Opsonophagocytosis of *Pneumocystis carinii*

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**Summary.** The interaction of *Pneumocystis carinii* purified from rat lungs with rat peritoneal macrophages and human circulating polymorphonuclear leucocytes was studied by amplified chemiluminescence and examination of stained cytospin preparations. A polyclonal rat antiserum to *P. carinii* was opsonic with both types of phagocyte. Complement had no opsonic properties alone but produced a synergic effect in combination with antibody.

**Introduction**

*Pneumocystis carinii*, a eukaryotic organism of uncertain taxonomic status, can cause a severe pneumonia in patients immunosuppressed for various reasons. Two main morphological stages of the parasite are known—trophozoite and cyst. Defects of either humoral or cell-mediated immunity predispose to *P. carinii* pneumonia (PCP). Chronic granulomatous disease, in which the defect is one of phagocyte function, is very seldom associated with PCP.

PCP is the commonest life-threatening secondary infection associated with the acquired immune deficiency syndrome (AIDS). It is not yet clear why there is such a strong association. Loss of CD4+ lymphocytes may result in defects in B cell help and a lack of cytokines such as interferon-γ and interleukin 2, with subsequent depression of natural killer cell and macrophage function. A loss of specific anti-pneumocystis antibody coincided with the development of PCP in a cohort of AIDS patients, and recovery of PCP antibody titres occurred in those who responded to chemotherapy.

The exact role of phagocytic cells and the relative importance of polymorphonuclear leucocytes (PMNL) and macrophages (MP) in immune defence against PC is unclear. Histological examination of human and animal cases of PCP shows the lungs to be infiltrated with mononuclear cells but few PMNL; the foamy alveolar exudate characteristic of this condition is believed to be composed of coalesced MP. Bacterial pneumonitis in rats induced by *Pseudomonas aeruginosa* (which induced a PMNL infiltrate) but not *Staphylococcus aureus* (which did not) protected against induction of PCP in the animals. Dissemination of *P. carinii* outside the lung is rare and circulating PMNL and monocytes may play a part in limiting infection to the lung. In AIDS patients with PCP, an increased proportion of PMNL in bronchoalveolar lavage specimens correlates with a worse clinical outcome. In-vitro studies have demonstrated phagocytosis of *P carinii* by MP and suggested a role for antibody in intracellular killing. Congenital absence of complement components is not associated with PCP and *P. carinii* cells mixed with specific antibody and complement in vitro, show no loss of viability, as determined by dye exclusion.

We have studied the interaction of PMNL and MP with *P. carinii* by amplified chemiluminescence and examination of fixed, stained preparations, and assessed the role of specific antibody and complement as opsonins.

**Materials and methods**

**Isolation of *P. carinii***

PCP was induced in 200-g female Sprague-Dawley rats (Harlan OLAC) by administration of oral betamethasone (Glaxo) 10 mg/L and tetracycline HCl (Sigma) 1 g/L in their drinking water. After 8–12 weeks, individual animals which were heavily infected with *P. carinii* were recognised by the presence of central cyanosis and raised respiratory rate. Lungs were excised and homogenised and *P. carinii* cysts and trophozoites were separated from contaminating rat cells by unit gravity sedimentation. Samples of *P. carinii* suspension in phosphate-buffered saline (PBS) with dimethyl sulphoxide 10% were stored at −70°C until the day of use. In the current study, two batches of purified parasites from separate animals were prepared and stored in this way. Cyst and trophozoite densities in these batches were estimated as previously described.

In batch 1, the trophozoite:cyst ratio was 13:1; in batch 2 the ratio was 31:1.

