by light microscopy and differential staining. The taxonomic position of *P. carinii*, however, is not clear even though morphologically it appears to be a protozoan.

In our study numerous organisms were observed as large as cyst forms and with a thick wall, darkly staining cytoplasm and one nuclear body which seemed to be a transitional phase from the trophozoite to the cyst form (Fig. 1, E; 3, tr). In some of the cells the nuclear material appeared to be in division (Fig. 1, E, F) eventually leading to a cyst with eight sporozoites (Fig. 1, H–J). It is interesting that the "comma"-shaped bodies positioned as a "parenthesis" which has been frequently referred to by others using methenamine silver stain alone, were found to be independent from the sporozoites inside the cyst when examined by the different stains used in this study (Fig. 3). These "bodies" seem to be part of cyst wall or at least closely related to the wall of the cyst. We have found that by treating the cyst forms with acid alcohol, the silver impregnation onto the cyst wall is inhibited and staining of these "parenthesis"-shaped bodies is blocked. Whether this structure could be related to the expulsion of mature sporozoites from the thick-walled cyst is a conjecture and the true mechanism of excystment is quite unclear. Trophozoites are pleomorphic and vary greatly in size. The cytoplasm stains lightly with Giemsa or polychome methylene blue stains and appears reticular, vacuolated and with a poorly defined cell wall. Most of the trophozoites have paired nuclei located eccentrically. It has been suggested that the trophozoite undergoes binary fission (6), but this was not clearly observed in our study. The proposed life cycle of *P. carinii* from our observations is shown in Fig. 1 and is closely in accordance with the observation of others (6). Each cell in the life cycle was confirmed as *P. carinii* by the IFA method. All of the stages described here were ob-

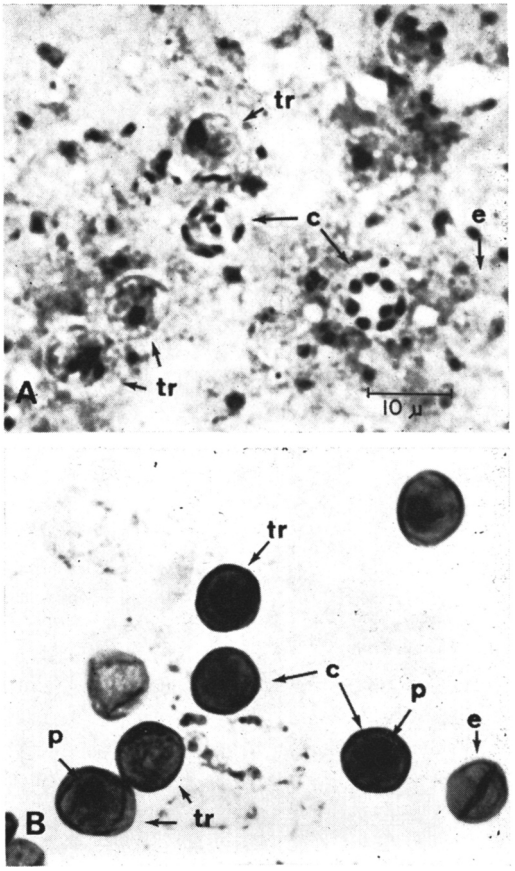

FIG. 3. Impression smear of lung stained with polychrome methylene blue (A) and same organisms stained with methenamine silver stain (B). Transitional stage with thick wall (tr); cysts with eight sporozoites (c); empty cyst (e). Note that the "parenthesis"-like body (p) is not related to the nuclear material or sporozoites.

served in human lung infested by *P. carinii* and morphologically identical to those seen in the rat lung. We have found that the dried cysts maintain their characteristic morphology after 7 mo at room temperature. Even though viability is unknown, it is possible that this cyst form might be the one maintaining a natural source of transmission.

Development of a reliable serologic test for human *P. carinii* infestation is needed for purposes of diagnosis and epidemiologic survey. In Europe, the complement fixation test using an extract of human lung tissue infested by *P. carinii* as antigen has been claimed useful (7). In the United States, however, this method was not used successfully (8). Whether this failure is due to immunosuppression of patients studied or the impurity of the antigen is not clear. The antigenic dissimilarity of *P. carinii* between the host species has been suggested. Barton *et al.* (9) suggested the antigenic difference between human and animal *P. carinii* by complement fixation and Goetz (7) found that rat and human stains of *P. carinii* would fix complement with only the homologous host serum. We were able to produce high antibody titer by immunization of rats with rat strain *P. carinii* and clearly demonstrated the specificity of the antiserum only against the rat strains of *P. carinii* by the indirect fluorescent antibody method.

Whether this type of species specificity of *P. carinii* exists among other animal hosts is not known. However, the antigenic characterization of *P. carinii* strains from different animal species is an important subject to be studied for possible subclasssification of *P. carinii* and also for the epidemiologic survey of human *P. carinii* infestation.

Summary. Rat and human strains of *Pneumocystis carinii* were found to be identical in morphology of all the developmental stages. "Trophozoites" varied greatly in size and had paired nuclei located eccentrically. Transitional forms were also observed in addition to "cyst" forms with eight "sporozoites" which have been described previously. By these observations, life cycle of *P. carinii* inside the pulmonary alveoli was proposed. Uninfected rats were immunized with the rat strains of *P. carinii*. The pooled rat antisera contained specific antibody to homologous *P. carinii* through dilution of 1:2048 as detected by indirect fluorescent antibody (IFA) method. Eight rat and five human strains of *P. carinii* were then studied by IFA method. All of the rat strains fluoresced while none of human strains gave fluorescence which suggested antigenic dissimilarity between these two strains. Both cysts and trophozoite forms identified by Giemsa staining fluoresced by IFA method.

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