Isolation and analysis of immune complexes from sera of patients with polymyalgia rheumatica and giant cell arteritis

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SUMMARY Serum samples were obtained from patients with polymyalgia rheumatica (PMR; n=10) or giant cell arteritis (GCA; n=7), or both. Samples were taken either before treatment or within one week of starting prednisolone. Immune complexes (IC) were concentrated by polyethylene glycol (PEG) precipitation then purified with either IgG anti-C1q-Sepharose or IgG anti-C3c-Sepharose. Complex components were separated by sodium dodecyl sulphate (SDS) gradient polyacrylamide gel electrophoresis then transferred to nitrocellulose by Western blotting. Identification of proteins was carried out using specific antisera. All the IC contained IgM (μ chain), some contained IgA (α chain), and IgG (γ chain). C1r, C1s, C1q, C3, C4, and C reactive protein (CRP), where tested, were found in most but not all IC. The occurrence of properdin, factor B, α2 macroglobulin (α2M), factor H (β1H), C1 esterase inhibitor, and C4 binding protein was also investigated. Immune complexes in PMR and GCA differed from those previously characterised in rheumatoid arthritis (RA) purified by anti-C1q-Sepharose which contained immunoglobulins and C1q only. No properdin or factor B were detected in RA IC purified with either anti-C1q-Sepharose or anti-C3c-Sepharose.

Polymyalgia rheumatica (PMR) and giant cell arteritis (GCA) frequently occur together in those over 50 years old. In PMR there is characteristic shoulder and pelvic girdle pain and morning stiffness. GCA commonly causes temporal headaches and visual problems but is associated with varying clinical syndromes which reflect the vessels involved. Both are associated with a raised erythrocyte sedimentation rate (ESR). The pathogenesis of the condition is unclear, but studies suggest there may be an immunological basis. The serum of patients with GCA contains raised IgG, total complement, C3, and C4 and ESR and CRP. Circulating IC have been investigated by several groups using various IC assays. Park et al using the PEG CC assay demonstrated raised IC levels in 44% of active untreated patients with PMR and GCA and in 23% of inactive treated cases. The 125I-C1q binding assay and PEG C4 assay also detected IC, but the 125I conglutinin binding assay did not. Espinoza et al using the Raji cell assay demonstrated raised IC levels in over 90% of patients. A solid phase C1q enzyme linked immunosorbent assay used by Malmvall et al, however, detected IC in only 2/15 cases of GCA investigated. Immunofluorescent studies of temporal artery biopsy specimens from patients with active arteritis show positive staining for immunoglobulins and the third component of complement. This could represent antibody binding to an arterial cell wall antigen or IC deposition from the blood.

Immune complexes are a heterogeneous population with varying sizes, immunoglobulin composition, and complement fixing abilities. The variations in composition affect removal, deposition, and the possible pathological activities of immune complexes. Each IC assay detects the presence of different components of the complex and therefore may reflect the presence of different IC populations. A knowledge of the nature and source of circulating IC in PMR/GCA may improve our understanding of the pathogenesis of the condition.

The aim of this study was to determine the composition of IC, where present, in PMR and GCA sera. Since the properties of IC depend on the immunoglobulins and complement components

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contained in the complexes, identification of components may increase our understanding of the nature of the disease.

**Patients and methods**

**Patients**

Fifteen patients with active untreated PMR/GCA were included in the study. All fulfilled the diagnostic criteria of Jones and Hazleman. Nineteen had PMR and six GCA, four of whom also had features of PMR. Only one patient was male. The mean age at onset was 68 years (range 52–81). Temporal artery biopsies were carried out in nine cases, and five showed the histological changes of GCA. Seventeen samples of sera were obtained and IC measured using three assays: C1q binding assay, PEG complemet consumption assay, and the PEG C4 assay. IC levels were raised in at least two of the three assays.

**Preparation of immune complexes**

The IC in two aliquots (750 μl) from each sample were concentrated by overnight precipitation at 4°C with an equal volume of 6% PEG in 0·1 M borate buffer pH 8·3, 75 mM NaCl. The precipitate was collected by centrifugation at 2000 g for 10 min at 4°C and then washed twice with 1·0 ml 3% PEG. The precipitate was dissolved in 1·0 ml complement fixation diluent.

IC were then isolated by incubation of an aliquot with 300 μl of 1·5 suspension of either anti-C1q-Sepharose or anti-C3c-Sepharose for two hours at 22°C. The Sepharose bound complex was washed with complement fixation diluent, then 500 μl of 0·5 M TRIS (trometamol) HCl buffer pH 6·8 containing 2·5% (w/v) SDS, 8% (v/v) glycerol, 0·01% (w/v) bromophenol blue, and 20 mM dithiothreitol was added, heated at 100°C for three minutes, and then loaded onto an SDS-polyacrylamide gel.

