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Pneumocystis carinii: Production of Antibody Either Specific to Trophozoite or to Cyst Wall

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Pneumocystis carinii was first found by Chagas in Brasil in 1909, and was described as a new species by Delanoë et Delanoë in France in 1912. After that, this organism was found from many kinds of animals including man. And it is now understood as an important agent of opportunistic infection, namely, it does not propagate so much in the lungs of normal healthy host but dose propagate much when the host lapses into an immunodeficient condition. This organism causes a fatal pneumonia, so called *Pneumocystis carinii* pneumonia, with marked hypoxia probably due to alveolar capillary block by abundance of the organism in almost all alveoli.

Pneumocystis carinii pneumonia was first noticed in Europe as interstitial plasma cell pneumonia of such debilitated children as premature or malnutrition. However, this pneumonia at present is considered as one of the important complications occurring after immunosuppressive therapy against leukemia, malignant lymphoma, cancer, autoimmune diseases, organ transplantation and so on.

The diagnosis of *P. carinii* pneumonia is confirmed with finding the organism by open lung biopsy, closed lung biopsy, transbronchoscopic biopsy, percutaneous needle lung aspiration, or examination of the sputum. However, those procedures except sputum examination are not always easy to apply to the patient who has serious respiratory insufficiency.

On the other hand, serodiagnosis of this disease is unfortunately not adequately established yet in spite of many workers have so far studied on it. The difficulty may come from the following two reasons: 1. Patients of this pneumonia usually lie under high grade of immunosuppressive condition. 2. Pure antigen, hence antibody also, can not be obtained since the culture of *P. carinii* is still difficult although Pifer *et al.* (1977) and Latorre *et al.* (1977) successfully reported.

The present study describes a new method to obtain pure antiserum against *P. carinii* by using affinity chromatography, and also describes the antigenic difference between cyst wall and trophozoite, and those between organisms from man and animal.

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Materials and Methods

1. Antigens

In this study, four kinds of antigens were used. Those were normal human lungs (NHL), normal rat's lungs (NRL), human lungs with *P. carinii* pneumonia (PcHL) and

rat's lungs with *P. carinii* dominant infection (PcRL).

NHL were obtained from legal autopsies. Only lungs with neither any pathological changes nor any pathogens were used for the experiment. PcHL were obtained in pathological autopsies from the patients who died by apparent *P. carinii* pneumonia in the course of immunosuppressive therapy against their underlying diseases. The lungs with other pathogens like bacteria and fungi, were also excluded from the material.

The rats used in the present study were all Wistar-strain young adults weighing around 200 g. NRL were obtained from healthy rats as soon as they arrived us from an animal dealer, and were checked the absence of *P. carinii* and other pathogens.

PcRL were obtained from *P. carinii* provocated rats by administration of cortisone acetate 25 mg twice a week and 0.05 % solution of tetracycline hydrochloride in drinking water daily for seven to ten weeks. Those lungs were stored in a deep freezer at −70 C until use.

Cysts of *P. carinii* were prepared from human and rat's lungs by cyst purification method (Ikai *et al.*, 1979).

2. Extraction of antigens

Each of the four kinds of lung materials stored at −70 C, was thawed under room temperature at use. About 30 g to 50 g of the lungs were cut into pieces of about 1 cm^3 in size, and were lyophilized. Dried lungs were powdered in a bowel, then about 100 ml of 0.1 % NaCl were added. This turbid fluid was frozen then smashed at room temperature until it thawed. This freeze and smash procedure was repeated ten times. Although the trophic stages of the organism were almost completely broken by this procedure, the cystic stages were usually remained without change. The fluid was then centrifuzed at 10,000 rpm for 30 minutes at 4 C. The supernate was dialyzed against distilled water overnight at 4 C, and was lyophilized. The crude antigens (NHL-extract, PcHL-ext., NRL-ext. and PcRL-ext.) thus obtained were stored in a desiccator.

3. Immunization of rabbits

Nine male, white rabbits weighing 2.5± 0.3 kg were divided into three groups (1, 2 and 3) of three each. Group 1 was immunized with the emulsion of 3 mg NHL-ext. powder in 0.5 ml distilled water and 0.5 ml Freund's complete adjuvant. The emulsion was subcutaneously given, dividing in 0.1–0.2 ml, to the back of the rabbits, once a week, for 10 weeks. Group 2 was immunized with the emulsion of 3 mg PcHL-ext. powder and Freund's complete adjuvant as the same manner as group 1. Group 3 was immunized with the emulsion of 10^6 cysts in 0.5 ml distilled water and 0.5 ml Freund's complete adjuvant by the same way as group 1 and 2.