**Immune serum**

Specific antiserum to *P. carinii* was raised in 800-g male Lewis rats. One ml of PBS containing 10⁹ *P.
carinii (cysts and trophozoites) was emulsified with 1 ml of complete Freund's adjuvant (Oxoid) and each of three rats was inoculated subcutaneously at four separate sites with 0.1 ml of emulsion. After 2 weeks, the procedure was repeated but with incomplete Freund's adjuvant. After a further 2 weeks, the rats were exsanguinated under terminal ketamine anaesthesia by the axillary pocket technique. The blood was allowed to clot at 37°C, and separated serum was pooled and heated to 56°C for 30 min. One-ml volumes were stored at -20°C until the day of use.

Antibody titres were determined by indirect immunofluorescence (IFA). Purified P. carinii were dried on to IFA multiwell slides, fixed in methanol for 2 min and dried. Dilutions of immune serum in PBS were applied to the wells for 30 min. After a 5-min wash in PBS, fluorescein-conjugated caprine anti-rat IgG (Sigma) diluted 1 in 10 in PBS was applied to each well for 30 min. Slides were then washed in PBS and viewed with an ultraviolet epifluorescence microscope. Antibody titres were the highest dilution that yielded + ++ cyst fluorescence. Two batches of immune serum were prepared; the first was used in preliminary experiments, and the second, which had a titre of 400, was used to generate the experimental data presented here. Absorbed immune serum was prepared by diluting 0.5 ml of immune serum with 2 ml of PBS, separating this into two equal aliquots and suspending 10^9 P. carinii in one. Both aliquots were then incubated on a roller at 37°C for 60 min. Parasites were pelleted by centrifugation (13 000 g, 5 min) and the absorbed serum was aspirated. Absorbed and control immune sera were stored at -20°C until the day of use.

Phagocyte preparation

PMNL were separated from the peripheral blood of apparently healthy adults; 20 ml of blood, anticoagulated with 400 units of preservative-free heparin, was mixed with 10 ml of Dextran 110 injection BP (Fisons) in a syringe clamped in a vertical position. After 45 min at room temperature, the top layer of plasma, leucocytes and platelets were aspirated and centrifuged at 300 g for 5 min. Residual red cells were lysed by suspending the cell pellet in 10 ml of a solution of 17 mm ammonium chloride and 140 mm Tris(hydroxymethyl)aminomethane (pH 7.65) and incubating at 37°C on a roller for 15 min. Cells were harvested and washed three times in Hank's Balanced Salt Solution supplemented with 20 mm N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) pH 7.3 (HBSS) and resuspended in the same medium supplemented with 1% fetal calf serum (FCS; Gibco) to a concentration of 2 x 10^6/ml; PMNL suspensions were incubated on a roller at 37°C for 1 h before use.

MP were elicited in the peritoneal cavity of male Lewis rats by injection of 20 ml of Thioglycollate Broth (Oxoid). After 5 days, animals were killed and the peritoneal cavity was lavaged with 20-ml volumes of HBSS supplemented with gelatin (Difco) 0.1% (gHBSS). Harvested cells were layered on to 10-ml volumes of Histopaque 1077 (Sigma) and centrifuged at 700 g for 30 min. The top layer of cells, free of PMNL and erythrocytes, was aspirated and washed twice in gHBSS and resuspended in the same medium supplemented with FCS 1% to a concentration of 4 x 10^6 MP/ml; cells were incubated on a roller at 37°C for 1 h before use. MP purity was always >99% as judged by examination of Giemsa-stained cytospin preparations.

Opsonisation

One-ml samples of P. carinii suspensions were thawed, washed with PBS to remove dimethyl sulphoxide and resuspended to their original volume in PBS. They were incubated at 37°C for 20 min with different concentrations of immune serum or freshly reconstituted guinea-pig complement (SeraLab Ltd, Crawley Down, Sussex), or both, as indicated in the Results. HBSS was used as the diluent. After centrifugation (13 000 g, 5 min) P. carinii cells were washed twice in HBSS, resuspended to a final volume of 0.5 ml and kept on ice until used.