**SDS gradient polyacrylamide gel electrophoresis (PAGE)**

The gels (125×140×0·75 mm) contained 10–22% total acrylamide gradient with 2·5% (w/v) N,N-methylenbisacrylamide and an upper gel containing 4·5% total acrylamide and 2·5% N,N-methylenbisacrylamide and were polymerised by 0·017% (w/v) ammonium persulphate and 0·015% (v/v) N,N,N',N'-tetramethylethylenediamine. The upper gel had three wells, the two end wells were 5×20 mm and the centre well 115×20 mm. The buffer system used was as described by Laemmli and Favre.

Two gels were run in parallel using the LKB 2001 vertical electrophoresis apparatus. The centre wells were loaded with IC purified by either anti-C1q-Sepharose or anti-C3c-Sepharose treated with SDS/DTT as described. 1251 standards containing phosphorylase A (relative molecular mass (Mr)=100 000), transferrin (Mr=78 000), bovine serum albumin (Mr=68 000), ovalbumin (Mr=43 000), carbonic anhydrase (Mr=29 000), soya bean trypsin inhibitor (24 000), and a lactalbumin (Mr=14 000) were loaded into the outside wells. Electrophoresis was carried out at 150 V until the marker dye reached the bottom of the gels.

A 2 cm strip of each gel containing one of the standard wells was removed, fixed in 40% (w/v) trichloroacetic acid, stained in 0·5% (w/v) Coomassie brilliant blue G in 40% (v/v) methanol and 5% (v/v) acetic acid,17 destained, and dried onto filter paper.

**Transfer to nitrocellulose**

The unfixed portion of each gel was placed in transfer buffer (25 mM TRIS, 192 mM glycine, 20% (v/v) methanol) for 10 min before being placed in the plastic holder with a 0·2 μm nitrocellulose sheet (Schleicher and Schuell, Anderman & Co Ltd, Kingston-Upon-Thames, Surrey, UK). The nitrocellulose was marked with the sample identification, gel orientation, and position of the standards. Transfer was carried out in a Bio-Rad transblot cell at 0·1 A overnight.

Unoccupied sites on the nitrocellulose were blocked using a buffer (50 mM TRIS/HCl pH 7·2, 5 mM ethylenediaminetetra-acetate, 150 mM NaCl, 0·05% Nonidet P40, 0·02% NaN3) containing 0·25% (w/v) gelatin for a minimum of five hours at 4°C.

**Identification of proteins**

Strips (6 mm) of nitrocellulose were cut avoiding the standards position. Individual strips were incubated in 5 ml of different specific antisera diluted 1:100 in blocking buffer for one hour at 4°C. A plastic box (280×150×40 mm) with compartments 20×150×40 mm (Engineering and Design Plastics, Cherry Hinton, Cambridge, UK) was used. The strips were washed with five changes of blocking buffer.

When the antisera had been raised in goats these strips were incubated in rabbit antigoat antisera (Atlantic antibodies, American Hospital Supplies UK Ltd) which had been passed through a human IgG-Sepharose column and then washed as before. All the strips were incubated in 125I goat anti-rabbit antiserum (Atlantic antibodies). 3·7 MBq was used in the labelling of approximately 3·5 mg of protein and was then diluted to 7×10^6 cpm/ml. The strips were washed, aligned with the piece of nitrocellulose containing the 125I standards, and autoradiographed at −70°C overnight.

**Isolation and analysis of immune complexes**

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Fig. 1  Autoradiographs of IC from the serum of a patient with non-arteritis disease purified by (a) anti-C1q-Sepharose or (b) anti-C3c-Sepharose. IC were obtained by PEG precipitation of serum and purified by affinity Sepharose. The components were separated by SDS gradient polyacrylamide gel electrophoresis then transferred to nitrocellulose sheets by Western blotting. Identification of proteins was made using specific antisera then an $^{125}$I labelled antiserum and autoradiography of the nitrocellulose. A non-specific band is found in all tests which is the rabbit IgG released from the affinity Sepharose by SDS/dithiothreitol treatment. Lanes: 1 = IgG; 2 = IgA; 3 = IgM; 4 = C1r; 5 = C1s; 6 = C1q; 7 = C3; 8 = C4; 9 = properdin; 10 = factor B; 11 = CRP; 12 = α, macroglobulin; 13 = factor H; 14 = C1 esterase inhibitor; 15 = C4 binding protein.
Initially, 10 specific antisera were used: rabbit antihuman IgG and Clq (Dako Patts, Dako Ltd, High Wycombe, UK), and C3c (The Behring Corporation) and goat antihuman IgA, IgM, C1r, C1s, C4, properdin, properdin factor B (Atlantic antibodies). Then six additional antisera were used: goat antihuman C reactive protein, α2 macro-globulin, factor H (β1H), C1 esterase inhibitor, and rabbit antihuman C4 binding protein (Miles Scientific Ltd, UK).

