CHARACTERISATION AND TYPING OF MICRO-ORGANISMS

Differences exist in the immunoblotting profiles of cyst and trophozoite antigens of *Pneumocystis carinii*

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Summary. The antigenic profiles of *Pneumocystis carinii* trophozoites and cysts were compared by immunoblotting with hyperimmune rat sera against cyst and trophozoite antigens. Strong bands corresponding to proteins of 50–60 kDa and 104 kDa were demonstrated in cyst and trophozoite antigens by all antisera. Additional prominent proteins of 81 and 63 kDa and less prominent proteins of 88, 73, 69 and 37 kDa were found only in trophozoite antigen. The latter proteins were recognised by anti-trophozoite and anti-cyst antisera but the 81- and 63-kDa proteins were associated specifically with trophozoites. With cyst-rich antigen, antibodies to the 50–60-kDa protein were detected in only two of 14 sera from *P. carinii* pneumonia (PCP)-positive rats. With trophozoite-rich antigen, 11 of 24 rats with PCP and one of 18 PCP-negative animals had antibodies to both the 50–60 kDa and 104-kDa antigens. Antibodies to the 81- or 63-kDa antigens were demonstrated in 15 of 24 PCP-positive animals and none of the PCP-negative animals. The use of trophozoites rather than cysts increased the sensitivity of immunoblotting. As trophozoites predominate in PCP, antibody to trophozoite-specific antigens rather than common cyst and trophozoite antigens is likely to be a more useful marker of current infection.

Introduction

Investigations in *Pneumocystis carinii* pneumonia (PCP) have focused on the resistant and easily identified cyst rather than the fragile trophozoite,¹ even though the latter can account for up to 99% of the parasites in a bronchoalveolar lavage sample.² It has been assumed also that cysts and trophozoites are antigenically identical. Reports that trophozoites react in immunofluorescence tests with antisera against cysts^{3,4} indicate that they share common antigens, but such evidence does not exclude the possibility of unique cyst or trophozoite antigens. Nevertheless, the convention has been to characterise the antigens and antisera used in serological tests in terms of numbers of cysts or reactivity with cysts. Trophozoites have been largely ignored in such analyses.¹

Identification of specific antigens that are important in the immune response to *P. carinii* may allow the development of more specific immunological tests. Most Western immunoblotting studies have investigated material that either contains all stages of the parasite or has been prepared by methods that preferentially destroy trophozoites. Only one study

has attempted to investigate cyst and trophozoite antigens separately.⁴ In the present study, the immunoblotting profiles of cysts and trophozoites were studied to identify the usefulness of trophozoite antigens as markers of *P. carinii* infection in the rat model.

Materials and methods

P. carinii antigens

PCP was induced in male Sprague–Dawley rats weighing 150 g (Harlan Olac Ltd, Bicester) by twice-weekly intramuscular injection of 25 mg of hydrocortisone acetate (The Boots Company, Nottingham). Tetracycline (1 mg/ml) was added to the water supply to protect against bacterial infection. The rats were immunosuppressed for 6–8 weeks and developed auto-infection. They were killed by intraperitoneal injection of pentobarbitone sodium (Veterinary Drug Co., York), and exsanguinated. The serum was stored at –20°C. Giemsa, toluidine blue O⁵ and indirect immunofluorescence (IFA) with rat anti-*P. carinii* antiserum and fluorescein-conjugated anti-rat globulin (Dako, Denmark) were used to identify *P. carinii* cysts and trophozoites in lung imprints, washings and gradient fractions.

P. carinii was eluted from minced, heavily infected lung by repeated stirring for 30 min in sterile phosphate-buffered saline, pH 7.3, containing mixed antibiotics (PBSM).⁶ Eluates containing most parasites and least host cell contamination were washed three times in PBSM by centrifugation at 2000 *g* for 15 min and resuspended in PBSM containing 1 mM ethylene diamine tetraacetic acid (EDTA). Supernates from the first wash were centrifuged at 20 000 rpm for 20 min to deposit any remaining parasites. The ultracentrifuged (UC) pellet was stored at -20°C . The resuspended low speed deposits were forced three times through a fine bore needle and gross particulate contamination was removed before digestion with collagenase (Type A; Sigma) 1.5 mg/ml at 37°C for 1.5 h with gentle agitation. Discontinuous gradients of three 5-ml layers of Percoll (Pharmacia) in PBSM containing 1 mM EDTA (density 36%, 16% and 8%, respectively) were loaded with 5 ml of sample and centrifuged at 2000 *g* for 15 min. The interphases and intervening Percoll layers were collected individually from the top of the gradients by angled pipette, washed three times in PBSM and the relative numbers of cysts and trophozoites were counted. If separation was inadequate, fractionation was repeated on gradients of the same composition. Fractions were classified according to the relative numbers of cysts and trophozoites and stored at -20°C . Fourteen preparations of *P. carinii* antigens were made and examined: five UC pellets (100% trophozoite); five containing $\geq 90\%$ trophozoites; two containing 50–60% trophozoites and two containing 80% cysts.