Chemiluminescence

PMNL (0.5 ml; 2 x 10^6 PMNL/ml) were mixed with 0.9 ml 0.02 mm 5-amino-2,3-dihydro-1, 4- phthalazinediarnide (Luminol; Sigma) in HBSS in a plastic cuvette. Baseline light output was measured at 37°C with an LKB 1250 luminometer (Bio-Orbit, Turku, Finland) with a water-jacketed cuvette holder. Opsonised or unopsonised P. carinii cells (0.2 ml) were added to the cuvette and serial luminometer readings were recorded at 4-min intervals over 32 min. MP CL was assayed in an identical manner except that samples of MP contained 4 x 10^6 cells/ml and 0.2 mm lucigenin (bis-N-methylacridinium nitrate; Sigma) in gHBSS was used in place of luminol. For luminol-dependent PMNL CL, peak values were used to assess opsonisation; for lucigenin-dependent MP CL, reaction kinetics were slower with a broad peak and the initial slope of the CL curve was used. Both parameters have been used for this purpose.4,5,16

Phagocytic indices

P. carinii cells (0-2 ml), phagocytes (0-5 ml; 2 x 10^6 PMNL/ml or 4 x 10^6 MP/ml) and HBSS/gHBSS (0.3 ml) were mixed and incubated at 37°C. Phagocyte-to-parasite ratios for each set of experiments are indicated in the Results. After 5 and 10 min, 0.1-ml samples were mixed with 0.2 ml of ice-cold HBSS/gHBSS. A sample of this suspension was spun onto a glass microscope slide with a Shandon Cytospin 2 centrifuge (Shandon Scientific, Runcorn, Cheshire). Slides were dried on a warm block, fixed in methanol for 5 min and stained in Giemsa (BDH; 1 in 60 in distilled water) for 60-120 min. On each slide, 200
phagocytes were assessed and the percentage containing one or more recognisable parasites was determined; in the great majority, only one intracellular organism could be discerned.

**Effect of complement on viability**

*P. carinii* cells mixed with immune serum 20% and freshly reconstituted guinea-pig complement 20% were incubated at 37°C for 20 min. After washing twice in HBSS, samples from each were mixed with fluorescein diacetate and ethidium bromide and then viewed by epifluorescence microscopy. The proportions of viable (green-fluorescing) and dead (red-fluorescing) *P. carinii* cells were determined. Complement-free controls were treated in an identical manner.

**Electronmicroscopy**

*P. carinii* cells opsonised with immune serum (1%) and complement (20%) were mixed with PMNL (PMNL: *P. carinii* cell ratios as for CL experiments) and incubated at 37°C for 1 h. Glutaraldehyde (BDH) was added to a final concentration of 2%. After overnight fixation, samples were pelleted and washed in cacodylate buffer (BDH). Osmium tetroxide (BDH) was added to a final concentration of 2% and the mixture was held at 4°C for 1 h. Cells were rinsed in distilled water, dehydrated in several changes of increasing concentrations of alcohol and mounted in araldite resin. Ultrathin sections were cut by a Cambridge Huxley Mark 2 ultramicrotome (Cambridge Medical Instruments Ltd, Melbourne, Royston, Herts) and placed on copper grids. After staining with uranyl acetate 2.5% for 5 min followed by Reynold's lead citrate for a further 5 min, sections were dried and examined by transmission electronmicroscopy with a JEOL-100C instrument (JEOL UK Ltd, Grove Park, Colindale, London).

**Results**

**Effect of opsonins on *P. carinii* viability**

The effect of immune serum and complement on *P. carinii* viability was assessed by fluorescein diacetate-ethidium bromide staining. Viability was unaffected by exposure to these opsonins, remaining >95%.

