Immunoochemical quantitation of complement components of Clq and C3 in sera and synovial fluids of patients with bone and joint diseases

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SUMMARY

The amount of the initiating complement component (Clq) in the classical pathway and the first essential component (C3) in the alternative complement pathway were measured with a single radial immunodiffusion (SRID). A high ionic strength was used corresponding to that of 0.25 M NaCl and 0.01 M EDTA to avoid nonspecific binding of Clq with immune aggregates. Measurements were made on sera and/or synovial fluids from 165 patients with various bone and joint diseases. Values of Clq and C3 in synovial fluids were also expressed as ratios to that of albumin in the same specimens to avoid the influence of differences in volume of synovial fluid in various diseases, and this appeared to provide a reliable index reflecting pathological conditions. Both serum Clq and C3 levels were raised highly in rheumatoid arthritis, gout, and osteomyelitis, but the extent of the elevation of C3 was less conspicuous. Values of Clq and C3 in synovial fluids also markedly increased in rheumatoid arthritis.

Abundant evidence suggests that immune mechanisms participate in the pathogenesis of rheumatoid arthritis (Maini, 1977a). Tissue damage in rheumatoid arthritis is considered to be caused by type II, antibody-dependent cytotoxic, and type III, complex-mediated, and probably by type IV reactions classified by Coombs and Gell (1975). Furthermore, in rheumatic disease the matrix of bone and joint composed of collagen, elastin, and other materials (e.g., basement membrane) may degenerate or dissolve mainly as a result of enzymatic degradation leading to loss of normal framework and architecture.

The complement (C) system not only plays important roles in host defence mechanisms but also is implicated in disease processes involving cytotoxic and immune-complex mediated hypersensitivities. Clq is the triggering component in the classical pathway of the C system and can bind not only to antigen-antibody complexes but presumably nonspecifically to denatured, soluble, altered immunoglobulins and other polyanionic substances (Agnello et al., 1970), and succeeding C components are activated. Interestingly, Clq contains hydroxyproline, hydroxylysine, and abundant glycine residues in its molecule. Analyses of its carbohydrates have shown that Clq had galactosylglucose residues linked to hydroxylysine, which are similar to those of basal membrane collagen and of membrane type collagen (Yonemasu et al., 1971; Reid et al., 1972; Calcott and Müller-Eberhard, 1972; Shinkai and Yonemasu, 1979). The haemolytic activity is readily destroyed on digestion by bacterial collagenase (Reid et al., 1972), and recent extensive chemical studies have disclosed that Clq is an unusual globular protein and that about half its molecule is composed of triple helix with collagen-like sequences (Reid and Porter, 1976). Furthermore, the production and secretion of Clq by fibroblasts (Cazenave et al., 1976; Reid and Solomon, 1977) and a competitive inhibition of collagen-induced platelet aggregation by Clq (Suba and Csako, 1976; Wautier et al., 1977) have been reported. On the other hand C3 is the essential component in the alternate pathway of C system and has been investigated in cases of rheumatoid arthritis (Ruddy and Austen, 1970; Versey et al., 1973; Ruddy et al., 1975).

The present study was undertaken to quantify serum Clq and C3 levels in rheumatoid arthritis.
(RA) and to compare the findings with those seen in other bone and joint diseases such as gout, transient synovitis (TS), osteomyelitis, and osteosarcoma. Although Kushner and Somerville (1971) reported a method of estimating the degree of synovial inflammation, no reliable methods of measuring absolute amounts of synovial fluid components were reported. So we tried to quantify synovial fluid C1q and C3 from various hydroarthritic patients, and amounts of these components were expressed as ratios to the concentration of albumin in the same synovial fluid, which is considered to be a typical blood transudate.

Materials and methods

Patients
All were Japanese seen at the Department of Orthopedics, Osaka University Hospital or the Koseinenkin Hospital.

The clinical diagnosis of classical, definite, or probable rheumatoid arthritis was made according to the diagnostic criteria of the American Rheumatism Association (Ropes, 1959). Cases of classical and definite rheumatoid arthritis were collectively studied as RA. In one group of patients with transient synovitis without systemic disease and without recurrence a standard diagnosis could not be established. For these patients we used the term 'transient synovitis' (TS).