Control antigen

Lungs from rats which had received immunosuppression to induce PCP, but were negative for *P. carinii* by Giemsa and toluidine blue O staining at post-mortem examination, were processed as described for *P. carinii*-infected lung. To ensure recovery of enough protein for control antigen, only two fractions were collected: one from the sample application level to the 16% interphase; the other comprised the 16% and 36% layers (equivalent to trophozoite and cyst rich fractions above). After washing three times in PBSM they were examined by Giemsa and toluidine blue O staining and by IFA for *P. carinii* and stored at -20°C .

Antisera

Nine rats were immunised: three with a fraction containing 80% cysts; three with a fraction containing $\geq 90\%$ trophozoites; and three with a fraction containing 50% cysts and trophozoites. Hyperimmune sera were prepared by inoculating rats with standardised amounts of protein with complete Freund's adjuvant. Antigen protein concentration was measured at 280 nm and 100 μg in 0.5 ml of PBS was then mixed with an equal volume of adjuvant and

injected intramuscularly at four sites (c. 250 μL /site). Booster injections of 100 μg of protein, the first in incomplete Freund's adjuvant, the others without adjuvant, were given three times at 4-week intervals; the animals were exsanguinated 2 weeks later. Sera from immunosuppressed rats were also used; 24 from animals that developed PCP and 18 from animals with no histological evidence of PCP.

Immunofluorescence

Antibodies were measured in hyperimmune sera by an indirect immunofluorescence technique reported previously.⁶ The antigen was prepared from unfractionated *P. carinii* washings. Hyperimmune sera were tested at dilutions ranging in two-fold steps from 1 in 32 to 1 in 1024. After addition of fluorescein-conjugated anti-rat immunoglobulin (Dako) their specificity for cyst and trophozoite antigens was assessed and compared.

SDS-PAGE and immunoblotting

Antigens were boiled for 5 min in a lysis buffer containing SDS 2%, 0.06 M Tris-HCl (pH 6.8), 2-mercaptoethanol 5%, glycerol 10% and bromophenol blue 0.001%. Electrophoresis was performed at a constant current of 6 mA for 16 h with a 3.5% stacking gel and a 12% separating gel in a discontinuous SDS buffer system.⁷ A mol. wt standard mixture containing myosin, β -galactosidase, phosphorylase B, bovine albumin, egg albumin and carbonic anhydrase (Sigma) was run as a marker on the gels. Separated proteins were transferred to nitrocellulose sheets⁸ (pore size 0.45 μm) in an LKB 2005 Transphor Electroblothing unit at 4°C for 2 h at 0.8 A. Lanes containing the mol. wt standards were stained with amido black (Sigma) and used to construct a calibration curve. The remainder of the sheet was blocked in PBS containing non-fat milk 5%. Thereafter, strips were incubated for 16 h at room temperature with a 1 in 100 dilution of hyperimmune rat serum or a 1 in 20 dilution of immunosuppressed rat serum in PBS containing Tween 20 0.05% (PBST) and non-fat milk 5% (PBSN). Strips were washed five times in PBST then incubated for 2 h at room temperature in a 1 in 750 dilution of goat anti-rat IgG peroxidase conjugate (Sigma) in PBSN. After five washes in PBST and two in PBS strips were immersed in substrate (4-chloro-1-naphthol 0.06% in methanol, hydrogen peroxide 0.014% in PBS) for 10 min.

