MICROBIAL PATHOGENICITY

Evidence of *Pneumocystis carinii* in cell line cultures infected with peripheral blood mononuclear cells isolated from AIDS patients with *P. carinii* pneumonia

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Summary. The detection of *Pneumocystis carinii* was investigated in an in-vitro system consisting of a human lung epithelial cell line (A-549) inoculated with infected peripheral blood mononuclear cells (PMBC) from HIV-infected patients with proven or suspected *P. carinii* pneumonia (PCP), and from HIV-negative patients with other lung infections. Supernates from cultures were sampled daily and evaluated for the presence of *P. carinii* by Giemsa and immunofluorescence staining. *P. carinii* was isolated from 98 (95.1%) of 103 culture supernate samples from patients with proven pneumocystosis and 45 (66.1%) of 68 from patients with suspected PCP 40 or 72 h after PBMC inoculation. This system has been shown to support the growth of *P. carinii* but did not seem to be adequate for the production of large numbers of organisms, although long-term survival *in vitro* for up to 3 weeks was observed. Recovery of *P. carinii* from infected PBMC strongly supports previous observations about its ability to disseminate haematogenously and could represent a further advance in understanding the pathogenesis and diagnosis of PCP.

Introduction

Pneumocystis carinii causes lethal pneumonia (PCP) in immunosuppressed patients and is a leading cause of mortality in patients with acquired immunodeficiency syndrome (AIDS). Pneumocystosis is diagnosed currently by direct visualisation of the organism in infected lung tissue or in respiratory fluids by conventional cytochemical or immunocytochemical staining. Immunological assays have also been employed. However, these methods are of little indicative value in *P. carinii* disease and current opinion is that the detection of antibodies or circulating soluble antigen cannot be considered to be alternatives to invasive diagnostic procedures.

In the last few years, new techniques have been developed for the diagnosis of PCP. In particular, detection of *P. carinii* in blood by the polymerase chain reaction (PCR) has provided a highly sensitive and specific tool that has facilitated diagnosis and elucidated the natural history and epidemiology of pneumocystosis.^{1–4}

Considering the potential diagnostic value of the detection of the organism in the blood and because

approaches to this problem have never involved in-vitro procedures, a modified culture system consisting of a human lung epithelial cell line (A-549) with inocula derived from peripheral blood mononuclear cells (PBMC) from AIDS patients with PCP was employed to determine whether it could be useful in detecting *P. carinii* infection.

Accumulating evidence suggests that attachment to host cells, particularly alveolar epithelial cells, is an important step for the growth and replication of the parasite and appears to be mediated by cell-surface glycoproteins, exogenous fibronectin and components of the parasite cytoskeleton.⁵ At no time in its life cycle does *P. carinii* seem to be an intracellular pathogen.

A protective host response against *P. carinii* involves both functional CD4+ T lymphocytes and alveolar macrophages (AM). Studies *in vitro* have shown that the organism can be ingested and digested by normal AM and killed by macrophage-derived biochemical mediators, including cytokines, which may contribute to the control of pneumonia.⁶ In-vitro studies have also demonstrated that conditions that lead to defects in the host's immune response, such as AIDS or corticosteroid treatment, may impair the phagocytic and cytolytic activities of AM by altering membrane Fc receptor functions.

The detection of *P. carinii* in cultured PBMC could be useful for the diagnosis of PCP, and also for providing insights into the relationship between this organism and circulating human monocytes in the pathobiology of this infection.

Materials and methods

Clinical specimens and PBMC isolation

Single or multiple blood samples were obtained as part of routine viral surveillance from 64 outpatients attending the AIDS clinic or patients admitted to the Institute of Infectious Diseases of University "La Sapienza", Rome, between September 1991 and February 1993. The average age was 34.7 years (range 28–51 years) for male patients and 31.2 years (range 24–44 years) for female patients. Thirty-eight were AIDS patients with morphologically and clinically proven PCP or extrapulmonary pneumocystosis (one case); 17 were patients who presented with respiratory symptoms, severe lung involvement and gasometric features consistent with PCP, in whom neither bronchoalveolar lavage (BAL) nor induced sputum could be obtained to make a definitive laboratory diagnosis (these subjects were not known to be on prophylaxis or treatment for PCP and no other pathogen was isolated from their samples by routine examination); nine were AIDS patients who were febrile and had other causes of pneumonias (*Mycobacterium tuberculosis*, four patients; *Streptococcus pneumoniae*, three; cytomegalovirus, two). Forty healthy afebrile volunteers without respiratory diseases were also included in the study.

PBMC were isolated from 10 ml of heparinised whole blood by Ficoll Hypaque density gradient centrifugation (Lymphoprep, Nycomed, Oslo, Norway) in plastic 50-ml centrifugation tubes at 400 *g* at room temperature for 30 min, within 2 h after collection. The top band at the sample-medium interface consisted of mononuclear cells and the lower band of polymorphonuclear cells. The band of mononuclear cells was washed twice with 10 ml of phosphate-buffered saline and centrifuged at 200 *g* for 8 min.