The total number of patients examined was 165. The study population for serum analyses consisted of patients with RA (44 cases), probable rheumatoid arthritis (17 cases), gout (14 cases), TS (28 cases), osteomyelitis (7 cases), and osteosarcoma (5 cases). The study population for synovial fluid analyses consisted of patients with RA (17 cases), TS (24 cases), and degenerative joint diseases (DJD) (50 cases).

Serum and Synovial Fluid
The blood samples collected by venepuncture were allowed to clot at room temperature for 60 min and then to retract at 4°C for 120 min. Synovial fluids were aspirated for therapeutic purposes. Serum or clear synovial fluid free from cell constituents was separated by centrifugation of 3000 g for 15 min or 20 000 g for 20 min, respectively, and stored in small aliquots at −70°C until used.

Immunochemical Quantitation of Serum C1q and C3
This was carried out by means of a single radial immunodiffusion (SRID) according to the method of Mancini et al. (1965) with some modification. Antihuman C1q or antihuman C3 serum was prepared according to the method described previously (Yonemasu, 1975) or by the method described by others (Nilsson and Müller-Eberhard, 1965; Arroyave and Tan, 1976). The sensitivity of these methods was about 2 μg Clq/ml and 10 μg C3/ml. To eliminate confusion from possible binding of C1q with coexistent immune complexes or altered immunoglobulins SRID was performed with 1% agarose in 0.05 M Tris and 0.05 M glycine-buffered 0.22 M NaCl containing 0.01 M EDTA and 0.1% Nazide (pH 8.0), the ionic strength of which corresponded to that of 0.25 M NaCl. At this ionic strength there was no apparent nonspecific binding of Clq with immune aggregates or soluble altered immunoglobulins (Agnellon et al., 1970).

The average serum C1q and C3 levels of 346 Japanese healthy adults (from 15 to 75 years of age) were those obtained in previous work (Yonemasu et al., 1978) and were found to be 136.5 ± 1.22 μg/ml for C1q and 1.081 ± 0.173 mg/ml for C3.

Immunochemical Quantitation of Synovial C1q, C3, and Albumin
1 ml of synovial fluid was mixed with 5 μl of testicular hyaluronidase (Sigma Chemical Co., St. Louis, Mo.), at a concentration of 1 mg/ml, and incubated at 37°C for 60 min. Preliminary experiments showed no effects on values of serum C1q, C3, and albumin obtained with SRID by this hyaluronidase digestion. Thus the synovial C1q, C3, and albumin of the hyaluronidase digestion were assayed in a manner similar to that of SRID as described above.

Statistical Analysis of Data
Mean concentrations of serum C1q and C3 in these diseases were compared with those of normal healthy adults, and statistical analyses were carried out by Student’s t test and P values were determined. Values of synovial fluid albumin, C1q, and C3 in various hydroarthroses were compared with each other by the F test and then analysed by Student’s t test similarly. The degree of correlation between C1q and C3 was determined by Kendall’s rank correlation coefficient (τ).

Results

Serum C1q
Serum C1q levels were measured in 115 patients with various bone and joint diseases and are presented in Table 1. The values in RA and osteomyelitis were conspicuously elevated (higher than 150% of that of healthy adults) and increased moderately in probable rheumatoid arthritis and gout. Statistically, the values of serum C1q in RA—probably rheumatoid arthritis, gout, and osteomyelitis—deviated greatly from those of normal healthy adults...
(P<0.001), while that for TS showed probably significant deviation (P<0.05) and that for osteosarcoma no significant deviation.

**SERUM C3**
Serum C3 levels were measured in the same 115 patients from whom serum Clq was assayed, and the results are presented in Table 2. The levels in RA, gout, and osteosarcoma increased moderately (higher than 120% of that of healthy adults) and slightly in osteomyelitis. The values for RA and gout deviated statistically significantly from that of the normal healthy adults (P<0.001 and P<0.01, respectively). Although the level for osteosarcoma showed probably significant deviation (P<0.05), those for probable rheumatoid arthritis, TS, and osteomyelitis were not significantly deviated.