In all groups, blood samples were collected from the auricular vein one week after the tenth injection. In order to collect antisera as much as possible, further booster injections with the same manner and blood collections were repeated 4 to 5 times with 1 week interval in each procedure. Those sera were all stored at −20 C until use.

4. Combination of NHL-ext. with AH-Sepharose 4B

AH-Sepharose 4B (Pharmacia Fine Chemicals) was activated by the method of Cambiaso *et al.* (1975). Fifteen grams of AH-Sepharose 4B were swollen in 200 to 300 ml of distilled water for 10 to 15 minutes with gentle stir. After removing the excess water and washing with 2 to 3 liters of 0.5 M $NaHCO_3$ through glassfilter, AH-Sepharose 4B was put in a beaker. Then, 140 ml of 0.5 M $NaHCO_3$ and 20 ml of 25 % glutaraldehyde (final concentration 2.5 %) were added to it. This gel was stirred gently for 10 to 15 minutes at room temperature until gel became yellowish. After that, glutaraldehyde was removed through glassfilter, and the activated AH-Sepharose 4B was washed with 2 to 3 liters of 0.5 M $NaHCO_3$.

The activated AH-Sepharose 4B was again put in a beaker, and about 100 ml of 0.5 M $NaHCO_3$ containing 2 g of NHL-ext. was added to it. This mixture was stirred gently at 4 C overnight. After removing the excess

NHL-ext. through glassfilter, NHL-ext.-combined AH-Sepharose 4B was washed with 2 to 3 liters of saline. The product thus obtained was stored in 300 to 500 ml of saline containing with 0.02% NaN_3, at 4C until use.

When the quantity of NHL-ext. is proportionally scant to that of activated AH-Sepharose 4B, it is necessary to block the uncoupling activated AH-Sepharose 4B with 0.2 M monoethanolamine in 0.1 M sodium borate buffer (pH 8.5) for 16 hours at 4C. However, such blocking procedure is unnecessary when the quantity of NHL-ext. is excess, for example 2g or more as in the present medium.

5. Purification of antibodies

In order to purify anti-Pc antibody, crude anti-PcHL-ext. antibody and crude anti-Pc cyst antibody were treated through affinity chromatography which contained NHL-ext.-combined AH-Sepharose 4B. Firstly, γ-globulin was separated from each antiserum of rabbits by $(NH_4)_2SO_4$ precipitation method. Each of final precipitate of γ-globulin (mainly IgG) was resolved in 30 to 50 ml of saline and was dialyzed against saline at 4C overnight. Since these γ-globulins contain both anti-Pc antibody and anti-NHL-ext. antibody, the latter antibody should be removed as the next step.

Each of those γ-globulin solution was treated by repeated passing through NHL-ext.-combined AH-Sepharose 4B gel in a special column which had perista mini-pump SJ-1211 type (Atto Corp.) recycle system at 4C overnight. Then, uncombined γ-globulin was collected from the chromatographic glassfilter tube. NHL-ext.-combined AH-Sepharose 4B coupled with anti-NHL-ext. antibody was washed with 1 to 2 liters of saline on glassfilter, and was put back to the chromatographic column again. After that, anti-NHL-ext. antibody was removed from NHL-ext.-combined AH-Sepharose 4B by adding 5 to 10 ml of 8 M urea. NHL-ext.-combined AH-Sepharose 4B was finally washed with 1 to 2 liters of saline. The gel can be used several times by this regeneration method. The procedure mentioned above was repeated six times or more on one sample to obtain pure antibody. Finally those sera were concentrated to about 50 mg/ml of protein content in saline with minicon B15 system (Amicon Corp.). They were stored at 4C after adding 0.02% NaN_3.

Anti-Pc cyst antibody was purified by the same manner as mentioned above since the cyst suspension still might have small amount of host elements even though cysts were obtained by purification method.

Hereafter, the author abbreviated the purified anti-PcHL-ext. antibody as T antibody since it is considered to be anti-trophozoite antibody, and the purified anti-Pc cyst antibody as C antibody.

6. Techniques for demonstration of antibodies

Double diffusion test (DD test) in agar gel of 0.9% agar in veronal buffer saline (pH 8.6) was performed. Central well of 3 mm diameter was filled with 5 μl of antibody, and peripheral wells, 3 mm diameter and 5 mm apart from the center one, were filled with 5 μl of antigen solutions, each of which contained 0.5 mg of NHL-ext., PcHL-ext., NRL-ext. and PcRL-ext.. The incubation of the plate was carried out firstly at room temperature for 24 hours then at 4C for 2 days both in a moist chamber.