**Effect of opsonins on *P. carinii* phagocytosis**

The interaction of *P. carinii* cells with immune serum, complement and human PMNL assessed by luminol-amplified CL is shown in fig. 1 and table I. Both antibody and complement were required for optimal PMNL CL production; antibody or complement alone resulted in lower PMNL CL peaks. Absorption of immune serum with purified *P. carinii* cells reduced PMNL luminol-amplified CL by 57%. A similar pattern of results was obtained with a microscopic assay of phagocytosis (table I); the addition of complement, not opsonic alone, significantly increased the opsonic activity of heat-inactivated immune serum. Ingestion of *P. carinii* by PMNL was associated with marked vacuolation of the phagocytes and the appearance of *P. carinii* inside phagosomes (fig. 3).

With luminol as amplifier, CL signals from MP stimulated with opsonised *P. carinii* were almost undetectable. Lucigenin-dependent MP CL signals were measurable but were considerably weaker with less defined peaks than luminol-amplified CL signals from stimulated PMNL (fig.2). Better discrimination
Table I. Phagocytosis of *P. carinii* by human PMNL assessed by microscopy and luminol-amplified CL

<table>
<thead>
<tr>
<th>Opsonic conditions</th>
<th>Phagocytic index* (%) at</th>
<th>Peak PMNL CL† (mV)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Immune serum 20%, complement 20%</td>
<td>36-5</td>
<td>33-5</td>
</tr>
<tr>
<td>Immune serum 20%</td>
<td>16-5</td>
<td>18-5</td>
</tr>
<tr>
<td>Immune serum 1%, complement 20%</td>
<td>17-5</td>
<td>17-5</td>
</tr>
<tr>
<td>Immune serum 0.2%, complement 20%</td>
<td>8-5</td>
<td>10-0</td>
</tr>
<tr>
<td>Complement 20%</td>
<td>4-5</td>
<td>5-5</td>
</tr>
<tr>
<td>Unopsonised</td>
<td>4-0</td>
<td>3-5</td>
</tr>
</tbody>
</table>

*P. carinii: PMNL ratio, 27:1; trophozoite:cyst ratio, 31:1; data representative of two separate experiments.
†P. carinii:PMNL ratio, 58:1; trophozoite:cyst ratio, 13:1; data representative of five separate experiments.

between the opsonic activity of antibody and complement was found by analysing the initial slope of the CL curve rather than its peak. Immune serum alone, even at concentrations as low as 0-2%, did effect opsonisation of *P. carinii*, and this was enhanced by the addition of complement (table II). Absorption of the rat immune serum with purified *P. carinii* cells reduced lucigenin-amplified MP CL by 74%. There was good correlation between the results obtained by lucigenin-dependent MP CL and those obtained by microscopy (table II). Again, MP ingestion of *P. carinii* cells was accompanied by extensive vacuolation.

Discussion

Evidence is lacking that *P. carinii* is susceptible to the direct action of antibody or complement. The principle host defence mechanism appears to be intracellular killing by phagocytic cells. *P. carinii* does not appear to be an intracellular parasite; once within a phagocyte, killing and digestion occur readily.† What remains unclear is the relative importance of different phagocytic cells (polymorphs, monocytes, macrophages) in defence against *P. carinii* infection, and the role of antibody, complement, T cells and cytokines in focusing and augmenting this phagocytic activity.

Epidemiological evidence suggests that both specific humoral and cell-mediated immunity play a part in preventing the development of PCP. Both children with isolated congenital defects of immunoglobulin production and children with T-cell abnormalities are susceptible to PCP. The cellular infiltrate in these conditions is predominantly mononuclear, with lymphocytes, macrophages and, in infantile marasmic PCP, plasma cells. Although *P. carinii* cells have been shown to adhere readily to the surface of MPs and other cells in vitro, pneumocystis antibody is required

Table II. Phagocytosis of *P. carinii* by rat peritoneal MPs assessed by microscopy and lucigenin-amplified CL

