Detection of *Pneumocystis carinii* by *in situ* hybridization in the lungs of immunosuppressed rats

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Abstract: *In situ* hybridization was performed to detect rat *Pneumocystis carinii* in the lung sections. Rats were immunosuppressed by weekly subcutaneous injection of 10 mg/kg methylprednisolone. On the 6th, 8th and 9th week of immunosuppression, the lungs were removed and fixed in 10% neutral formalin. A 22 base oligonucleotide probe complementary to *P. carinii* 5S ribosomal RNA was commercially synhesized and its 3' terminal was labeled with biotin. *In situ* hybridization was performed utilizing manual capillary action technology on the Microprobe system. *P. carinii* were detected along the luminal surface of alveolar pneumocytes, in exudate of alveolar cavities, and also in secretory material of bronchioles. In the 6th week group, positive reaction was observed focally in the peripheral region of the lung sections, but the reaction was observed diffusely in the 8th or 9th week groups. In comparison with Grocott's methenamine silver stain, *in situ* hybridization technique can detect the organism rapidly, and can detect trophic forms very well. Furthermore, no nonspecific reaction with other pathogenic fungi and protozoa was recognized. Therefore, *in situ* hybridization can be a good technique to detect *P. carinii* in the lungs of infected rats.

Key words: *Pneumocystis carinii, in situ* hybridization, oligonucleotide probe, rat, lung

INTRODUCTION

Pneumocystis carinii (*P. carinii*) is an important cause of pneumonia in immunosuppressed state. Accurate and rapid diagnosis is essentially necessary in this pneumonia. For histopathologic diagnosis, Grocott's methenamine silver (GMS) stain has been largely used to detect its cystic forms. However, this GMS stain, which revealed black colour in polysaccharide moieties of most fungi (Drury and Wallington, 1980), is not specific for *P. carinii.* Recently, monoclonal antibodies

with high specificity for *P. carinii* have been developed and used with favorable results (Wazir *et al.,* 1994).

In situ hybridization is a useful technique for detection of various organisms including virus, bacteria, fungi and protozoa in routinely processed tissue sections (Brigati *et al.,* 1983; Bashir *et al.,* 1994; Montone, 1994). This method allows specific nucleic or ribonucleic acid sequence to be detected in morphologically preserved cells or tissues. Nowadays, non-radioactive nucleic acid probes, sensitive detection system, and capillary action technology have permitted the development of effective, sensitive and rapid *in situ* hybridization assays within one hour (Park *et al.,* 1991).

Several types of nucleic acid probes are

available for *in situ* hybridization. Especially, oligonucleotide probes which are short segment of DNA with known nucleotide sequences have recently been introduced. Until now, some oligonucleotide probes for *P. carinii* were designed and revealed high reactivity and specificity in the diagnosis of human *P. carinii* (Hayashi *et al.*, 1990; Montone, 1994).

The aim of the present study is to detect *P. carinii* rapidly in lung sections of immunosuppressed rats using one hour *in situ* hybridization technique, and to evaluate the applicability of a known oligonucleotide probe (Montone, 1994) to domestic *P. carinii* in Korea.

MATERIALS AND METHODS

1. Oligonucleotide probe synthesis

A probe of 22 base oligonucleotides (Montone, 1994) complementary to nucleic acid 1 to 22 of *P. carinii* 5S ribosomal RNA was commercially synthesized and 3′ terminally labeled with biotin. By GenBank analysis (BLAST search), the probe sequence was approved to have less than 78% homology to a fungus, *Aspergillus parasiticum* and 61% to rat (Altschul *et al.*, 1990). This probe was received lyophilized, reconstituted to 1 $\mu g/\mu l$ in Tris-EDTA buffer and stored in 10 μl aliquots at -20°C before use in hybridization reaction.