Immunoelectrophoresis (IEP) was performed on 1% agarose in veronal buffer saline (pH 8.6). Wells were filled with 0.05 ml of antigen solution containing 5 mg of NHL-ext., PcHL-ext., NRL-ext. and PcRL-ext.. After electrophoresis was done by 2 mA/cm for 90 minutes in the same buffer system, T antibody was poured into each trough. Precipitin patterns were allowed to develop in a moist chamber at room temperature for 24 hours then at 4C for 2 days.

Indirect fluorescent antibody technique (IFA) was performed between purified antibodies (T and C) and antigens on tissue sections or purified cysts on smear. For IFA test on tissue section, Hamashima and Kyogoku's method (1965) was mainly used. The details are as follows: *P. carinii* infected

lungs of rats provocated the organism by Frenkel's method (1966) for ten weeks, were cut into 2 to 4 mm^3 in size, and were immediately fixed in cold 95% ethyl alcohol at −70 C for one hour then kept at 4 C overnight. Dehydration with absolute ethyl alcohol at 4 C for 3 days changing alcohol every day and dealcoholization with xylene three times at 4 C for 20 minutes each were performed. The lungs were put into paraffin three times at 57 C for 5 minutes each then embedded. The embedded lungs were stored at −20 C until use. The tissue sections of about 5 μ thick were stretched on warm water, transfered onto a glass slide, and dryed at 37 C for 30 minutes then in a desiccator for 15 minutes under room temperature. Deparaffinization with xylene three times 3 minutes for each, and dexylenization with absolute ethyl alcohol two times, 90%, 70% and 45% ethyl alcohol one minute for each were performed. The glass slide was then moved into cold phosphate buffered saline (pH 7.2) and washed by changing PBS four times. One drop of antiserum diluted to 3 to 5 mg/ml of protein content, was put on the section, and it was incubated at 37 C for one hour. A glass slide was washed with cold PBS several times for at least one hour at 4 C. Then one drop of fluorescein isothiocyanate conjugated anti-rabbit IgG (Fujizoki Parmaceutical Co.) which contained 11 mg/ml of IgG and 1.3 of fluorescein per protein ratio, was put on the glass slide and incubated at room temperature for one hour. The glass slide was washed with cold PBS, and embedded in 10% glycerin in PBS.

On the other hand, *P. carinii* cysts obtained by cyst purification method (Ikai *et al.*, 1979) were spread on some glass slides, and dried quickly. Those slides were fixed in acetone at 37 C for 10 minutes, then dried at 37 C for 30 minutes. IFA test was carried out by the same way as described above. Those materials were examined with fluorescent microscope Model BHF (Olympus Co.).

7. Absorption of purified antisera

Ten mg of NHL-ext. and PcHL-ext. was added to each of 0.1 ml of purified antiserum at room temperature for 3 hours followed by at 4 C overnight. Then sera were centrifuged at 2,000 rpm for 15 minutes and supernates were used in DD test and IEP test.

Results

1. Double diffusion test of four kinds of antigens with anti-NHL-ext. antiserum or anti-PcHL-ext. antiserum

Prior to use of purified antiserum, anti-NHL-ext. antiserum or anti-PcHL-ext. antiserum were attempted to react with extracts of NHL (B), PcHL (A, E, F, G), NRL (C) and PcRL (D). Fig. 1 is a pattern resulted between anti-NHL-ext. antiserum (H) and four kinds of extracts, showing multiplicity of precipitin bands in each antigen-antibody system including faint cross-reactivity between human and rat's lung antigenicity. The same finding was obtained when anti-PcHL-ext. antiserum (I) was used. Specific Pc antigen and antibody reaction seems to be hidden under the reaction between antigen and antibody responsible for the lung component (Fig. 2).

2. Double diffusion test of four kinds of antigens with purified T antibody

As mentioned before, T antibody was obtained by affinity chromatography by which the activity of anti-NHL-ext. antibody was completely absorbed out.

As shown in Fig. 3, single band was produced not only between T antibody (J) and PcHL-ext. (A), but also between T antibody and PcRL-ext. (D). And no band was seen between T antibody and NHL-ext. (B) and between T antibody and NRL-ext. (C). Thus, this is evidently resultant of Pc antigen-antibody reaction.

3. Double diffusion test of three more human lung extracts of *P. carinii* pneumonia with purified T antibody

Case 1 corresponds to PcHL-ext. (A) mentioned above. Three other autopsied human lungs (case 2–4, E, F, G) containing *P. carinii* were used for the experiment. As shown in Fig. 4, a clear single band between each of antigen and antibody was seen with

apparent fusion one another.

From the fact that C antibody did not show a precipitin band with any of NHL-ext., PcHL-ext., NRL-ext. and PcRL-ext., the antigen and antibody responsible for it must be derived from the trophozoite (T antigen and T antibody).

