OCCASIONAL ARTICLES

Why have Pneumocystis carinii trophozoites been ignored?

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Introduction

The recent classification of Pneumocystis carinii as a fungus was the result of detailed investigations.[1] Equally fundamental observations remain to be made on this organism's life cycle and pathogenicity. Although trophozoites and cysts of *P carinii* have been described in infected lung,[2] their role and relative importance have not been clearly established. Despite this uncertainty investigators have mainly concentrated on cysts. The possibility that trophozoites are clinically more important and antigenically different deserves further examination. In this paper we consider how and to what extent they have been overlooked and the implications for the development of diagnostic tests for *P carinii* pneumonia (PCP).

Reagents

The specificity and sensitivity of laboratory tests greatly depend on the quality of the reagents. *P carinii* cysts are easily identified because their size and shape are consistent and they stain in a predictable way with widely used stains such as Grocott's methenamine silver, toluidine blue O, or cresyl violet. In contrast, trophozoites are pleomorphic, variable in size, and do not stain with these stains. They stain with Giemsa or Diff-Quick but are difficult to identify and are best demonstrated by electron microscopy (figure).

The precise relation between cysts and trophozoites is unknown. Trophozoites may develop into cysts[2] or may replicate independently.[3] The latter could explain why they are often more numerous. Indeed, it has been estimated that cysts may represent less than 1% of *P carinii* parasites in an alveolar lavage.[4] This suggests that trophozoites are more important than cysts in the growth of the parasite, and in tissue culture studies trophozoites provide the most sensitive indicator of growth.[5] Furthermore, the number of cysts does not correlate with the severity of PCP[6] and drugs effective in PCP seem to have greater effect on trophozoites than cysts.[2,7]

Test reagents for *P carinii* are prepared from parasites purified from tissue culture[8] or more commonly from infected lung. Necropsy samples of human lung are scarce so infected rat lung produced by immunosuppression is widely used.[9] Because of their resistant nature and easy identification most purification procedures favour cyst separation[10–12] with little attention devoted to the fate of trophozoites.[11] Cysts can survive combinations of mechanical and enzyme treatments, differential centrifugation, and gradient separation techniques, but enzymes affect their morphology and immunological activity. Thus pronase and trypsin treatments can produce cysts which react differently in indirect immunofluorescence (IFA) tests and noticeably reduce numbers of trophozoites,[9] but collagenase does not seem to affect either cysts or trophozoites.[11]

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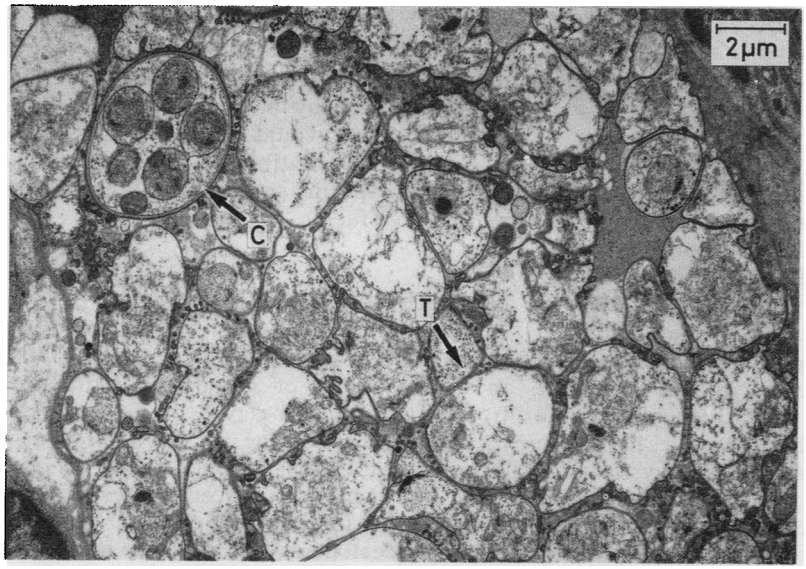

Rat lung infected with P carinii; single thick walled cyst (C) with developing intracystic bodies and numerous trophozoites (T) Scale bar = 2 μm.

Serological studies

Serological tests have used cyst rich fractions. Trophozoites have been shown to persist in such fractions,[11] but only cyst numbers are usually evaluated because they are easier to show and quantity. Differences in purity and quality of cysts or trophozoites might therefore explain the differences in reactivities which have been reported. It is assumed that cysts and trophozoites are antigenically identical but the evidence for this is not convincing. Although trophozoites have been found to react with antisera against cysts,[11] this could be explained by trophozoites contaminating cyst fractions used to prepare the antisera.

