Pneumocystis carinii Pneumonia Monitored by *P. carinii* Shedding in Sputum by the Polymerase Chain Reaction

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This study investigated the efficacy of applying the polymerase chain reaction (PCR) technique to the monitoring of therapy to *P. carinii* pneumonia (PCP). The PCR method was sensitive enough to determine *P. carinii* shedding in sputum samples which was not detectable by Diff-Quik (International Reagent Corp., Kobe, Japan) stain. By PCR, *P. carinii* disappeared in sputa on days 8 to 15 after treatment. Clinical improvements correlated with the loss of detectable *P. carinii*. This study represents a potential usefulness of PCR to monitor *P. carinii* shedding in sputum during treatment.
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Key words: AIDS, renal transplantation, therapeutic marker

Introduction

Pneumocystis carinii pneumonia (PCP) is one of the most prevalent opportunistic infections and a frequent cause of death in immunodeficient or immunosuppressed patients, such as the acquired immunodeficiency syndrome (AIDS) or organ transplant patients. More than 60% of AIDS patients suffer from PCP at some time in the course of the disease (1). It is estimated that approximately 52,000 AIDS patients in the United States have developed PCP during 1981 through 1988. The current data predict that approximately 150,000 cases of PCP will occur among AIDS patients over the next 3 years in the United States (2).

Accordingly, clinical studies on therapy and prophylaxis for PCP have been explored in recent years. It has been shown that anti-*P. carinii* drugs such as trimethoprim-sulfamethoxazole, pentamidine isethionate and dapsone are effective in more than 60% of patients (3), and that an adjunctive therapy with corticosteroids is also effective in some cases (4). Furthermore, administration routes and dosages have been well investigated (5, 6). On the other hand, sensitive diagnostic methods for PCP have not been developed until recently. Monoclonal antibodies have been used to detect *P. carinii* organisms in clinical specimens by several groups (7, 8). However, monoclonal antibodies as well as the classic cytological methods are not always useful to detect *P. carinii* accurately in clinical samples. Therefore, a sensitive and accurate monitoring of *P. carinii*

during the anti-PCP therapy has not been possible because of the lack of sensitive and reliable markers for the morbidity of this disease. Accordingly, AIDS patients with PCP are now usually treated for 3 weeks and renal transplant patients are treated for 2 weeks (3). These empirical regimens are based on retrospective studies and do not consider the severity of each individual's pneumonia. Moreover, several severe side effects such as drug allergy, myelosuppression and/or nephrotoxicity are sometimes caused by anti-*P. carinii* drugs, and these side effects become more prevalent with longer treatments. Therefore, it is urgent to establish a sensitive and reliable laboratory marker of PCP and to determine an appropriate time period for the anti-PCP therapy for individual cases by monitoring the marker as well as the clinical findings.

We, as well as another group, have recently developed sensitive methods for the diagnosis of *P. carinii* infection by amplifying ribosomal DNA (rDNA) sequences by the polymerase chain reaction (PCR) (9, 10). In this study, we examined *P. carinii* shedding in sputum by using the PCR technique, and tested its usefulness, compared to the cytological method, as a therapeutic marker during treatment.

Materials and Methods

Preparation of sputum samples

After obtaining informed consent, expectorated sputa were collected repeatedly at various points during the anti-PCP

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therapy from the patients described in the case reports. Induced sputa were obtained when expectoration was difficult. The specimens were treated with 33 mM dithiothreitol at 37°C for 10 minutes (11). The mixtures were then washed twice with phosphate-buffered saline, and the pellets were further processed for PCR and the cytological test.

Polymerase chain reaction

The processed sputum was suspended in a solution containing 0.25% Nonidet P-40 (Iwai Kagaku Co. Ltd, Tokyo, Japan), 0.25% Tween-20 (Wako Pure Chemical Industries Ltd, Tokyo, Japan), and 200 mg/ml proteinase K (Boehringer Mannheim GmbH, Germany). After incubation at 60°C for 6 hours and then boiling for 10 minutes, the suspension was subjected directly to PCR.