4. Immunoelectrophoresis of four kinds of antigens with crude and purified antibody

IEP patterns between anti-NHL-ext. antiserum (in trough) and NHL-ext. (B), PcHL-ext. (A), NRL-ext. (C) and PcRL-ext. (D) (Fig. 5) and those between anti-PcHL-ext. antiserum (in trough) and NHL-ext. (B), PcHL-ext. (A), NRL-ext. (C) and PcRL-ext. (D) (Fig. 6) are considered mostly to be reactions responsible for host tissue antigen and antibody. However, when the purified T antibody (in trough) was used (Fig. 7), a single band was clearly seen between T antibody and PcHL-ext. (A), and between T antibody and PcRL-ext. (D). Neither precipitin band was found between T antibody and NHL-ext. (B), nor between T antibody and NRL-ext. (C). The antigen taking part in this precipitin band showed slight mobility to anodic direction in pH 8.6 veronal buffered saline.

5. Immunoelectrophoresis of three more human lung extracts of *P. carinii* pneumonia with purified T antibody

IEP patterns between T antibody and each of PcHL-extracts from three other clinical cases (E, F, G) showed clear precipitin bands (Fig. 8). These results were well corresponded with those obtained in DD test (Fig. 3 and 4). Furthermore, these DD and IEP reactions were inhibited by absorption with adding PcHL-ext. to T antibody, but not inhibited with NHL-ext.. C antibody did not show any reaction with four kinds of antigens.

Thus, the purified antibody obtained by affinity chromatography in this experiment is considered to be *P. carinii* origin and that mostly from trophozoite.

6. Indirect fluorescent antibody test

As the next step, IFA test was carried out. The reactions of T antibody on a tissue section of *P. carinii* infected rat's lung were shown in Fig. 9 (magnification 200×) and Fig. 10 (400×). Tissue sections stained with toluidine blue O were also shown in Fig. 11 (200×) and Fig. 12 (400×) for comparison. The fluorescein activity was noted not only on trophozoites but also on cysts, which seemed to come from intracystic contents rather than the cyst wall.

The final step is the comparison of antigenicity between T antigen and C antigen using IFA test. As described before (Ikai *et al.*, 1979), the harvest by cyst purification method do not contain the trophozoite at all, and intracystic bodies also could not be found by Giemsa stain probably due to degeneration and lysis during this disruptive procedure. However, the cyst wall was usually remained intact as shown in Fig. 13.

The fact that C antibody showed marked positive fluorescein reactions with purified cysts derived not only from human lungs but also from rat's lungs, (Fig. 14) although T antibody did not react with the same material, suggests that the antigenicity of trophozoite derived antigen is somewhat different from that of cyst wall derived one.

Discussion

In the present study, two different methods were used for preparation of *P. carinii* antigen. One was freezing and thawing method of *P. carinii* infected human lungs, and the other was cyst purification method by passing of millipore filter system (Ikai *et al.*, 1979). It became evident that the *P. carinii* antigen obtained by the former method was trophozoite derived one (T antigen), and that by the later method cyst wall derived antigen (C antigen). By the immunization of rabbits with these two types of antigens, T antibody (anti-trophozoite antibody) and C antibody (anti-cyst wall antibody) were obtained. The present study proposed a new technique for purifying these antibodies by absorption of the antibodies responsible for the host element with NHL-ext.-combined AH-Sepharose 4B.

All of DD test, IEP test and IFA test apparently demonstrated antigen-antibody reaction responsible for *P. carinii*. The fact that T antibody and C antibody did not show any cross-reaction, suggests that antigenicity of cyst wall is entirely different from that of trophozoite. And it is auther's speculation that soluble antigen is only derived from components of the trophozoite, not from the cyst wall.

The extractions of *P. carinii* antigen from autopsied human lungs were attempted by many investigators. Dvořáček *et al.* (1953), Bárta *et al.* (1955) and Bárta (1966, 1969) extracted the antigens with 96% ethyl alcohol. Vivell (1954, 1955), Jirovec (1954), Kučera (1967) and Meuwissen and Leeuwenberg (1972) made the antigens by homogenation or by freezing and thawing method. It is presumed, from the present study that their soluble antigens are trophozoite derived antigen. They tried complement fixation test or intradermal reaction with those antigens.

On the other hand, trials to collect the cysts as purely as possible have also been performed by several workers by different techniques as follows: Norman and Kagan (1972) by sucrose density gradient, Meuwissen *et al.* (1973, 1977) pronase digestion, Lim *et al.* (1973) trypsin digestion, Ikai *et al.* (1979) millipore filter, and Walzer *et al.* (1979) collagenase and hyaluronidase followed by ficoll-hypaque density gradient respectively. They carried out IFA test and titration of antibody with those cysts as antigen. Those antigens are mainly cyst wall antigen.