2. Tissue preparation

Adult albino rats of Sprague-Dawley strain were immunosuppressed by a weekly subcutaneous injection of 10 mg/kg methylprednisolone (Depo-Medrol, Upjohn Korea Ltd.). Prophylactically, tetracycline 1 mg/ml in drinking water was given to prevent a bacterial infection. On the 6th, 8th and 9th week of immunosuppression, the lungs were removed. Before fixation in 10% neutral buffered formalin, impression smears and Diff-Quik stains were performed to evaluate an infection density. Five micrometer sections were cut from the paraffin blocks, and the sections were placed on positively charged ProbeOn Plus glass slides (Fisher scientific). To observe the cystic forms of *P. carinii*, Grocott's methenamine silver stain was also performed.

3. *In situ* hybridization

All *in situ* hybridizations were carried out using manual capillary action technology on the Microprobe staining system (Fisher Scientific) with the modified method of Park *et al.* (1991). The glass slides were placed in the Microprobe slide holder in order to make a 150 μm gap and all of the subsequent reagents were placed on and drained from the slides by capillary action. All of the procedures were summarized in Table 1. First, Lung sections were deparaffinized using Auto Dewaxer (Research Genetics). The sections were immersed in Redusol (Biomeda) or Endo/blocker (Biomeda) to block the endogenous alkaline phosphatase and peroxidase activities respectively, and rinsed in absolute ethanol. The pepsin (2 mg/ml) predigestion was performed to increase the tissue penetration of the probe. After pepsin digestion, the tissue sections were treated with absolute formamide (AMRESCO) for chemical denaturation. The oligonucleotide probe was diluted to 1 $\mu g/ml$ with Brigati probe diluent (Research Genetics). The probe solution was applied to the slides, and the tissues were heated at 110°C to denature the secondary rRNA structures. The hybridization of tissue and probe was performed by exposing the slides to an oven whose temperature was gradually decreasing from 110°C to 85°C. After hybridization, the tissue slides were washed in 2 × SSC (post-hybridization washing buffer) containing nonionic detergent. In the present study, two detection systems were used. First, the biotinylated hybrids were detected with a streptavidin alkaline phosphatase, and then, preincubated in an alkaline phosphatase enhancer not only to wash out unbound phosphatase but also to increase the activity of the enzyme before the chromogen reaction. Second, the hybrids were also detected with streptavidin horseradish peroxidase. Therefore, two kinds of chromogens were used; a mixture of naphthol AS-MX phosphate and fast red TR salt (NASFRTR) for phosphatase, and stable diaminobenzidine (DAB) for peroxidase. The NASFRTR chromogen produced a bright red colour, and DAB showed brown colour. The slides were washed with

Table 1. Procedure for *in situ* hybridization.

Reagent	Time	Temperature
Dewaxing agent	1 Min (2 times)	110°C
Dewaxing agent	1 Min (2 times)	RT[a]
Absolute alcohol	6 Washes	RT
Blocking agent	2 Min	RT
Pepsin	3 Min	110°C
Formamide	2 Min	110°C
Probe	1 Min	110°C
Cooling		RT
Probe	2 Min	105°C
Cooling		RT
Probe	0.5 Min	105°C
Cooling	1 Min	RT
Probe	0.5 Min	95°C
Cooling	2 Min	RT
Probe	0.5 Min	85°C
Cooling	3 Min	RT
Probe	0.5 Min	85°C
Cooling	4 Min	RT
2 × SSC	3 Washes	RT
Detection system	6 Min	45°C
Enhancer	1 Wash	RT
Chromogen	10 Min	45°C
Chromogen	10 Min	45°C
Distilled water	1 Wash	RT
Hematoxylin	0.5 Min	RT
Distilled water	2 Washes	RT

[a]Room temperature

distilled water, counterstained with hematoxylin and covered with Universal mount (Research genetics) to avoid decolorization of NASFRTR.

