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Detection of *Pneumocystis carinii* DNA by PCR amplification in various rat organs in experimental pneumocystosis

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Pneumocystosis is usually a disease of the lungs, but the number of cases of extrapulmonary pneumocystosis has greatly increased during the AIDS epidemic. Much remains unknown about the frequency and mechanisms of dissemination. In the present study, a systematic search for *Pneumocystis carinii* by PCR with primers specific for mitochondrial rRNA was performed in the lung, liver, spleen and kidney of 12 immunosuppressed rats and two immunocompetent rats. The amplified products were analysed by Southern hybridisation with a digoxigenin-11-dUTP labeled probe. *P. carinii* DNA was found in lungs in all 14 rats and in at least one organ other than lung in 11 immunosuppressed rats and the two control rats. We suggest that extrapulmonary dissemination may not be an exceptional phenomenon in the course of pneumocystosis, but rather part of the natural evolution of the disease.

Introduction

Pneumocystis carinii pneumonia is a major cause of morbidity and mortality in HIV-infected patients. Recently, an increasing number of cases of extrapulmonary and disseminated pneumocystosis, or both, has been reported in AIDS patients [1]. This raises issues about the natural history of the disease; what is the frequency of the dissemination; is this dissemination always associated with a pulmonary infection; and does the proliferation occur only in the immunosuppressed host or does *P. carinii* proliferate in extrapulmonary sites in the normal host? A few experimental studies on extra-pulmonary dissemination of *P. carinii* have been done [2, 3]. Involvement of various organs has been observed in some cases, but the number of *P. carinii* organisms seems to be very low in sites other than lung. Therefore, a very sensitive technique is required to detect *P. carinii* in these sites. Recently, the polymerase chain reaction (PCR) has been reported as a specific diagnostic method for *P. carinii* pneumonia [4–9]. The results of various studies indicate that PCR is more sensitive than conventional methods of diagnosis (staining methods or immuno-

fluorescence assays) for the detection of *P. carinii* in human samples. Thus, the present study compared PCR to Giemsa and methenamine silver stains in a systematic search for *P. carinii* in the lung and in various organs of untreated control rats and immunosuppressed rats.

Materials and methods

Experimental animals

Fourteen female Sprague-Dawley rats (Iffa Credo, Lyon, France), all weighing *c.* 200 g were used. Twelve rats, housed in isolation cages, were provided with drinking water containing dexamethasone (2 mg/L) [10], to induce immunosuppression, and tetracycline (500 mg/L) to prevent bacterial infections. The remaining two rats, caged in the same room as the immunosuppressed rats, did not receive dexamethasone and served as sentinel controls. All the rats were fed a normal protein diet. Twelve weeks after the beginning of immunosuppression the mean weight of the control rats was 310 g (range 300–320 g). The mean weight of the immunosuppressed rats was 163 g (range 150–180 g) and they showed clinical signs of severe infection (hair loss, shivering, dyspnoea). At this time the rats were anaesthetised with ether and exsanguinated by intracardiac puncture. Lungs, liver, spleen and kidney were collected with extreme care to avoid cross-

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contamination between tissues by using separate surgical materials. Organs were stored at -80°C . Smears prepared from each organ were stained with Giemsa and methenamine silver methods.

DNA preparation

To avoid cross-contamination of the extra-pulmonary tissues with infected lung tissues, the other tissues were processed several weeks after lung tissues and new materials and solutions were used. Tissue samples of each organ (0.25 g) were homogenised in separate Waring blenders and washed three times in phosphate-buffered saline (PBS) (pH 7.2) to remove as much blood as possible. The blenders were cleaned and autoclaved each time before use. The homogenates were incubated at 55°C overnight in a solution containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.5 mM MgCl_2 , gelatin 0.1 mg/ml, Nonidet P-40 0.45%, Tween-20 0.45% and proteinase K at a final concentration of 60 $\mu\text{g}/\text{ml}$. DNA was extracted with phenol-chloroform, precipitated with ethanol, and dissolved in water.

DNA amplification and hybridisation

The primers described by Wakefield *et al.* [10] were used for amplification of part of the mitochondrial gene encoding the large subunit of rRNA: pAZ102-E (5'-GATGGCTGTTTCCAAGCCCA-3') and pAZ102-H (5'-GTGTACGTTGCAAAGTACTC-3'). One μg of DNA extracted from each organ was amplified in a reaction mixture (100 μl) containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM MgCl_2 , gelatin 0.01%, 0.25 mM each of dATP, dTTP, dCTP and dGTP, 100 pmol each of the oligonucleotide primers and 2.5 units of Taq-polymerase (Perkin Elmer Cetus, Norwalk, CT, USA). After an initial 5-min denaturation step, DNA was amplified for 40 cycles in a Trio-Thermoblock thermocycler (Biometra, Göttingen, Germany). Each cycle consisted of 1.5 min denaturation at 94°C , 1 min annealing at 56°C and 1 min extension at 72°C . The amplification of lung tissues took place on different days from those on which the other tissues were amplified. Standard protocols to avoid contamination were followed [11], i.e., DNA extraction and amplification procedures were performed in separate rooms within the laboratory and aerosol-barrier pipette tips were used for all reagent transfers and negative controls (water) were run in each experiment. The amplified products (355 bp) were electrophoresed in agarose 1.5% gels and visualised after ethidium bromide staining. They were then Southern-blotted on to a Hybond N⁺ nylon membrane (Amersham), and hybridised with an internal oligonucleotide probe, pAZ102-L1 (5'-ATAAGGTGAGGAGTTCGAGAG-3') [10]. The probe was labelled with digoxigenin-11-dUTP by a non-radioactive DNA labelling kit (Boehringer Mannheim, Mannheim, Germany). The hybrids were detected by enzyme-linked immunoassay.