### Results

The dried gels had very few visible protein bands after staining with Coomassie brilliant blue. Individual proteins were identified using specific antiserum and an 125I labelled probe after Western blotting (Fig. 1). The relative molecular mass of the proteins was calculated from the autoradiographs by reference to the mobility of the 125I labelled standards. A number of positive staining samples were identified (Table 1), and where multiple protein bands were present this is shown in Table 2. The overall results are shown in Table 1, where the number of positive samples identified is shown for both the anti-C1q-Sepharose and the anti-C3c-Sepharose adsorbents. Minor differences were observed, but in general the results for either adsorbent were similar. In some samples differences were observed in both the number or size of protein bands identified. For example 11/17 samples had three protein bands identified with the C1q antiserum (Mr 37 000, Mr 32 500, and Mr 24 500) (see lane 6, Fig. 1), whereas the other six samples had only either one or two of these protein bands visible. Similarly, multiple protein bands were observed on

### Table 1

Identification of proteins on nitrocellulose blots using specific antisera

<table>
<thead>
<tr>
<th>Antiserum to:</th>
<th>Anti-C1q-Sepharose</th>
<th>Anti-C3c-Sepharose</th>
</tr>
</thead>
<tbody>
<tr>
<td>n=17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>3</td>
<td>2</td>
</tr>
<tr>
<td>IgA</td>
<td>7</td>
<td>9</td>
</tr>
<tr>
<td>IgM</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>C1r</td>
<td>16</td>
<td>13</td>
</tr>
<tr>
<td>C1s</td>
<td>16</td>
<td>14</td>
</tr>
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<td>C1q</td>
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<td>14</td>
</tr>
<tr>
<td>C3</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>C4</td>
<td>13</td>
<td>12</td>
</tr>
<tr>
<td>Properdin</td>
<td>4</td>
<td>5</td>
</tr>
<tr>
<td>Factor B</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>n=10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C reactive protein</td>
<td>10</td>
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<tr>
<td>α2 Macroglobulin</td>
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<td>2</td>
</tr>
<tr>
<td>Factor H</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>C1 esterase inhibitor</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>C4 binding protein</td>
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<td>0</td>
</tr>
</tbody>
</table>

IC precipitated by PEG from serum were purified using either anti-C1q or anti-C3c-Sepharose. The components were separated by SDS gradient PAGE then transferred to nitrocellulose by Western blotting. Proteins were identified using specific antisera and an 125I labelled probe. The numbers represent the number of samples which gave positive, i.e., visible, bands on autoradiography of the nitrocellulose sheets.

### Table 2

Identification of protein subunits with antiserum to C1r, C1s, C1q, C3 and C4 on Western blotting of IC from the serum of patients with polymyalgia rheumatica or giant cell arteritis, or both (n=7), or polymyalgia rheumatica only (n=10)

<table>
<thead>
<tr>
<th>Antiserum to:</th>
<th>No of bands</th>
<th>PMR/GCA</th>
<th>PMR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Anti-C1q-Sepharose</td>
<td>Anti-C3c-Sepharose</td>
</tr>
<tr>
<td>C1r</td>
<td>1</td>
<td>1</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>C1s</td>
<td>1</td>
<td>4</td>
<td>4</td>
</tr>
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<td></td>
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<td>2</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
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<td>4</td>
</tr>
<tr>
<td>C1q</td>
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<td>4</td>
</tr>
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<td></td>
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<td>1</td>
</tr>
<tr>
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<td>3</td>
<td>2</td>
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</tr>
<tr>
<td></td>
<td>Total</td>
<td>7</td>
<td>5</td>
</tr>
<tr>
<td>C3</td>
<td>1</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>1</td>
<td>5</td>
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<td>1</td>
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<tr>
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<td>1</td>
</tr>
<tr>
<td>Total</td>
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<td>9</td>
</tr>
<tr>
<td>C4</td>
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<td>2</td>
</tr>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>3</td>
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<td>1</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

The numbers represent samples with positive staining.
the immunoblots for other complement components such as Clr, C1s, C3, and C4, and the relative numbers identified and sizes are shown in Table 2. Six additional antisera were used to investigate a further 10 immunoblot pairs (Table 1). C reactive protein (CRP) was present in all the sera (except one) tested, but no C4 binding protein (C4bp) was identified. The anti-Clq-Sepharose IC showed positive staining for \( \alpha_2 \) macroglobulin in 7/10 samples, factor H in 3/10 samples, and C1 esterase inhibitor in 5/10 samples. Similar results are shown for the anti-C3c-Sepharose IC (Table 1).