RESULTS

In situ hybridization with biotinylated oligonucleotide probe showed distinctive positive reactions in all individuals of the pneumocystosis among rats immunosuppressed for 6, 8 and 9 weeks (Fig 1, 4, 5, 6) but not in the normal lungs without immunosuppression (Fig. 6A). The positive signals appeared as a diffuse granular pattern within alveolar exudate (Fig. 4, 6). In most cases, positive signals were largely noted in trophic forms and slightly inner bodies of cystic forms of *P. carinii*, but not in the thick wall of cystic forms. *In situ* hybridization with the same probe to fungi such as *Aspergillus* species in the human lung and brain (Fig. 7), and *Candida* species in the human esophagus (Fig. 8), and to protozoa such as cysts of *Toxoplasma gondii* in the mouse brain (Fig. 9) and trophozoites of *Entamoeba histolytica* in the intestinal mucosa (Fig. 10) were all negative. *In situ* hybridization for rat *P. carinii* using two other oligonucleotide probes complementary to *Aspergillus* and *Toxoplasma* ribosomal RNA were also negative (data not shown).

A typical field of infected lung tissue stained with hematoxylin and eosin is shown in Fig. 2. There were alveolar interstitial thickenings, and the alveolar lumens were filled with the eosinophilic materials. Diff-Quik stain on the impression smears of the lungs showed a lot of cystic and trophic forms of *P. carinii* in rats immunosuppressed for 6, 8 and 9 weeks (data not shown). GMS stain showed numerous cysts along the alveolar wall and within alveolar exudate (Fig. 3).

In the 6th week of immunosuppression, there were focal involvements of the lung with *P. carinii* mainly in the peripheral region (Fig. 1). *P. carinii* were detected along the alveolar septa. Although peripheral region showed an alveolar interstitial thickening, collapse of alveoli and cellular infiltrates, most alveoli conserved their architecture, and intralveolar frothy material was not observed (Fig. 1, 6B). In the 8th week of immunosuppression, *in situ* hybridization revealed most alveoli were involved with *P. carinii* (Fig. 4, 5, 6C). The thickening of alveolar septa and alveolar exudate were observed in more than half of alveolar area. Positive signals were detected with a linear or aggregated pattern along the luminal surface of alveolar pneumocytes (Fig. 5) and with a granular pattern in alveolar exudate (Fig. 4). In the 9th week, most alveoli were filled with exudate. *P. carinii* were found in all alveolar lumen where the intralveolar frothy material was accumulated (Fig. 1B, 6D). As the infection progressed, positive signals gradually increased within the secretory material of bronchiole (Fig. 6).