Sensitivity of the PCR

To determine the level of detection of the PCR, a 20- μl sample of a human bronchoalveolar lavage (BAL) specimen known to contain *P. carinii* was placed on a microscope slide. The smears were stained with Giemsa and methenamine silver. PCR was performed as described above on another 10- μl sample of the same BAL specimen and on 10-fold serial dilutions (1 in 10^1 –1 in 10^{10}) of this sample. The amplification products were visualised on an agarose 1.5% gel containing ethidium bromide.

Results and Discussion

P. carinii DNA was detected by PCR in the lungs of all 14 rats and in at least one other organ in 13 of them (Table 1). The results for the kidney are particularly interesting as *P. carinii* DNA was found in this tissue in the two control rats (Fig. 1). On the contrary, stains failed to show the presence of *P. carinii* organisms in any specimens from the two immunocompetent rats. In the 12 immunosuppressed rats, *P. carinii* trophozoites were observed on the Giemsa-stained smears prepared from nine lungs and one liver. Cysts were detected by methenamine silver stain in the smears prepared from 10 lungs, two spleens, one liver and one kidney (Table 1). Thus we have confirmed by PCR the healthy carrier state of the sentinel control rats exposed to *P. carinii* infected-rats, as previously described by Sepkowitz *et al.* [12]. These results suggest almost systematic extra-pulmonary dissemination of *P. carinii* to several organs in both the immunosuppressed and

Fig. 1. PCR detection of *P. carinii* DNA in the kidney of 12 immunosuppressed rats and two control rats. Southern hybridisation of electrophoresis gel with digoxigenin-11-dUTP-labelled oligonucleotide probe; +, positive control (*P. carinii* DNA from rat lung); –, negative control (water). The immunosuppressed rats are numbered 1–12 and the two control rats are numbered C1 and C2.

Table 1. Detection of *P. carinii* by Giemsa stain (GS), methenamine silver stain (MSS) and PCR in the 12 immunosuppressed rats and the two sentinel control rats

Rat no.	Lung			Spleen			Liver			Kidney		
	GS	MSS	PCR	GS	MSS	PCR	GS	MSS	PCR	GS	MSS	PCR
1	+	+	+	-	-	+	-	-	+	-	-	-
2	-	-	+	-	-	+	-	-	-	-	-	-
3	+	+	+	-	+	+	+	+	+	-	+	+
4	-	+	+	-	-	+	-	-	+	-	-	-
5	+	+	+	-	-	-	-	-	+	-	-	-
6	+	+	+	-	-	+	-	-	+	-	-	+
7	-	-	+	-	-	-	-	-	-	-	-	-
8	+	+	+	-	+	+	-	-	-	-	-	-
9	+	+	+	-	-	+	-	-	+	-	-	+
10	+	+	+	-	-	-	-	-	-	-	-	+
11	+	+	+	-	-	-	-	-	-	-	-	+
12	+	+	+	-	-	-	-	-	-	-	-	+
Control 1	-	-	+	-	-	+	-	-	-	-	-	+
Control 2	-	-	+	-	-	-	-	-	-	-	-	+
Total	9	10	14	0	2	8	1	1	6	0	1	8

immunocompetent rats. The present study also confirmed that PCR, in contrast to conventional staining techniques, is able to detect a very low level of infection in various organs. Amplification products were observed after PCR of an undiluted BAL specimen and in specimens diluted 1 in 10^1 , 1 in 10^2 , 1 in 10^3 and 1 in 10^4 . This indicates a limit of detection of 10 organisms (data not shown).

The number of human cases of extra-pulmonary dissemination of *P. carinii* has increased since the beginning of the AIDS epidemic, growing from 12 published cases before the epidemic to 80 published cases [1]. A relationship between the prophylactic use of aerosolised pentamidine and the development of extra-pulmonary pneumocystosis has been suggested [1]. The risk for extra-pulmonary dissemination of *P. carinii* in HIV-infected patients is estimated to range from 0.5 to 3% [1], but this rate is certainly underestimated because: (i) extra-pulmonary signs are infrequent and premortem diagnosis is hardly ever suspected even when such signs are present; (ii) autopsy studies are not systematically performed; and (iii) histological studies at necropsy only very rarely include a staining technique for *P. carinii* detection and DNA amplification has never been performed. The findings of the present experimental study suggest that the proliferation of the parasite is lower in extra-pulmonary sites than in the lungs, where it is readily detectable in immunosuppressed rats by conventional techniques. Therefore, despite the small number of patients documented with extra-pulmonary dissemination of the parasite, the dissemination, in contrast to its clinical expression, is not necessarily a rare event. Clinical signs appear when *P. carinii* proliferation is massive, and can be detected by classical techniques.