Histopathological examination of antigenicity of *P. carinii* was first described by Brzosko and Nowoslawski (1965). They examined fluorescent reactions on lung section of *P. carinii* pneumonia with FITC-conjugated antihuman IgG and IgM. PAS staining was also done on the same material. They concluded from the results of those two staining methods that glyco- or mucoproteins of *P. carinii* might be the active antigenic components of the organism.

Attempts to obtain the anti-*P. carinii* antiserum in rabbits have already been performed by Minielly *et al.* (1970), Lim *et al.* (1971, 1973), Kim *et al.* (1972), Kagan and Norman (1976), Pifer *et al.* (1977, 1978), and Walzer *et al.* (1979). Minielly *et al.* (1970) immunized rabbits with an antigen extracted from PcHL in saline, then purified the antibody by absorption with NHL. Their antigen and antibody are thought to be T antigen and T antibody. They examined the reaction between their antibody and *P. carinii* in tissue sections of infected human lungs with IFA test, and found positive results. However, they did neither mention about the result of DD test and IEP test nor about origin of the antigenicity.

Kim *et al.* (1972) immunized rabbits with cysts collected from rat's lungs, and purified the obtained antibody by absorption with normal rat's lungs. Their antibody showed positive fluorescence against cysts and trophozoites collected from rat's lungs, whereas the cysts from human lungs were nagative. So, they stated that antigenicity of *P. carinii* in rat's lungs was different from that in human lungs. Frenkel (1976) separated *Pneumocystis* in man from that in rat, and gave a new specific name, *Pneumocystis jiroveci*, to it. However, the present author failed to demonstrate any immunological difference between *Pneumocystis* derived from man and rat.

Lim *et al.* (1971) produced antiserum in rats which were treated with cortisone acetate for 8 weeks, then kept for another 8 weeks without cortisone to allow the antibody titer to rise. They found positive fluorescent reactions between *P. carinii* in rat's lungs and their antiserum. Because they did not find various morphological pneumocystic forms rather than the cystic form by IFA test, they stated that antigenic differences might exist among various developmental stages. Lim *et al.* (1973) also immunized rabbits with relatively cleaned cysts collected from rat's lungs and human lungs by sucrose density gradient method, and absorbed the obtained antiserum with mouse powder and rat's serum or human serum. They examined antigen-antibody reactions by IFA, DFA

and DD tests. DD test showed a single band between antiserum and sonically disrupted soluble antigen of *P. carinii*. IFA test was also positive between *P. carinii* and the antiserum. Cross reactions of *P. carinii* in rat's and human lungs were found, but higher titer was noted in the homologous system. From those data, they suggested that antigenicity of *P. carinii* from different hosts might not be entirely identical. It is suspected that their antibody cotained T antibody and C antibody. Their data partly corresponded with the present study except no immunological difference between *Pneumocystis* derived from man and rat was found in the later.

Kagan and Norman (1976) immunized rabbits and monkeys with the antigens which were extracted from PcHL, NHL, PcRL and NRL by Vivell's method (Vivell, 1955). The anti-PcHL antiserum and anti-PcRL antiserum were examined with each of the antigens mentioned above after absorbing with NHL and NRL. Since they did not find any specific band to *P. carinii* in that system, they presumed that *Pneumocystis* cysts *per se* were poor antigens and that component present in infected lungs were closely host related. Their results are quite different from many other investigators' including the present author. The present study used a different method in preparing antigens and absorbing antisera from them, and demonstrated two types of antigens (T antigen and C antigen), hence two types of antigen-antibody reactions. The antigenicity of *P. carinii* proved not to be poor as the results.

Recently, Pifer *et al.* (1977) and Latorre *et al.* (1977) reported on successful cultivation of *P. carinii in vitro*. Pifer *et al.* (1977) immunized rabbits with the cultivated *P. carinii*. They measured antibody titer by IFA test, and found positive fluorescence in as much as 1 : 1,024 to 1 : 2,048 dilutions of the antiserum. They utilized that antiserum in diagnosis of *P. carinii* pneumonia in man. However, they did not mention about the difference of the antigenicity between cyst wall and trophozoite.

Walzer *et al.* (1979) reported a separation method of *P. carinii* from the lung tissue by digestion with collagenase and hyaluronidase followed by ficoll-hypaque density gradient. They immunized rabbits with the cysts or trophozoites thus collected from the rat's lungs, and examined antigen-antibody reaction by IFA test. Although they found positive fluorescence between *P. carinii* and their antiserum, they did not examined the cross reaction between *P. carinii* from rat's lungs and from human lungs. They also did not describe the difference of antigenicity between cyst wall and trophozoite.

Nowadays, cultivation of *P. carinii* is still difficult, and so, it is very important to establish another method to obtain pure antigen and pure antibody of *P. carinii*.