DISCUSSION

For histopathological detection of *P. carinii*, Grocott's or Gomori's methenamine silver
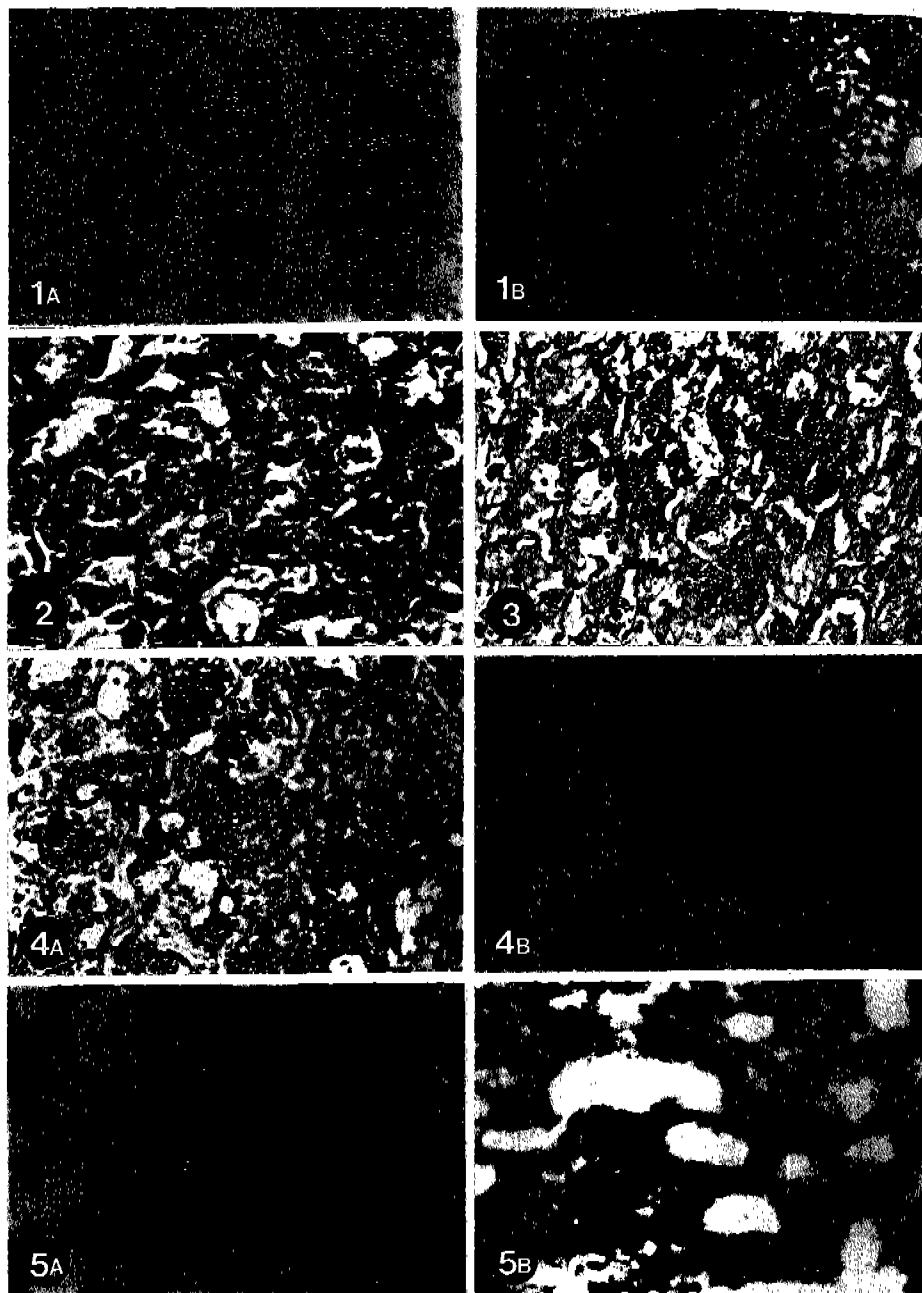
Fig. 1. *In situ* hybridization using red chromogen, a mixture of naphthol AS-MX phosphate and fast red TR salt (NASFRTR). **A.** Focal positive reaction, 6 week group (× 40); **B.** Diffuse positive reaction, 9 week group (× 20). **Fig. 2.** Hematoxylin and eosin stain. Typical microscopic appearance of *P. carinii* pneumonia showing alveolar interstitial thickening and frothy exudate in the alveolar lumens, 8 week group (× 200). **Fig. 3.** Grocott's methenamine silver stain. Numerous cysts are seen apparently, but trophic forms are not stained, 8 week group (× 200). **Fig. 4.** *In situ* hybridization. Granular positive reaction within alveolar exudate, 8 week group. **A.** Red chromogen, NASFRTR; **B.** Brown chromogen, diaminobenzidine (× 200). **Fig. 5.** *In situ* hybridization. Linear positive reaction along the luminal surface of alveoli, 8 week group. **A.** Red chromogen, NASFRTR; **B.** Brown chromogen, diaminobenzidine (× 200).
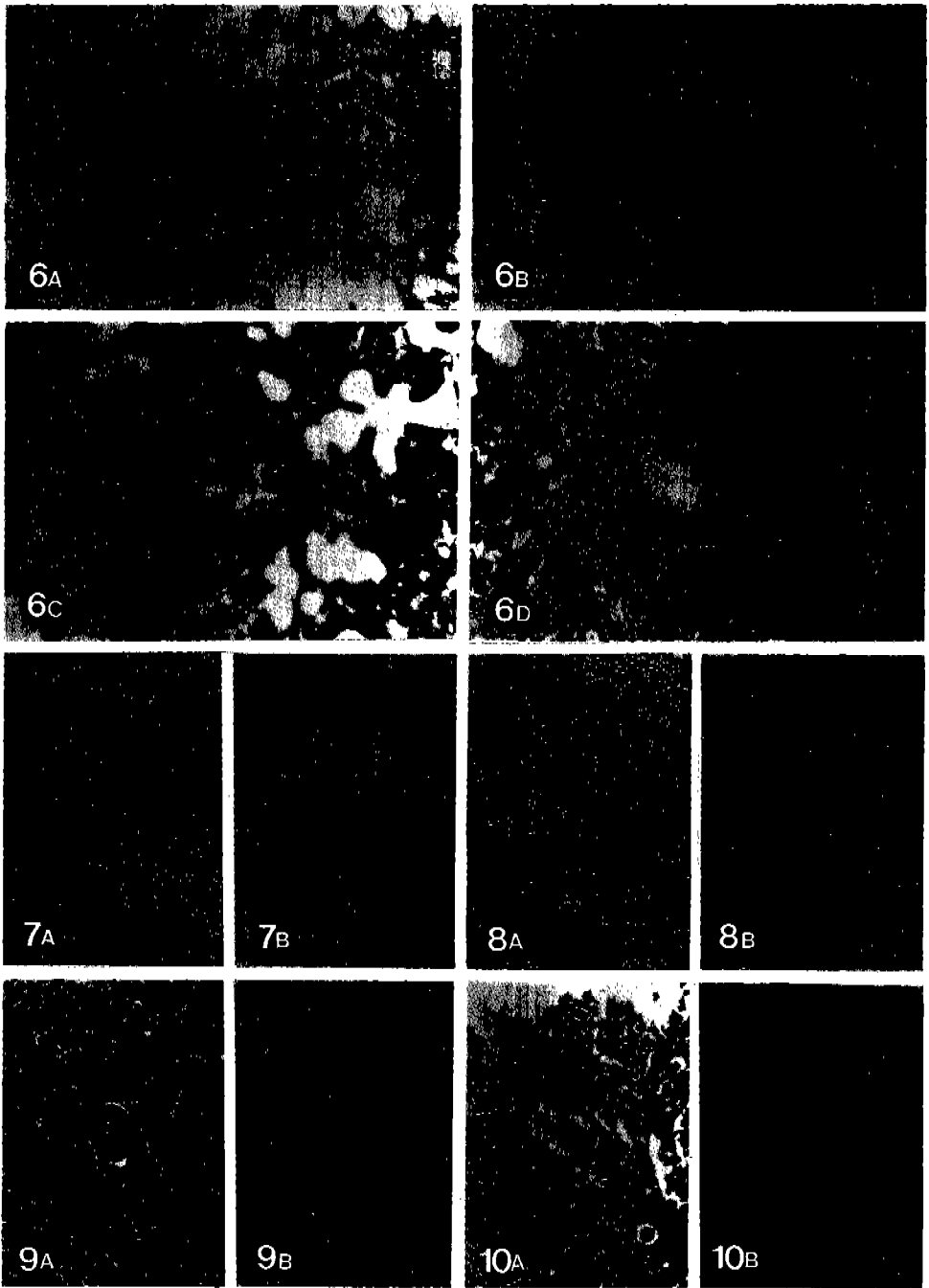
Fig. 6. *In situ* hybridization. Positive signals gradually increased within the secretory material of bronchiole and within alveolar exudate according to the lapse of immunosuppression time. **A.** Control group; **B.** 6 week group; **C.** 8 week group; **D.** 9 week group (× 100). **Fig. 7.** Aspergillosis in human lung. **A.** PAS stain; **B.** *In situ* hybridization with *P. carinii* oligoprobe, negative (× 100). **Fig. 8.** Candidiasis in human esophagus. **A.** PAS stain; **B.** *In situ* hybridization with *P. carinii* oligoprobe, negative (× 200). **Fig 9.** Toxoplasmosis in mouse brain. **A.** Hematoxylin and eosin stain; **B.** *In situ* hybridization with *P. carinii* oligoprobe, negative (× 200). **Fig. 10.** Amebiasis in human colon. **A.** Hematoxylin and eosin stain (× 200); **B.** *In situ* hybridization with *P. carinii* oligoprobe, negative (× 400).