The 5S rDNA sequence of *P. carinii* was amplified by the PCR method as described in a previous paper, using a pair of primers, '5S sense' (5'-AGTTACGGCCATACCTCAGA-3') and '5S antisense' (5'-AAAGCTACAGCACGTCGTAT-3'), which generates a 120-bp product (9). These primers hybridize to sequences within the termini of 5S rDNA that are not conserved among related organisms thus allowing for highly sensitive and specific detection of *P. carinii* (9). Amplification was done by 40 cycles with denaturation at 94°C for 1.5

minutes, annealing at 55°C for 2.5 minutes and extension at 72°C for 1.9 min. The products were subjected to a 12% polyacrylamide gel electrophoresis and stained with ethidium bromide.

Cytological test

Specimens were stained with Diff-Quik (International Reagent Corp., Kobe, Japan) according to the manufacturer's instructions. A specimen was scored positive when a cluster of typical organisms (cysts or trophozoites) were found.

Case Reports

Patient 1

A 21-year-old hemophiliac with HIV infection was admitted to our hospital on August 31, 1990, because of a two-week history of progressive dyspnea and a low grade fever (37.8°C). The physical examination showed diminished breath sounds over the lower third of both lung fields. The chest film showed increased bronchovascular markings bilaterally. Partial pressure of arterial oxygen (PaO₂) on room air was 82.1 mmHg. Although *P. carinii* cysts or trophozoites were not detected by the cytological test, 5S rDNA sequences were detected in sputum by PCR on September 3 (day 0). Induced sputa were

Fig. 1. Clinical courses of patients 1 and 2 and *P. carinii* shedding in sputa. Patient's detailed information is described in their case reports. Administration of anti-*P. carinii* drugs began on day 0. *P. carinii* shedding in sputum was detected by PCR and the cytological test (CT) as described in materials and methods. ST and DIV indicate trimethoprim-sulfamethoxazole and intravenous drip infusion, respectively.

obtained repeatedly during the anti-PCP therapy and the shedding patterns of *P. carinii* were examined by PCR and the cytological test. As presented in Fig. 1, Patient 1, the patient was treated with 3 mg/kg/day of intravenous pentamidine for 11 days. The chest radiograph returned to normal on day 4. As corresponding to the chest film, clinical symptoms became improving, on day 5. The negative conversion of the shedding occurred, at least, between day 5 and day 8. Although his temperature began to increase gradually from day 8, it decreased naturally after the cessation of the administration of the drug on day 12. Aerosolized pentamidine was added for 4 days from day 14. The patient improved clinically and was discharged from the hospital. He received aerosolized pentamidine twice a month as a prophylactic and remained free from PCP over the ensuing 26 months.

Patient 2

A 34-year-old woman who received renal transplantation in 1987 was admitted to our hospital on October 27, 1990 because of a two-day history of cough, hemoptysis, fever, and dyspnea on exertion. She had been controlled by immunosuppressive therapy with 10 mg/day of prednisone, 50 mg/day of azathioprine, and 90 mg/day of cyclosporine. PaO₂ on room air revealed 44.0 mmHg. The patient's clinical course is shown in Fig. 1, Patient 2. *P. carinii* cysts and trophozoites were detected in one of the three consecutive examinations of sputa by the cytological test on admission. Expecterated or partly induced sputa were obtained repeatedly during the anti-PCP therapy and shedding patterns of *P. carinii* were examined by PCR and the cytological test. Although oral trimethoprim (240 mg/day) and sulfamethoxazole (1200 mg/day) were started (day 0), the therapy was hard to continue because of severe nausea. The patient deteriorated on day 5 and received mechanical ventilation. The chest film revealed severe bilateral infiltrates with multiple confluent patchy densities in the right upper lobe (Fig.