A new absorption technique with NHL-ext.-combined AH-Sepharose 4B proposed here is very useful to absorb and remove the anti-NHL-ext. antibody without contamination of NHL-ext..

Summary

In the present status of difficulty to obtain pure antigen of *P. carinii* the purification of antibody was performed by absorption with affinity chromatography.

Two kinds of crude antigens were used. One was trophozoite derived soluble antigen which was extracted by freezing and thawing the *P. carinii* infected human lungs. The other was purified cyst *per se* collected from human lungs by cyst purification method.

Two kinds of antisera obtained by immunization of rabbits with the crude antigens, were thoroughly treated by affinity chromatography to remove antibodies responsible for the host elements. Thus, the purified T antibody (trophozoite derived) and C antibody (cyst wall derived) were produced.

Double diffusion test and immunoelectrophoresis confirmed the purity of T antibody by showing a clear single precipitin band between this antibody and *P. carinii* infected human and rat's lung extracts, and also by not showing any band between this antibody

and normal human and normal rat's lung extracts. C antibody did not show any precipitin band with *P. carinii* infected human and rat's lung extracts.

In indirect fluorescent antibody test, T antibody showed positive reaction to *P. carinii* on tissue section of infected rat's lung, probably with trophozoites and intracystic elements concerned, whereas negative on cyst smear preparation. However, the smeared cysts showed marked positive fluorescein when C antibody was used.

From the facts mentioned above, it can be said that the antigenicity of trophozoite is different from that of cyst wall. In addition, the antigenicity either of T antigen or C antigen, was not different between *P. carinii* of human origin and rat's origin.

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Pneumocystis carinii: 抗 trophozoite ならびに抗 cyst wall 抗体の産生

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Pneumocystis carinii の免疫学的研究は今迄多くの研究者により試みられてきた．しかし純培養が困難なため純粋な抗体の作成はまだ成功していない．

今回 affinity chromatography を用いて *P. carinii* に対する純粋な抗体を作成するとともに，その抗原性についても検討した．用いた抗原は *P. carinii* 肺炎患者肺抽出抗原 (PcHL-ext.)，正常人肺抽出抗原 (NHL-ext.) と *P. carinii* シスト抗原である．

抽出抗原の作成法は，まず *P. carinii* 肺炎で死亡した患者肺および疾病以外の原因で死亡し，肺には病理変化や感染のない正常肺を凍結乾燥し，乳鉢で粉状にして 0.1 % NaCl を加えた後 10 回凍結，打砕，融解を繰り返した．10,000 rpm で 30 分遠沈し透析後凍結乾燥した．この方法では，*P. carinii* の trophozoite は破壊されるが，シスト数は減少せずシストの破壊はみられない．一方，*P. carinii* のシストは，シスト純化法 (猪飼ら，1979) で *P. carinii* 肺炎患者肺より集めた．