stains have been widely used. It is well known that these methods can stain only the cyst wall of *P. carinii* as black colour, and are time consuming (Drury and Wallington, 1980). Toluidine blue O and cresyl echt violet stains are other simple, rapid and easy cell-wall staining methods. The disadvantage of these staining methods is that they stain only cystic forms but not trophic forms nor intracystic bodies. Occasionally the stained round cysts of *P. carinii* are confused with fungi such as *Histoplasma, Candida* and *Cryptococcus* which show the same staining characteristics. Giemsa or Diff-Quik stain can detect both trophic and cystic forms of *P. carinii*. These methods are very rapid, and can be applied to the lung imprint smear, the bronchoalveolar lavage fluid (BAL) and sputum. However, Giemsa or Diff-Quik stain are not suitable for the tissue sections, because they also stain host cells or many other microorganisms simultaneously. Recently, an immunohistochemical technique using with monoclonal antibody has been applied. This method can detect trophic forms as well as cystic forms in several specimens (Kovacs *et al.*, 1989; Wazir *et al.*, 1994). It was revealed that a significant antigenic difference exists among different host isolates of *P. carinii.* (Gigliotti *et al.*, 1986; Kovacs *et al.*, 1989). Therefore, the nonreactive strain due to antigenic variation remains unstained.