A-549 cell culture

PBMC from each blood sample were then inoculated on to 48-h confluent A-549 cell monolayers grown in triplicate 25-cm² flasks (ICN, Costa Mesa, CA, USA) in Ham's F12 medium (Slavo, Siena, Italy) 90%, supplemented with fetal calf serum 10%, antibiotics (ampicillin 200 U/ml, streptomycin 200 µg/ml, amphotericin B 0.5 µg/ml) and incubated at 37°C in a humidified CO₂ 5% atmosphere. After inoculation, cultures were sampled daily by removing one-tenth of the volume of each flask and pooling supernates as reported previously.⁷

The pooled supernates from ongoing cultures were centrifuged at 5000 *g* and then checked for *P. carinii* nuclei and cyst counts by a modified Giemsa stain⁸ and immunofluorescence staining with monoclonal anti-

bodies (MAbs) to human strains of *P. carinii*.⁹ The viability of the micro-organisms was determined by a combination stain with fluorescein diacetate and ethidium bromide¹⁰ adapted for *P. carinii*. To optimise the quantitation system, the number of *P. carinii* was calculated from each supernate sample by determining the mean number of cysts and trophozoites grown in triplicate flasks during the 3-week culture period. Statistical differences were calculated by analysis of variance.

Controls for A-549 cell cultures

To determine the conditions under which growth of human PBMC-derived *P. carinii* could be observed *in vitro*, a number of uninfected cultures were also inoculated with *P. carinii* extracts (inoculum size = 1.5×10^6 cysts) from frozen stored post-mortem human lung tissue prepared according to the method of Walzer *et al.*,¹¹ and with non-infected human lung treated in the same manner as the *P. carinii*-infected tissue. For *P. carinii* inocula as well as for normal lung tissues, erythrocytes were lysed with ammonium chloride 0.85%. Cultures were monitored for the presence of bacteria and fungi by microscopy and culture.¹²

Results

In separate experiments, two non-commercial well-characterised MAbs were evaluated to establish their sensitivity and specificity. *P. carinii* cysts were identified clearly by indirect immunofluorescence in respiratory secretions as well as in cultured PBMC from patients with PCP. Specificity studies were done on each of the two MAbs with normal human lung extract and fungi such as *Cryptococcus neoformans* and various *Candida* spp., including *C. albicans*. No non-specific fluorescence was observed. These MAbs have also been shown to be as sensitive and specific as a commercial Monofluo kit (Diagnostic Pasteur, France).⁹ *P. carinii* trophozoites were counted by microscopy with a modified Giemsa stain, which although less rapid than Diff Quik, allowed the assessment of organism growth without observation of background material.⁷

When an A-549 cell culture was infected with human lung-derived *P. carinii*, counts of both cyst and trophozoite forms peaked from day 1 to day 3 from the beginning of the culture. Cyst and trophozoite counts decreased from day 3 but cysts predominated in large clumps and persisted at levels below the inoculum size for up to 1 week of culture. These data indicated that an inoculum size of 1.5×10^6 cysts could be used to evaluate *P. carinii* growth in culture and to ensure uniformity of results.

In the cell cultures infected with human PBMC, cultures provided evidence of infection in 36 (94.7%) of the 38 AIDS patients with proven pneumocystosis and in nine (52.9%) of the 17 patients with suspected

Figure. Immunofluorescence stain showing clusters of *P. carinii* organisms in a 3-day culture supernate of cell line A-549 inoculated with PBMC from a patient with PCP ($\times 400$).

Table. Numbers of *P. carinii* organisms grown in cultured PBMC from different population groups

Patient characteristic	Number (%) of positive patients	Number (%) of positive cultures	Mean (SE) organism yield during 3 weeks of culture		
			1-7 days	8-14 days*	15-21 days†
AIDS, clinically and microbiologically proven PCP (38)	36 (94.7)	98/103 (95.1)	$2.16 (1.15) \times 10^5$	$1.64 (0.2) \times 10^4$	$1.22 (2.0) \times 10^3$
AIDS, clinically suspected PCP (17)	9 (52.9)	45/68 (66.1)	$4.45 (5.65) \times 10^4$	$9.01 (2.6) \times 10^3$	19.89 (5.03)
AIDS, febrile, with other pneumonias (9)	0 —	0/42 —	—	—	—
Control subjects (40)	0 —	0/58 —	—	—	—

Note: point data are expressed as the means of *P. carinii* forms from pooled samples of triplicate flasks.

*Organism counts significantly different between first and second week, $p < 0.01$. †Organism counts significantly different between second and third week, $p < 0.001$.