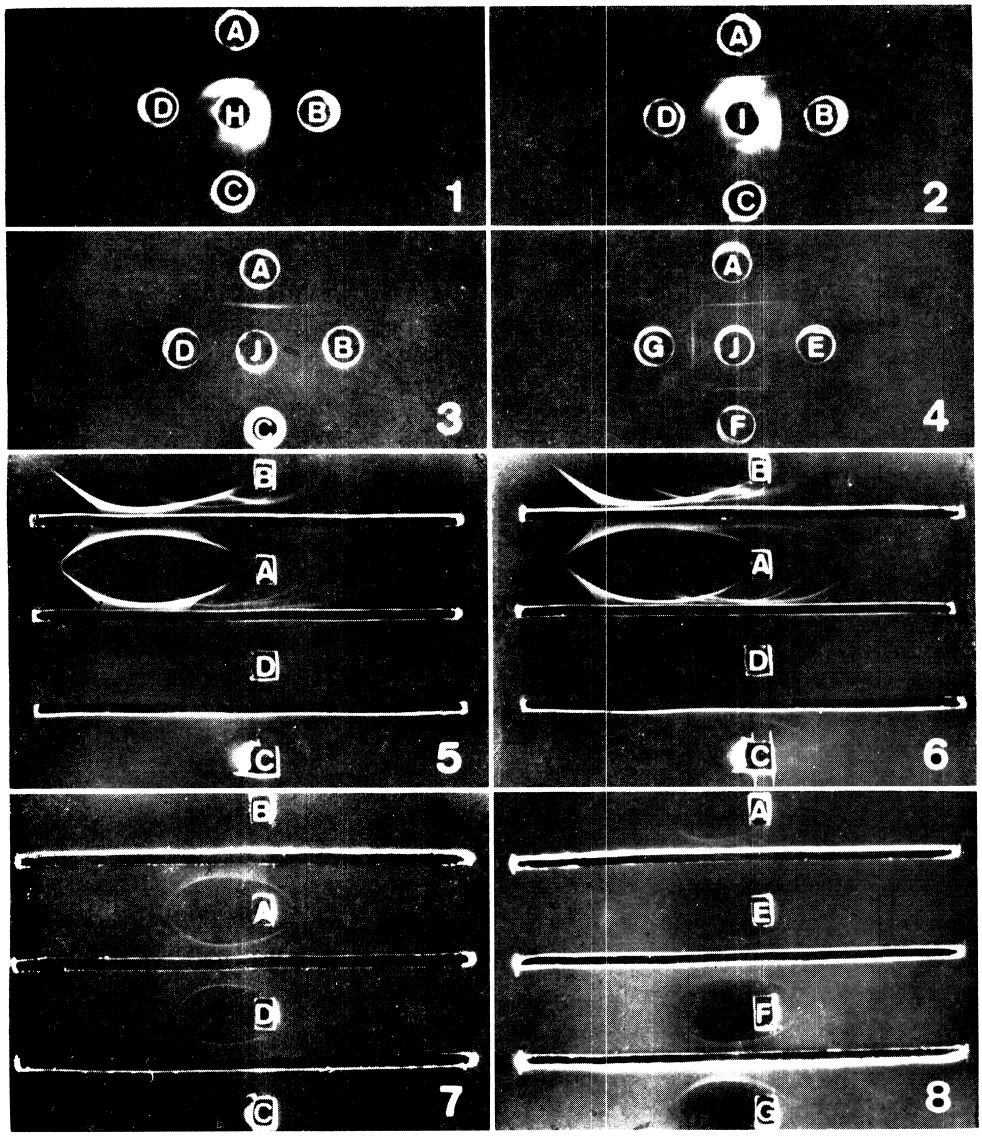
免疫法は抽出抗原ではそれぞれ 3 mg ずつ，シストでは 10^6 個を Freund's complete adjuvant とともに兎に毎週皮下注射した．作成した抗血清のうち抗 PcHL-extract 血清と抗 *P. carinii* シスト血清は次に述べる方法で作成した affinity chromatography で繰り返し吸収操作を行い純化した．

Affinity chromatography には Sepharose 4B を用いた。NHL-ext. と Sepharose 4B を glutaraldehyde 法で結合させ，これに perista mini-pump を用いて抗血清を繰り返し循環して反応させ抗血清中の抗 NHL-ext. 抗体を吸収除去した．

こうして得られた純粋な抗血清を二重拡散法，電気泳動法，間接螢光抗体法で検討した．純化する前の抗 PcHL-ext. 血清では抗 NHL-ext. 血清と同様 Figs. 1, 2, 5, 6 に示すように NHL-ext. と PcHL-ext. との間に多数の沈降線を認め，またラット肺より抽出した抗原 (PcRL-ext. および NRL-ext.) との間でも沈降線が認められ，この中から *P. carinii* に対する特異沈降線を指摘することはできなかつた．一方吸収し純化した血清では Figs. 3, 7 に示すように PcHL-ext. と PcRL-ext. との間に 1 本の沈降線を認め，これらは同一の抗原抗体反応によるものと考えられた．また他の 3 例の Pc 肺炎患者肺抽出抗原とでも Figs. 4, 8 に示す如く同様の沈降線を認め，*P. carinii* の存在しない NHL-ext. や NRL-ext. との間には反応を認めなかつた．一方抗シスト血清 (以後 C 抗体と略) ではすべての抗原に対し沈降線は認めなかつた．