Several molecular biological techniques have been also applied for detection of *P. carinii.* including PCR (Leibovitz, 1995; Lu, 1995), DNA hybridization with a radioactively labeled nick translated probe (Tanabe *et al.*, 1988), and *in situ* hybridization with a nick translated probe (Wakefield *et al.*, 1988) or a biotinylated oligonucleotide probe in human lung tissue (Hayashi *et al.*, 1990; Montone, 1994). *In situ* hybridization offers the advantage of preserving tissue morphology and being sensitive enough to permit detection of even a small number of organisms. *In situ* hybridization technique was originally developed by Pardue and Gall (1969), and John *et al.* (1969). At this time, radioisotopes were the only available labels. Radioisotopes have some problems such as safety measure and extensive time required for autoradiography. Therefore in spite of the high sensitivity and wide applicability, this technique has been limitedly used. Recently, the general application of *in situ* hybridization was possible due to not only the modification of nucleic acid probes with nonradioactive labels such as biotin or digoxigenin (Langer *et al.*, 1981; Kesseler, 1991), but also introduction of capillary action technology (Brigati *et al.*, 1988). In this study, authors used these methods to reduce the consumption and waste of high-priced probes and reagents, and to shorten the reaction time from deparaffinization to mounting. Therefore, every *in situ* hybridization assay could be performed rapidly within less than one hour.