<table>
<thead>
<tr>
<th>Opsonic conditions</th>
<th>Phagocytic index* (%) at</th>
<th>MP CL† (mV/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Immune serum 20%, complement 20%</td>
<td>20-5</td>
<td>45-0</td>
</tr>
<tr>
<td>Immune serum 20%</td>
<td>20-5</td>
<td>36-0</td>
</tr>
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<td>Immune serum 1%</td>
<td>18-0</td>
<td>20-5</td>
</tr>
<tr>
<td>Immune serum 0.2%</td>
<td>13-5</td>
<td>17-0</td>
</tr>
<tr>
<td>Complement 20%</td>
<td>8-5</td>
<td>8-0</td>
</tr>
<tr>
<td>Unopsonised</td>
<td>8-0</td>
<td>7-0</td>
</tr>
</tbody>
</table>

*P. carinii: MP ratio, 39:1; trophozoite:cyst ratio, 31:1; results representative of two experiments.
†P. carinii:MP ratio, 27:1; trophozoite:cyst ratio, 13:1; results representative of three experiments.
Fig. 3. Electronmicrographs showing PMNL containing *P. carinii* cysts. (A) PMNL containing a cyst; (B) similar to (A) with indications of lysosomal fusion with the phagosome; (C) PMNL containing a trophozoite with typical tubular expansions (filopodia), indicated by arrowheads, extending from its external membrane. Bar, 1 μm.
for ingestion and intracellular killing by freshly isolated alveolar or peritoneal MP.\(^{11}\) These studies indicate that complement is not necessary for antibody-mediated ingestion. However, Von Behren and Pesanti,\(^{10}\) in another in-vitro study, demonstrated that newly explanted alveolar MP did not ingest \textit{P. carinii} but both freshly isolated peritoneal MP and alveolar MP which had been cultured for 2 days could phagocytose \textit{P. carinii} in the absence of serum opsonins. As antibody or complement were not employed in these experiments, it is impossible to say whether opsonisation would have improved this phagocytic activity. Our results with PMNL and MP confirm the importance of antibody for the phagocytosis of \textit{P. carinii}. Complement appeared to enhance antibody-mediated opsonisation but alone was not an opsonin for \textit{P. carinii}.

Given the links between cell-mediated immunity and protection against PCP, it would be interesting to investigate the effect of stimulation of phagocytes by cytokines such as interferon-\(\gamma\), interleukin 2, tumour necrosis factor and colony stimulating factors, both on their capacity to ingest \textit{P. carinii} and their requirement for opsonins to enhance phagocytosis.

As well as being protective, phagocytic cells can mediate tissue damage in infection through the release of lysosomal enzymes and highly reactive oxygen species.\(^{18}\) CL is a measure of the generation of these oxygen moieties which occur both intracellularly and extracellularly.\(^{19}\) Mason \textit{et al.}\(^{9}\) have recently reported an association between increased numbers of PMNL in bronchoalveolar lavage samples from patients with PCP and respiratory failure and risk of death. They and others\(^ {9}\) have commented on the similarities between PMNL in PCP and in other conditions such as adult respiratory distress syndrome, and postulated a role for PMNL in the pathogenesis of lung damage in these conditions. These speculations and the possible role of antibody and complement in promoting PMNL-mediated lung damage in PCP require further study. In rats, the influx of PMNL has been shown to protect against PCP.\(^ {12}\) The balance between harm and protection may depend on the timing and degree of these events. Pulmonary fibrosis develops in rats allowed to recover from PCP by gradually withdrawing corticosteroids.\(^ {20}\) Fibrosis also occurs in the lungs of some patients who recover from PCP. Several factors may contribute to fibrosis including drugs and inhalation of high oxygen concentrations,\(^ {1}\) but PMNL-mediated damage should also be considered.

We thank Professor Firth for making available electronmicroscope facilities in the Department of Anatomy and H. Jagessar for cutting and staining sections, and for technical assistance. Betamethasone was a gift from Glaxo Ltd. M.B.T. was a Wellcome Training Fellow.

References