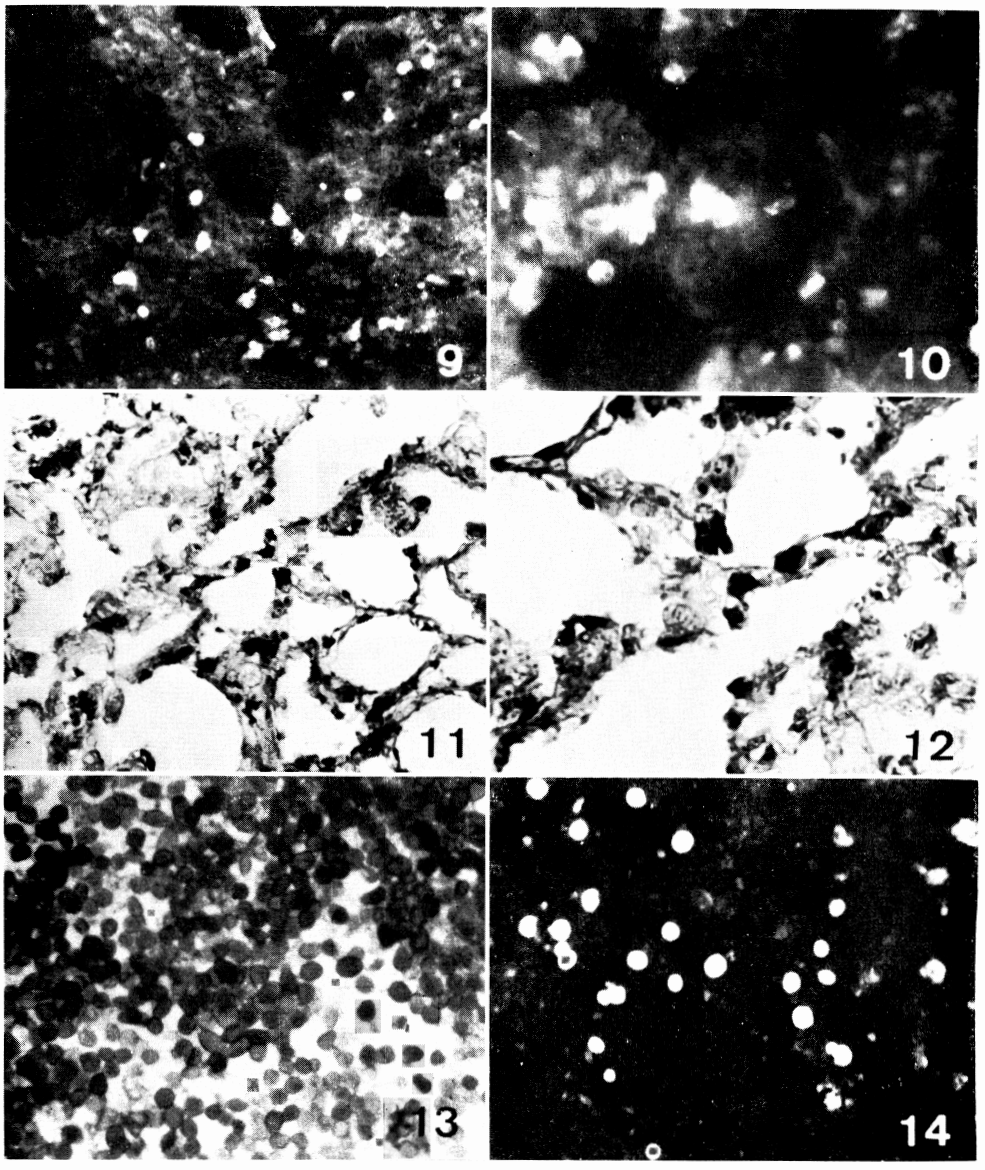
次に *P. carinii* 感染ラット肺の組織切片標本とシスト純化法で集めたシスト塗抹標本を抗原として用い間接螢光抗体法を行つた．純化した抗 PcHL-ext. 抗体 (以後 T 抗体と略) では Figs. 9, 10 で示すように組織切片標本で螢光に染つた *P. carinii* を認めた．シスト塗抹標本ではラットより集めたシストでも，人より集めたシストでも T 抗体では陰性であり，C 抗体では Fig. 14 で示すように陽性であつた．

以上の結果より T 抗体は *P. carinii* の抗 trophozoite 抗体であり，C 抗体は抗 cyst wall 抗体であると考えられこれら二者の抗原性は明らかに異つていた．しかし，人の *P. carinii* とラットの *P. carinii* とは免疫学的に差異を認めなかつた．



Explanation of Figures

Figs. 1–4 Double diffusion test.
Figs. 5–8 Immunoelectrophoresis (cathode right and anode left).
Figs. 1–8 A, E, F and G : PcHL-extracts (case 1, 2, 3 and 4)
B : NHL-extract C : NRL-extract D : PcRL-extract H : Anti-NHL-extract antiserum
I : Anti-PcHL-extract antiserum J : T antibody
Fig. 5 Anti-NHL-extract antiserum in three troughs.
Fig. 6 Anti-PcHL-extract antiserum in three troughs.
Figs. 7–8 T antibody in three troughs.



Explanation of Figures

Fig. 9 Positive fluorescent reaction of *P. carinii* with T antibody in tissue section of rat's lung. (×200)

Fig. 10 High magnification of the same specimen shown in Fig. 9. (×400)

Fig. 11 *P. carinii* stained by toluidine blue O in tissue section of the same rat's lung as Fig. 9. (×200)

Fig. 12 High magnification of Fig. 11. (×400)

Fig. 13 Purified *P. carinii* cysts stained by toluidine blue O. (×400)

Fig. 14 Positive fluorescent reaction of *P. carinii* cysts with C antibody. The purified cyst suspension was diluted and spread on a glass slide at use. (×400)