Variability in antigen composition could also explain the results obtained when comparing parasites from different species. Rat and human *P carinii* have been reported to be antigenically similar,[13] antigenically distinct,[14] or to have specific and common antigens.[15] Workers have been cavalier in their use of rat

Table 1 Results of P carinii antigenaemia using sera from immunised rabbits

Reference	Method	Immunogen	Groups examined	Results and conclusions
1 Pifer *et al*[21] 1978	CIE	Human and rat cysts, in vitro culture, trypsinised, gradient separation, sonicated	123 PCP/cancer 100 cancer 120 normal	Positive in 67% PCP, 15% cancer and 0% normal controls
2 Meyers *et al*[22] 1979	CIE	As (1)	28 BMT/PCP 80 other pneumonia 25 BMT 50 BMT donors	Positive in 79% PCP, 71% other pneumonia, 44% BMT, 2% donors not diagnostic
3 Maddison *et al*[17] 1982	a) ELISA b) CIE	Rat cysts, collagenase treated, gradient separation, HCl soluble	20 PCP 1 control	All negative by ELISA—1/20 reactive CIE
4 Maddison *et al*[18] 1982	CIE	As (3)	102 PCP 153 contacts 99 healthy subjects 24 AIDS/PCP	Positive in 13·7% PCP, 3% contacts 1% healthy, 51% AIDS/PCP, insensitive
5 Maddison *et al*[19] 1984	CIE	As (3)	32 AIDS/PCP 135 controls	Positive in 41% PCP; 20% healthy controls; unreliable indicator
6 Tanabe *et al*[23] 1985	CIE	Rat cysts, collagenase treated, filtration, sonicated	13 PCP	23% positive PCP, 0% controls
7 Jarowenko *et al*[20] 1986	CIE LPA	Rat cysts in vitro culture	7 RT/PCP 78 RT/asymptomatic 100 healthy subjects	CIE positive in 28% PCP, 1% asymptomatics and 0% healthy subjects, LPA positive in 100% PCP, 62% of asymptomatics and 5% normal controls
8 Tanabe and Furuta[24] 1987	ELISA CIE	Cysts from athymic nude rats extracted as (6)	10 PCP	ELISA 80% positive; CIE 20% positive
9 Pifer *et al*[25] 1988	CIE	Rat cysts (1)	12 AIDS/PCP 106 AIDS/clinical PCP	Positive in 75% confirmed PCP, 30% others

BMT, Bone marrow transplant; RT, renal transplant; LPA, latex particle agglutination; CIE, counterimmunoelectrophoresis.

and human parasites, enzyme treated or untreated and purified on gradients of different compositions. These antigens and antisera may not be interchangeable.

Reports on the usefulness of IFA for antibody detection in human serum have varied widely.[16] There is little agreement on the best source and method of antigen preparation for IFA, and apparently similar antigens give different results. Soluble antigens derived from disrupted cysts have been used in enzyme linked immunosorbent assays (ELISA) for antibody detection.[17–20] The good correlation between IFA and ELISA suggests that similar wall antigens are involved. Although the ELISA is more sensitive than IFA, it is no more specific to PCP.[18 20] It is perhaps relevant that ELISA antigens are assessed on concentration of cysts, yet numbers do not correlate with the protein concentrations of the final preparations.[17] This may be due to contaminating tissue proteins, but the contribution of trophozoites could be important. The activity of IFA and ELISA antigens prepared from trophozoite rich fractions remains to be studied.

Current infection

Identification of *P carinii* specific antigen in serum allows current infection to be diagnosed. Initially, promising results were obtained using sera from immunised rabbits (table 1), but these tests have proved insensitive. Reliable differentiation of clinical PCP from subclinical infection is not possible,[17–25] but antigenaemia has been found in patients with PCP before the appearance of antibody and the onset of acute pneumonia.[24] Although trophozoites have been shown to be more numerous than cysts, particularly in early PCP, anti-trophozoite specific antisera have not been evaluated in antigen detection systems.