Table 3 separates the patients' samples into those with polymyalgia rheumatica alone \((n=10)\) and those with giant cell arteritis \((n=7)\) and then examines the relative distribution of the various immune complex components. Few differences were seen in the components which were often present, e.g., IgM and Clr, and where some differences occurred the numbers were too small for any firm conclusions to be drawn.

**Discussion**

Immunoglobulins and complement deposits have been demonstrated in the media and adjacent to the internal elastic lamina in some involved temporal arteries. It is not known, however, whether such deposits were the result of passive deposition of immune complexes from the circulation or from the combination of specific antibodies with antigens in situ. Sera from patients with giant cell arteritis or polymyalgia rheumatica, or both, have been found to contain increased levels of circulating immune complexes. Although there was not a high correlation between immune complex levels and disease activity, isolation and analysis of the immune complex may lead to further understanding of this disorder.

Circulating immune complexes are frequently detected in patients with inflammatory disorders, and characterisation of the immune complex antigens has been carried out in order to identify antigens that may elicit the inflammatory reaction in affected tissues. Several investigators have attempted to identify immune complex antigens in inflammation. We have for the first time analysed immune complex preparations from patients with giant cell arteritis.

In this study we have used a new and rapid method for the analysis of immune complexes which allows small volumes of sera to be processed and the individual components of the immune complexes to be identified. This method is ideal for the investigation of the composition of immune complexes in the serum of patients with PMR/GCA as the IC present are only raised before steroid therapy and thereafter usually fall rapidly. Consequently only small amounts of sera containing IC are available for analysis. Pretreatment or first treatment samples were taken and assayed for IC by three independent methods. Only those sera containing raised IC by two or three of these methods were analysed further. The IC isolated contained a mixture of immunoglobulins, complement components, acute phase proteins, and proteinase inhibitors.

All the IC investigated contained IgM but only a few contained IgG, and both of these immunoglobulins can activate the classical complement pathway. IgA was present in more IC than IgG, and although IgA does not activate the classical complement pathway, some subclasses can activate the alternative complement pathway.

A number of complement components were present in the IC. Clq was obviously present in all samples where the anti-Clq affinity adsorbent was used to purify the complexes and was also present in most of the complexes purified by the anti-C3c-Sepharose adsorbent. Clr, C1s, C3, and C4 were also present in many samples. C1 esterase inhibitor (C1 activator), which binds tightly and rapidly to cause dissociation of Clr and C1s from the IC, was also found and tended to be more common in patients with PMR. Factor B was identified in only three IC, all purified with anti-C3c-Sepharose. In the alternative complement pathway it forms the C3 convertase C3bBb with activated C3 (C3b). Properdin, which binds to C3b and stabilises the
C3bBb complex and maintains the C3b positive feedback loop. Factor H was identified in several IC, it is a control protein for the classical and alternative complement pathways, potentiating the action of C3b inactivator on C3b while preventing the binding of other proteins to C3b. Two IC contained both factor H and properdin. Other investigations of IC components showed CRP was present in all the IC studied. CRP is an acute phase protein, which is raised in untreated PMR and GCA, and it has been suggested that CRP may be capable of inhibiting alternative complement pathway activation and stopping C3b binding to surfaces. α2 Macroglobulin, a serum protease inhibitor, was found in some IC, which might suggest that these complexes are in the process of inactivation and are therefore inert.

It is possible that some serum components could bind non-specifically to the IgG-Sepharose (e.g., Clq or rheumatoid factor) via the Fc portion of the coupled IgG. Obviously the use of (Fab)2 antibodies would overcome this problem. The levels of non-complexed Fc binding materials, however, are minimised by the PEG precipitation and washing, and we have not found that PMR/GCA sera contain raised levels of rheumatoid factor.

There are some differences in the components identified in IC purified using the different affinity matrices. This was expected and is likely to reflect differences in the degree of complement activation, the complement activation pathway, the presence of inhibitors, the degree of solubilisation, and differences in the removal of the complexes.

There were also some differences between those patients who had polymyalgia rheumatica only and those who had giant cell arteritis. The presence or number, or both, of the polypeptide chains of C1r, C1q, C3 and C4, properdin, factor B, α2M, factor H, and C1 esterase inhibitor appeared to be different, though it was hard to determine the significance of these differences or draw any firm conclusions because of the small numbers. The IC in PMR and GCA differed from the IC previously analysed by the same method, in the sera of patients with rheumatoid arthritis where the RA IC did not contain properdin and factor B, and had a population purified by anti-C1q-Sepharose unable to activate the classical complement pathway indicated by the absence of C3 and the presence in only 1/7 IC of C1r or C1s.

The present study indicates that the method we have developed allows the rapid and reproducible analysis of immune complexes in multiple samples which are available in small amounts. In addition, it is possible to separate the immune complexes by this method and store nitrocellulose strips to allow identification of further components as new antisera become available.

References
Smith, Kyle, Cawston, Hazleman

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