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, even though the latter can account for up to 99% of the parasites in a bronchoalveolar lavage sample. It has been assumed also that cysts and trophozoites are antigenically identical. Reports that trophozoites react in immunofluorescence tests with antisera against cysts 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.

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. 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 pentabarbitone sodium (Veterinary Drug Co., York), and exsanguinated. The serum was stored at −20°C. Giemsa, toluidine blue O 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 tetracetic acid (EDTA). Supernates from the first wash were centrifugated at 20000 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 centrifugated 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% trophozoites); five containing ≥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 ≥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 Electroblotting 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-naphthal 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

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

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 (≥ 90%). Fractions three and four contained a mixture of trophozoites and cysts. Fractions five and six were relatively enriched with cysts (≥ 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-
Table II. Bands detected by immunoblotting cyst and trophozoite-rich preparations with hyperimmune rat sera

<table>
<thead>
<tr>
<th>Antiserum</th>
<th>Antigen</th>
<th>Bands (kDa) detected by</th>
<th>both antisera</th>
<th>anti-trophozoite antisera only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>124 104 88 73 69 60 55 42 37</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-trophozoite</td>
<td>Cyst</td>
<td>- - - + + + + + + - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trophozoite</td>
<td>+ + + + + + + + + + - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>+ + + + +/ - - - - - - - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Anti-cyst</td>
<td>Cyst</td>
<td>+ + + + + + + + + + + + - - -</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Trophozoite</td>
<td>+ + + + + + + + + + + + + + + +</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>UC</td>
<td>+ + + + +/ - - - - - - - - -</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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

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 fewer 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 >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 >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
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 lungs, but separation of trophozoites from cysts has proved difficult and has only rarely been pursued. Pure trophozoite preparations have reportedly been obtained from rat bronchoalveolar lavage, but lung homogenates were used in this study, as the yield of parasites is much greater. Like others we were unable to obtain pure trophozoite or cyst preparations 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 marker proteins of 104 and 50-60 kDa in cyst and trophozoite preparations, and an additional six bands with mol.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Number of rats</th>
<th>Number of rats with antibody to proteins (kDa)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyst</td>
<td>With PCP 14</td>
<td>0 0 0 0 2 12</td>
</tr>
<tr>
<td></td>
<td>Without PCP 11</td>
<td>0 0 0 0 0 11</td>
</tr>
<tr>
<td>Trophozoite</td>
<td>With PCP 24</td>
<td>2 16 9 12 13 4</td>
</tr>
<tr>
<td></td>
<td>Without PCP 18</td>
<td>1 1 0 0 1 17</td>
</tr>
<tr>
<td>UC</td>
<td>With PCP 20</td>
<td>5 2 9 12 8 3</td>
</tr>
<tr>
<td></td>
<td>Without PCP 13</td>
<td>0 0 1 1 0 11</td>
</tr>
</tbody>
</table>

PCP, *P. carinii* identified at necropsy; UC, ultracentrifuge pellet (small trophozoites).
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, 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 10000 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. 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. 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. 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 recovered rats, whereas proteins of 116, 50 and 45 kDa were recognised equally by normal, suppressed rats, but only with trophozoite-rich antibody to 60-92-kDa antigens has been reported in sera from exposed and rats that recovered from PCP animals but in none of the 18 PCP-negative animals. We and others have detected dominant 116-, 50- and 45-kDa antigens identified by sera from exposed and rats that recovered from PCP.

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