Monoclonal antibody technology has enabled production of *P carinii* specific antibodies despite crude starting antigen.[26–30] These antibodies have been used in IFA tests for simple, rapid, and highly specific demonstration of *P carinii* in clinical specimens.[28–32] The monoclonal antibodies so far available have been selected to react with cyst wall antigens. They seem to be capable of detecting at least 95% of human isolates from patients with AIDS and non-AIDS patients.[28] These workers suggest that only visualisation of characteristic cysts should be considered diagnostic. In one study, however,[32] free trophozoites and a few cysts were found in one patient and only thin walled cysts in another of 19 *P carinii* positive bronchoalveolar lavage samples. Thus if the diagnosis of current infection depends on the presence of cysts, cases could be missed.

Antigenaemia was detected in only 11% of heavily infected rats using anti-rat *P carinii* monoclonal antibodies in a sandwich ELISA.[33] Antigen was detected in the supernate and pellet of lavage fluid taken during acute infection, but there was no correlation between absorbance values and the numbers of cysts present. Trophozoite populations were not evaluated and may be responsible for these discrepancies. It would be interesting to know if monoclonal antibodies that recognise trophozoite specific antigens would have been more successful.

New techniques

Immunoblotting (Western blot) has only recently been applied to *P carinii*. As with the development of monoclonal antibodies trophozoites must be present in the crude extracts applied to the polyacrylamide gels. Antigens chosen for immunoblotting based on the highest protein concentrations did not correlate with highest cyst numbers.[3 4] Yet little effort has been made specifically to investigate trophozoite antigen or even determine relative cyst and trophozoite numbers. Rat and human

Table 2 Results of Western blot analysis of P carinii antigens using normal and infection derived human and rat sera (Major bands underlined)

Antigen source	Bands detected (Kd)	Human sera		Rat sera			Reference No
		N	P	N	E	R	
Rats	30–32	+	+	+		+	36
	35–40	+	+		+	+	35, 36, 37
	45	+	+	+	+	+	34, 37
	50–60	+	+	+	+	+	33, 34, 36, 37
	60–92	+	+		+	+	34, 37
	110–116	+	+	+	+	+	33, 34, 35, 36, 37
	125	+	+	+		+	36
	170	+	+	+		+	33, 36
Man	40	+	+		*+	+	34
	66	+					34

N = normal healthy controls/rats with anti-*P carinii* antibody
P = Patients with PCP
E = Healthy rats exposed to *P carinii*
R = PCP induced in rats

antigen preparations have been probed using serum from immunised rabbits,[33 34] monoclonal antibodies,[30 33 35] and infection derived sera from rats or humans[33–37] (table 2).

With rat antigen, major bands at 45, 50–60, and 110–116 kilodaltons are most consistently shown, while detection of the other polypeptides is variable. Only the 45 kilodalton band may be specific for *P carinii* as bands at 55–60 and 110 have been found in normal rat lung antigen,[36] suggesting they are due to rat proteins or cysts in normal lung. In human *P carinii* an 82 kilodalton polypeptide seems to be important as 17 of 21 anti-human *P carinii* monoclonal antibodies reacted only with this single polypeptide.[30] Another study identified a 40 and to a lesser extent a 66 kilodalton polypeptide as the principal immunoreactive bands in human *P carinii*.[34] These bands were detected in six of seven *P carinii* isolates from patients with AIDS but in only one isolate of eight patients without AIDS. Cyst counts in these preparations were similar, but trophozoites were not counted. Higher numbers of trophozoites might have contributed to the antigenicity of the AIDS specimens. A further contributing factor may have been the storage of specimens: three of eight non-AIDS specimens had been stored at −70°C for 11 years and thawed and stored at −20°C on a number of occasions. Thus trophozoites may have been preferentially destroyed.

Analysis of *P carinii* by immunoblotting has not helped clinical decisions as studies have reported little difference in results obtained with serum from healthy subjects and patients with PCP,[34 36] or between recovered and rats with PCP.[36 37]

Conclusion

Reagents prepared from *P carinii* parasites have been of limited use in the serological diagnosis of PCP. For technical reasons cyst rich antigens have been selected for investigation and trophozoite antigens have not been properly evaluated. Cysts and trophozoites may not be antigenically identical and contamination of cyst antigens by trophozoites may explain some of the anomalies of serological testing. Future developments require a better understanding of *P carinii* antigens and more effort must be devoted to specific analysis of trophozoites. We believe that the more numerous trophozoites so often ignored in immunological studies may hold the key to effective diagnosis of PCP.

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