A search for extra-pulmonary dissemination of *P. carinii* by stains has been performed in two experimental studies. Denis *et al.* [2] developed three models, i.e., rats and mice which received corticoster-

oid treatment and SCID mice, all maintained in contact with *P. carinii*-infected mice. They observed extra-pulmonary dissemination, as noted in the present study, but the percentage of animals presenting with infection was lower than in the present study (<50%). Oz *et al.* [3] also used conventional techniques to search for extra-pulmonary foci of *P. carinii* in 40 ferrets and 30 rats. These animals were immunosuppressed with dexamethasone, as in the present experiments, but were killed 7–8 weeks after the beginning of immunosuppression, instead of 12 weeks as in this study. *P. carinii* was not observed outside the lungs in rats, but it was found in 10% of the ferrets, in liver and spleen.

Other authors have used PCR. Evans *et al.* [13] searched for *P. carinii* DNA in the lung, liver, spleen and kidney of five immunosuppressed rats with pAZ102-E and pAZ102-H primers. *P. carinii* was found exclusively in the lung. However, the immunosuppressive regimen was shorter (6 weeks instead of 12) and less vigorous (hydrocortisone instead of dexamethasone) than the regimen used in the present study. The limit of detection of the DNA amplification was surprisingly high (590 organisms) even when a nested-PCR was used. Reddy *et al.* [14] amplified the dihydrofolate reductase (DHFR) gene to detect *P. carinii* in two rats immunosuppressed with dexamethasone. *P. carinii* was found in the liver, but not in the spleen or in the kidney. By following the amplification protocol described by Wakefield *et al.* [10], Furuta *et al.* [15] detected *P. carinii* in several organs of two out of five simian immunodeficiency virus (SIV)-infected monkeys, with positive results in as many as 14 extra-pulmonary sites in one monkey. More recently, with primers specific for the major surface glycoprotein (MSG) gene, Chary-Reddy *et al.* [16] found *P. carinii* DNA in several non-pulmonary tissues of 10 immunosuppressed rats, including kidney (80%), liver (90%) and spleen (60%). Furthermore, reverse transcriptase-PCR analysis with DHFR primers

demonstrated active transcription in these tissues and thus the presence of viable *P. carinii* organisms. These results are consistent with those of the present study although the immunosuppression protocol (methylprednisolone) and the amplification target were different.

The extra-pulmonary locations of *P. carinii* in the sentinel control rats lends support to our hypothesis that the extra-pulmonary dissemination from the lung may be a part of the normal progression of the disease. A search for *P. carinii* in non-pulmonary tissues in non-immunosuppressed animals housed near corticosteroid-treated rats has not been performed in the six studies cited above. Oz *et al.* [3], Reddy *et al.* [14] and Chary-Reddy *et al.* [16] did not find *P. carinii* in lung or in other organs in immunocompetent animals, but the authors did not specify whether these animals were housed near the *P. carinii*-infected animals. Furthermore, Chary-Reddy *et al.* [16] used special rats which were viral antibody-negative and *P. carinii*-free. No control animals were mentioned in the three other studies [2, 13, 15]. Thus the present findings in immunocompetent rats can be compared only with those of Sepkowitz *et al.* [12]. In their study *P. carinii* DNA was detected by PCR in lung tissue and in sera from non-immunocompromised sentinel rats that were exposed to *P. carinii*-infected rats. After a few weeks of isolation apart from the corticosteroid-treated rats, *P. carinii* DNA became undetectable in lung and in serum. The results of Sepkowitz *et al.* may reflect subclinical infection or a high level of lung colonisation and a low level parasitaemia after aerial transmission of *P. carinii*. The precise nature of the *P. carinii* DNA detected by PCR in blood is not known at the present time, but the fact that *P. carinii* can be isolated in cell line cultures from the blood of AIDS patients with pulmonary pneumocystosis [17] lends support to the hypothesis of viable circulating organisms. Thus the presence of *P. carinii* in the non-pulmonary tissues observed in immunocompetent sentinel rats in the present study may be the result of *P. carinii* dissemination via the haematogenous route. Further experiments are required to clarify this, in immunocompetent animals as well as in immunosuppressed animals. Extra-pulmonary sites are thus important, not so much because they generate pathogenic effects, but rather because they might be potential sources of development or relapse of pulmonary pneumocystosis.

The present study compared the usual stains used for the detection of *P. carinii* with PCR and demonstrated the usefulness of DNA amplification for detection of *P. carinii* in extra-pulmonary rat tissues. It is likely that the spread of *P. carinii* from the lungs to various organs is the natural outcome of infection in the host. This hypothesis has to be tested in man by a

systematic search for *P. carinii* in extra-pulmonary tissues by PCR.

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