All prokaryotic and eukaryotic cells contain ribosomal RNA for protein synthesis. Because of its abundance in gene copy numbers, rRNA is an optimal target for *in situ* hybridization to detect the organisms. Therefore, DNA oligonucleotide probes complementary to specific rRNA sequence have been largely used for diagnosis of various fungal and protozoal disease including aspergillosis, toxoplasmosis and pneumocystosis (Montone, 1994; Montone and Litzky, 1995). In the present study, we used also an oligonucleotide probe complementary to *P. carinii* 5S ribosomal RNA. This probe was already proved to have high sensitivity in diagnosis of human *P. carinii* pneumonia (Montone, 1994). There was no difference in sensitivity when this probe was applied to detect rat *P. carinii* in the present study. The positive signal appeared as an aggregation of small cytoplasmic granules. This means that the signal was consistent with the distribution of ribosomes in cytoplasm of *P. carinii*. This finding was different from an immunohistochemistry using monoclonal antibody which appeared ring-like pattern according to cyst wall (Kovacs *et al.*, 1989; Hayashi *et al.*, 1990).

In the 6th week group, *in situ* hybridization revealed the positive signal at less than half of the alveolar area, but after 8 week of immunosuppression, most alveoli were involved. According to intensity of *P. carinii* infection described by Hong *et al.* (1992, 1994), it was moderate in the 6th week group, and severe in the 8th and 9th week groups. As the

immunosuppression period increased, an intensity of positive signals became strong gradually in secretory materials of the bronchioles as well as the alveolar exudate. This means that *P. carinii* discharged through mucus secrete increased.

The major disadvantage of *in situ* hybridization in diagnostic field is that the oligonucleotide probe is limitedly applied to lung impression smear, BAL fluid and sputum. The reason is that hybridization between this oligoprobe and rRNA was impossible, because the intact wall of *P. carinii* cysts and trophic forms is impermeable to the probe. Therefore, for the clinical usage of *in situ* hybridization, a further study is necessary to make the probe penetrate into the intact wall of *P. carinii*.

In conclusion, *P. carinii* was successfully detected in lung sections of the immuno-suppressed rats by *in situ* hybridization technique, and the probe designed by Montone was confirmed to be very useful in the diagnosis of Korean *P. carinii*. In comparison with GMS stain, *in situ* hybridization technique can detect the organism rapidly and can detect trophic forms very well, and there is no nonspecific reaction with known pathogenic fungi and protozoa. Therefore, *in situ* hybridization can be usefully applicable to diagnose the experimental *P. carinii* pneumonia in the routinely processed tissue specimens.

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=초록=

면역억제 흰쥐에서 조직내교잡법을 이용한 폐포자충의 검출

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흰쥐의 폐조직 절편에서 실험적으로 유발시킨 폐포자충을 검출하고자 조직내교잡법(*in situ* hybridization)을 수행하였다. 흰쥐는 methylprednisolone(데포-메드롤)을 10 mg/kg의 양으로 주 1회 피하주사하여 면역억제시켰고, 6, 8 및 9주에 희생시켜 폐를 적출한 후 포르말린에 고정하였다. 폐포자충의 5S rRNA에 상보적인 22 base 길이의 oligonucleotide probe를 주문제작(한국생공)하였고 3′ 부위에 biotin을 부착하였다. 조직내교잡법은 Microprobe staining system과 manual capillary action법을 이용하여 1시간 이내에 수행하였다. 폐포자충은 주로 폐포세포의 내강면, 폐포내 삼출물 및 세기관지의 분비물 등에서 검출되었다. 6주 면역억제시 양성반응은 주로 폐단면의 가장자리 부위에서 부분적으로 관찰되었으나, 8 내지 9주에서는 전체적으로 관찰되었다. Grocott의 methenamine silver 염색법에 비하여 조직내 교잡법은 폐포자충을 신속하게 검출할 수 있고, 영양형의 검출에는 특히 유리하며, 다른 알려진 병원성 진균 및 원충류와의 교차반응이 없어, 실험적으로 유발시킨 폐포자충에 의한 폐렴의 진단에도 유용하게 사용될 수 있을 것으로 생각된다.