Results

Fractionation of *P. carinii* cysts and trophozoites

The recovery of *P. carinii* cysts and trophozoites from a Percoll gradient is summarised in table I. Cysts and trophozoites could be counted simultaneously by IFA. Cysts were identified easily and the relative pro-

Table I. Distribution of *P. carinii* cysts and trophozoites from a discontinuous Percoll gradient

Fraction	Number of parasites counted	Number (%) of cysts	Number (%) of trophozoites
Unfractionated sample	1057	127 (12)	930 (88)
<i>Percoll gradient</i>			
1. (0–8% interphase)	838	10 (1.2)	828 (98.8)
2. (8% layer)	1661	60 (3.6)	1601 (96.4)
3. (8–16% interphase)	803	106 (13.2)	697 (86.8)
4. (16% layer)	414	266 (64.3)	148 (35.7)
5. (16–36% interphase)	656	566 (86.3)	90 (13.7)
6. (36% layer)	278	233 (83.8)	45 (16.2)

portions of cysts in each fraction were confirmed by toluidine blue O staining. Trophozoites were more difficult to count and numbers were probably underestimated. Giemsa staining was useful in estimating host cell contamination but was abandoned for confirming trophozoite counts because of difficulties in identifying parasites. We were unable to obtain preparations that contained only cysts or trophozoites. Fractions one and two were enriched with trophozoites ($\geq 90\%$). Fractions three and four contained a mixture of trophozoites and cysts. Fractions five and six were relatively enriched with cysts ($\geq 80\%$). Host cell contamination was present in all fractions but was particularly associated with fraction five.

Separation was generally consistent between individual gradients, and fractions with similar proportions of cysts and trophozoites were pooled. Refractionation on second gradients was not pursued because although fractions were cleaner, the yield of parasites was very poor. The pellets obtained by ultracentrifugation provided the purest trophozoite

preparations: although they contained host cell contamination material no cysts were observed. Irreversible clumping of parasites occurred when material was frozen and thawed, making separation impossible. Collagenase digestion and gradient fractionation did not seem to cause deterioration of the parasites; morphology was retained and reactivity in IFA was enhanced.

Hyperimmune rat sera

The final IFA antibody titres of sera from immunised rats ranged from 256 to 1024. There was no reactivity against rat cells; therefore, sera were not absorbed with normal rat lung tissue. Cysts and trophozoites were stained to the same degree by all antisera. Only one anti-trophozoite serum appeared to react more strongly with trophozoites, with only a patchy IFA pattern with cysts.

In Western blot analyses, all hyperimmune rat sera recognised more proteins in trophozoite-rich prepara-

Fig. 1. Western immunoblot of: **A**, $\geq 90\%$ trophozoite antigen; **B**, UC pellet antigen; **C**, cyst-rich antigen, all with hyperimmune rat sera. Lane 1, anti-trophozoite antiserum; 2, anti-cyst antiserum; 3, mixed antiserum.

Table II. Bands detected by immunoblotting cyst and trophozoite-rich preparations with hyperimmune rat sera

Antiserum	Antigen	Bands (kDa) detected by											
		both antisera										anti-trophozoite antiserum only	
		124	104	88	73	69	60	55	50	42	37	81	63
Anti-trophozoite	Cyst	+	+++	—	—	—	+	+	+	—	—	—	—
	Trophozoite	++	+++	+	+	+	++	++	++	+	+	++	+++
	UC	+	++	+/-	—	—	+	+	+	—	—	++	++
Anti-cyst	Cyst	+	++	—	—	—	+	+	+	+	—	—	—
	Trophozoite	++	+++	+	+/-	+/-	++	++	++	+	+	—	—
	UC	+	++	+/-	—	—	+	+	+	—	—	—	—

UC, ultracentrifuge pellet (small trophozoites). Intensity of bands: +++ very strong; ++ strong; + faint; +/- very faint; — absent.

tions than in cyst-rich preparations. Although preparations with similar proportions of cysts and trophozoites and equivalent protein content showed some variation in intensity of the bands, reproducible banding patterns were established for each antigen (fig. 1, table II). Cyst preparations produced fewest protein bands and all of them were common to bands found in the trophozoite preparations. Bands at 104 kDa and a broad band between 50 and 60 kDa were prominent and recognised equally by anti-trophozoite and anti-cyst antisera (fig. 1, table II). Bands in the 60–90 kDa range were detected in preparations containing $\geq 90\%$ trophozoites but not in cyst-rich preparations and were identified more strongly by anti-trophozoite than by anti-cyst antisera. Proteins of 81 and 63 kDa were particularly prominent (fig. 1, table II). UC pellet preparations produced similar but fewer bands than fractions containing $\geq 90\%$ trophozoites and again the 81- and 63-kDa bands were the most prominent and were recognised

most intensely by anti-trophozoite antisera (fig. 2, table II).