2 A). Intravenous pentamidine (3 mg/kg/day) was administered and a three-day course of the pulse therapy with 1.0 g/day of methylprednisolone followed by a tapering from 40 mg/day of prednisone was begun on day 5. *P. carinii* shedding in sputum was monitored by PCR (Fig. 3), and it became negative on day 15 when the patient improved clinically, whereas faint infiltrates were still noted on the chest film (Fig. 2 B). The chest film also became normal, on day 19 (Fig. 2 C). She was followed as an out-patient without the recurrence of PCP over the ensuing 24 months.

Discussion

P. carinii was detected by PCR in the sputum in both patients at various times during their anti-PCP therapy. However, using the cytological test, the organism could be detected only in a severe patient and only at one time (Patient 2; see Fig. 1). These results indicate that PCR is sensitive enough to disclose *P. carinii* shedding in expectorated or induced sputa, which can not be detected by the cytological test. To our knowledge, this is the first report of monitoring *P. carinii* shedding in sputum during the anti-PCP therapy by PCR. This study was accomplished by using the PCR technique which is suitable for identifying a low-copy-number of genetic sequences. Current cytological tests are not sensitive enough to pursue persistence of *P. carinii* during the clinical course, whereas PCR can detect a few organisms, even with a conventional ethidium-bromide-staining method (9). In both patients, the diagnosis of PCP was certain by clinical symptoms associated with the PCR-result. However, even though the PCR method is very sensitive, whether subclinical PCP exists or not is to be determined.

The successful anti-PCP therapy of Patient 1 illustrates the usefulness of PCR-monitoring during treatment. His temperature began to increase gradually from day 8 of pentamidine administration, which may have indicated a relapse of infection

Fig. 2. The chest radiographs at different points during the anti-PCP therapy of Patient 2. (A) Day 0, (B) Day 15, when *P. carinii* shedding disappeared in the sputum by PCR, (C) Day 19, when clinical manifestation improved.

Novel Marker of *P. Carinii* Pneumonia

Fig. 3. *P. carinii* shedding in the sputum of patient 2 detected by PCR. Lane 1: Day 0, Lane 2: Day 1, Lane 3: Day 2, Lane 4: Day 3, Lane 5: Day 5, Lane 6: Day 8, Lane 7: Day 10, Lane 8: Day 13, Lane 9: Day 15, Lane 10: Day 17. A 120 base-pair product was amplified when sputum samples contained the organism.

and necessitated an increase in drug dosage. However, because of the negative result of PCR, combined with clinical improvements other than his temperature, and no abnormality on the chest film, this was judged to be a drug fever and not a recurrence of PCP. Therefore, pentamidine was stopped on day 12, and this management successfully lowered his temperature. Furthermore, although Patient 2 at first deteriorated and then was treated successfully with the steroid-pulse combined with the anti-PCP therapy, the shedding patterns in the sputum monitored by PCR and the clinical data correlated well in the patient. These preliminary data indicate the usefulness of this method as a therapeutic marker. Further clinical studies will settle whether or not the PCR method is to be a reliable laboratory marker.

Shortening the period of anti-PCP therapy based on a reliable marker will greatly advance clinical treatment. Until now, PCP patients, even with different severities of the disease, have been treated for the same duration because of the lack of sensitive and specific microbiological methods. For instance, a three-week regimen is carried out routinely against AIDS patients with PCP (3). However, accurate monitoring of anti-PCP therapy will shorten the therapeutic period and be useful in reducing the side effects of unnecessarily long or inappropriate drug treatments. This will also save medical costs. Given the recommendation of an early adjunctive therapy with corticosteroids (4), their use will be popular regimens. Thus, sensitive microbiological monitoring will be more important because corticosteroids often mask the clinical manifestation. Finally, this monitoring tool awaits larger prospective studies to establish a proper period for therapy based on a reliable clinical marker of *P. carinii* pneumonia.

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