PCP. Culture supernate samples were positive 2-3 days after PBMC inoculation, with large clusters of organisms which were predominantly the cyst form (figure). Different growth patterns were observed in supernate samples collected after the first week of the culture. Generally, trophozoites appeared to predominate and occurred scattered in groups. Although many of the organisms remained adherent to the underlying cells and could not be counted accurately, the number of these decreased significantly between the first and the second week ($p < 0.01$), as well as between the second and the third week ($p < 0.001$) after inoculation (table). All positive cultures showed prolonged *P. carinii* survival for up to 3 weeks, after which most of the organisms were non-viable.

No evidence of *P. carinii* growth was observed on monolayers or in supernates inoculated with PBMC

from patients with other respiratory infections (non-PCP) or from control subjects, nor from cultures inoculated with normal human lung extract. The negative culture specimens underwent no further testing. No fungal or bacterial contaminants were observed.

Discussion

In the last 10 years, different immunological approaches have been used for the diagnosis of pneumocystosis. Antibody testing has been shown to be useful epidemiologically, but it is unreliable in diagnosing PCP, as the majority of healthy individuals as well as patients with *P. carinii* infection have substantial titres.¹³ Antigen detection, although initially encour-

aging, was also reported to be a poor diagnostic system because of the tight adherence of this pathogen to the host pneumocytes which could hinder its access to the bloodstream and subsequent release of antigen.¹⁴

With increasing reports of cases of disseminated pneumocystosis, which support the concept that organisms may reach and colonise disparate sites outside the lung,^{15,16} evidence is now emerging that *P. carinii* is liberated from the lung into the peripheral circulation through haematogenous or lymphatic pathways. Recently, *P. carinii* has been detected by PCR DNA amplification of blood from rats and human patients with PCP and with disseminated pneumocystosis, thus helping to answer a number of important questions about the pathogenesis of *P. carinii* infection.^{2,3}

Considering the potential importance of detection of the organism in the blood, a culture method was used which has been employed extensively for the short-term culture of rat- or human-derived *P. carinii*.^{17,18} In the current study, the cell line was infected with a different inoculum source, which consisted of PBMC from human patients with *P. carinii* infection instead of conventional inocula.

This system was able not only to confirm cases of known pneumocystosis, but also to recognise the typical organisms in high isolation rates in culture supernate samples from AIDS patients with suspected PCP in whom demonstrations of *P. carinii* could not be made conventionally and in whom specific therapy for pneumocystosis was followed by rapid clinical and radiographic improvement. *P. carinii* forms were not seen in cultures infected with PBMC from HIV-infected patients with other pneumonias or from control subjects, thus confirming previous reports which did not reveal positive PCR signals in patients with advanced AIDS without a history of PCP.³

Having demonstrated the presence of *P. carinii* in cultured human-derived PBMC, we were not able to determine whether organisms were attached to these cells or were within the PBMC. The interaction of these organisms with monocytes and T cells was reported previously.¹⁹ Transmission electronmicroscopy has shown that *P. carinii* trophozoites adhere to alveolar and peritoneal macrophages and T cells, and

are ingested and destroyed by macrophages after the addition of rabbit anti-*P. carinii* serum, thus suggesting that humoral and cellular mechanisms may be necessary in combination to control *P. carinii* disease. Some reports have also suggested that proteins such as fibronectin play an important role in attachment by serving as a bridge between the surface glycoprotein-A and host cell receptors.²⁰ Recently, *P. carinii* surface glycoprotein-A was demonstrated to be a chemotactic factor for normal human blood monocytes.²¹

Presently, most of the cell-culture systems employed for *P. carinii* propagation have yielded only transient proliferation and did not prove adequate for isolation of *P. carinii* in sufficient quantities to explore important basic biological questions. Much of this unsuccessful work is related to the quality of the inocula used, which may be contaminated or not of sufficient purity.^{7,15,22,23} A culture system employing cell monolayers on micro-carrier beads has been described recently. This method did not achieve *P. carinii* proliferation significantly higher than that seen in plate or flask culture, but the organisms harvested contained little host-cell contamination.²⁴

The system employed in the present report appears to support the growth of *P. carinii* and to avoid the technical problems resulting from contamination of host cells or additional infectious agents introduced with a conventional *P. carinii* inoculum. This method has been shown to be inadequate for the production of large numbers of organisms, even if cultured for 21 days. Humoral factors secreted by PBMC which allow *P. carinii* to grow in culture, or HIV infection of the monolayer cells by the PBMC somehow making the monolayer cells permissive to the organism's growth, might explain long term survival.

The possibility of identification of *P. carinii* in cultures infected with blood from patients with proven or suspected PCP represents a further advance in *P. carinii* diagnosis and better understanding of the transmission of the infection. A more extensive development of this in-vitro cultivation method could also allow a study of the putative differences between *P. carinii* strains isolated from the blood of infected individuals.²⁵

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