Hyperimmune rat sera produced weakly staining blots with control rat lung preparations. Only bands at 104 kDa and 50–60 kDa were identified and these were found in both control fractions and recognised equally by anti-trophozoite and anti-cyst antisera. The bands were very much weaker than in *P. carinii* preparations. Bands in the 60–90 kDa range were not identified in any of the control preparations.

Immunosuppressed rat sera

Sera from immunosuppressed rats produced weaker staining blots than sera from immunised animals and recognised a restricted range of proteins. With cyst-rich antigen, only two of 14 rats in which *P. carinii* was demonstrated in lung tissue had detectable antibodies: in both cases antibody recognised the 50–60-kDa protein (table III). With trophozoite-rich and UC

Fig. 2. Western immunoblot of: **A**, $\geq 90\%$ trophozoite antigen; **B**, UC pellet antigen. Lanes 1 and 2, anti-trophozoite antisera; 3 and 4, anti-cyst antisera; 5 and 6, mixed antisera.

Table III. Bands detected in cyst and trophozoite-rich *P. carinii* antigens with sera from immunosuppressed rats

Antigen	Number of rats	Number of rats with antibody to proteins (kDa)					
		124	104	81	63	50–60	None
Cyst	With PCP	14	0	0	0	2	12
	Without PCP	11	0	0	0	0	11
Trophozoite	With PCP	24	2	16	9	12	4
	Without PCP	18	1	1	0	1	17
UC	With PCP	20	5	2	9	12	8
	Without PCP	13	0	0	1	1	0

PCP, *P. carinii* identified at necropsy; UC, ultracentrifuge pellet (small trophozoites).

pellet antigens sera were much more reactive and proteins of 124, 104, 81, 63 and 50–60 kDa were recognised (table III). There was a strong association between recognition of bands and demonstration of *P. carinii* in the lung (table III). With trophozoite-rich antigen, antibodies to both the 104- and 50–60-kDa proteins were identified in serum from 11 of 24 PCP-positive rats and one of 18 rats with no evidence of *P. carinii* in the lung. Fifteen of 24 PCP-positive animals had antibodies to the 63- or 81-kDa proteins. This included six with antibodies only to the 63-kDa proteins, three with antibodies only to the 81-kDa protein and six with antibodies to both. None of 18 PCP-negative animals had antibodies to either of these proteins. In the case of the UC pellet antigen, demonstration of antibody to the 63- or 81-kDa proteins was more strongly associated with evidence of *P. carinii* in the lung (table III). Of 20 PCP-positive animals, only two had antibodies to both the 104- and 50–60-kDa proteins, whereas 16 had antibodies to the 63- or 81-kDa proteins, including seven with antibodies only to the 63-kDa protein, four with antibodies only to the 81-kDa protein and five with antibodies to both. Two of 13 PCP-negative rats had detectable antibody; one to the 63-kDa protein and the other to the 81-kDa protein.

Discussion

Various methods have been used to separate and purify *P. carinii* cysts and trophozoites from infected lung^{3,4,9,10} but separation of trophozoites from cysts has proved to be very difficult and has only rarely been pursued.^{4,11} Pure trophozoite preparations have reportedly been obtained from rat bronchoalveolar lavage^{4,11} but lung homogenates were used in this study, as the yield of parasites is much greater.^{10,11} Like others we were unable to obtain pure trophozoite or cyst preparations^{3,9} and used preparations that were enriched with trophozoites or cysts and relatively free of host cells.

The immunoblotting patterns of cyst and trophozoite antigens were significantly different. Hyperimmune rat sera recognised two major proteins of 104 and 50–60 kDa in cyst and trophozoite preparations, and an additional six bands with mol.

weights between 60 and 90 kDa were found only in preparations of trophozoites. This may reflect limitations in the antigenic repertoire of cysts or may be the result of poor solubilisation of resistant cysts compared to fragile trophozoites. Only one other study has attempted to isolate trophozoites and cysts and compare antigenic profiles by immunoblotting with anti-trophozoite and anti-cyst rabbit antisera.⁴ These workers also reported differences in the antigen profiles of cysts and trophozoites: two major bands of 110 and 45 kDa and a secondary band of 50 kDa were associated with cysts; proteins of 30 and 48 kDa and minor bands of 60, 50 and 90 kDa were associated with trophozoites. As anti-trophozoite and anti-cyst antisera showed the same pattern of reactivity, but a stronger intensity in species-specific reactions, the authors concluded that cysts and trophozoites had identical antigens present in different proportions. A high degree of cross-reactivity with the hyperimmune sera was found also in the present study but the 63- and 81-kDa proteins were detected only in trophozoite-rich antigen with anti-trophozoite antisera, suggesting that they are trophozoite specific.

The 104- and 50–60-kDa bands detected in cyst and trophozoite-rich antigens by anti-cyst and anti-trophozoite antisera are probably identical to the 116-, 50- and 45-kDa proteins which have been detected consistently in rat *P. carinii* in immunoblotting studies with sera from immunised rabbits.^{12,13} Bands corresponding to proteins of 60–90 kDa have been demonstrated also, but they varied in clarity and intensity and were overlooked in favour of the more dominant bands in a study in which the parasites were not gradient-derived.¹³ Although the 63- and 81-kDa bands were detected in all the preparations that contained $\geq 90\%$ trophozoites, the intensity of the bands varied; they were not detected in lung homogenates which had not been purified on Percoll gradients. This suggests that concentration is important, since the effect of Percoll gradient separation is to increase the numbers of parasites and reduce the host cell contamination in the preparations.⁹ The 63-kDa protein ran very close to the 50–60-kDa bands common to all preparations and was sometimes masked in intensely stained blots. The UC pellet preparation, which was completely devoid of cysts, confirmed the

specificity of the 63- and 81-kDa bands as markers of trophozoites.

There is some evidence that these bands may be specific markers of PCP. We and others have detected proteins of 50–60 and 110 kDa in control rat antigen preparations,¹⁴ suggesting they arise from rat proteins or cysts in normal lung. Bands in the 60–90 kDa range were not detected in control antigen preparations. In an interesting study, lavage fluid from infected rats, centrifuged at 10 000 *g* to remove any cysts, was probed with a pool of five anti-*P. carinii* monoclonal antibodies and bands of 66 and 90 kDa were found in addition to the major bands of 110–116 and 50–60 kDa.¹² Although a faint 66-kDa band was detected in lavage supernate controls, suggesting that the band may not be specific for *P. carinii*, these antigens may be useful markers of early PCP, since they were detected in the absence of cysts. Furthermore, the study confirmed the association of these bands with trophozoites, as soluble antigen is much more likely to be derived from them than from cysts.

In some studies, immunosuppression of rats to induce PCP prevented or suppressed antibody production and positive blots were obtained only when animals were allowed to recover.¹⁵ We were able to detect antibody in sera from severely immunosuppressed rats, but only with trophozoite-rich antigen. The pattern of antigen recognition varied in individual animals; the 104- and 50–60-kDa proteins that were detected are probably the same as the dominant 116-, 50- and 45-kDa antigens identified by rat sera in other studies.¹ Proteins of 81 and 63 kDa were also detected. Antibodies were found to one or other of these antigens in 15 of 24 PCP-positive animals but in none of the 18 PCP-negative animals. Antibody to 60–92-kDa antigens has been reported in serum from exposed and rats that recovered from PCP but not from normal rats, whereas proteins of 116, 50 and 45 kDa were recognised equally by normal, exposed and recovered rats.¹ These results indicate

that the latter antibodies are markers of past as well as current infection, and that antibodies directed against the 81- and 63-kDa antigens may be more specific markers of current infection. This pattern of antibody development is consistent with the 104- and 50–60-kDa antigens being common markers of cysts and trophozoites and the 81- and 63-kDa antigens being trophozoite specific, since trophozoites predominate in active infection.

Effective treatment of human PCP depends upon early diagnosis. The demonstration of *P. carinii* in bronchoalveolar lavage or induced sputum has improved with the availability of monoclonal antibodies and the polymerase chain reaction (PCR). Diagnosis with PCR has the advantage that it is sensitive and may be positive before there is a serological response. However, methods demonstrating *P. carinii* rely on potentially hazardous invasive procedures to obtain suitable specimens.¹⁶ Furthermore, the presence of *P. carinii* does not always equate with clinically significant disease,^{17–19} whereas the demonstration of a serological response can be more helpful.^{6,17,18} Although serological testing is simpler, the available tests are of limited value because of poor sensitivity or specificity.^{1,6} Many investigators have highlighted the need to identify specific antigens which might be exploited for diagnostic purposes. So far, Western immunoblotting studies have identified reactive proteins of 40, 66 and 82 kDa in human *P. carinii* but antibodies to these proteins are found in serum from healthy subjects as well as PCP patients, so that they are of limited diagnostic use.¹ The results described in this paper indicate that serological tests based on the detection of antibodies directed against trophozoite antigen might be diagnostically useful.

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