**ALBUMIN LEVELS IN SYNOVIAL FLUIDS**
Concentrations of albumin in synovial fluid were measured in 91 patients with various inflammatory conditions and the findings are shown in Table 3. The albumin concentrations in synovial fluid were practically equivalent, and no statistically significant differences between those 3 groups (RA, TS, and DJD) were found. These results indicate that the concentrations of synovial albumin were rather constant in various types of inflammatory conditions with different pathogenesis. Consequently, the ratio of the concentration of the synovial fluid components (for example, Clq, C3, etc., which may fluctuate under various pathological conditions) to that of the albumin in the same synovial specimen appeared to be a more reliable index.

**SYNOVIAL FLUID CLQ**
The quantitation of Clq in synovial fluids of the same 3 groups (RA, TS, and DJD) of the hydroarthrotic patients from whom synovial albumin was assayed, was carried out and data are presented in Table 4. The ratio of the concentration of Clq to that of albumin (Clq/albumin) was also calculated. In RA both the concentrations of synovial fluid Clq and its ratio to those of synovial fluid albumin were conspicuously higher than those in other inflammatory conditions and were statistically highly significantly deviated from the mean values in others. In DJD, Clq/albumin ratio was statistically significantly deviated (P<0.01), but the significance in statistical deviation of the concentration of Clq was only probable (P<0.05). In Fig. 1 Clq/albumin ratios in these synovial fluids were plotted. Only in RA were these ratios conspicuously higher than those in other inflammatory conditions.

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**Table 1** Concentrations of serum Clq

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age (average)</th>
<th>No. of cases</th>
<th>Mean±SE (µg/ml)</th>
<th>Standard deviation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy adults</td>
<td>15-79 (39-1)</td>
<td>346</td>
<td>136.5±1.2</td>
<td>22.6</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>RA</td>
<td>23-78 (48-6)</td>
<td>44</td>
<td>211.9±4.1</td>
<td>26.9</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Probable rheumatoid arthritis</td>
<td>30-59 (45-9)</td>
<td>17</td>
<td>164.5±5.1</td>
<td>21.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Gout</td>
<td>27-71 (40-3)</td>
<td>14</td>
<td>175.0±4.2</td>
<td>15.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TS</td>
<td>18-64 (40-1)</td>
<td>28</td>
<td>144.5±4.8</td>
<td>25.5</td>
<td>&lt;0.005*</td>
</tr>
<tr>
<td>Osteomyelitis</td>
<td>18-65 (41-9)</td>
<td>7</td>
<td>226.1±12.9</td>
<td>34.1</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>12-19 (14-4)</td>
<td>5</td>
<td>146.0±4.1</td>
<td>9.1</td>
<td>0.3&lt; &lt;0.4</td>
</tr>
</tbody>
</table>

* Statistically highly significantly deviated from the normal healthy adult level with P<0.001.

**Table 3** Concentrations of synovial fluid albumin

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age (average)</th>
<th>No. of cases</th>
<th>Mean±SE (mg/ml)</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>28-63 (48-7)</td>
<td>17</td>
<td>26.2±1.38</td>
<td>5.70</td>
</tr>
<tr>
<td>TS</td>
<td>26-77 (52-5)</td>
<td>24</td>
<td>23.9±1.33</td>
<td>6.50</td>
</tr>
<tr>
<td>DJD</td>
<td>45-73 (63-2)</td>
<td>50</td>
<td>24.3±0.69</td>
<td>4.82</td>
</tr>
</tbody>
</table>

**Table 2** Concentrations of serum C3

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>Age (average)</th>
<th>No. of cases</th>
<th>Mean±SE (mg/ml)</th>
<th>Standard deviation</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy adults</td>
<td>15-79 (39-1)</td>
<td>346</td>
<td>1.09±0.017</td>
<td>0.317</td>
<td></td>
</tr>
<tr>
<td>RA</td>
<td>23-78 (48-6)</td>
<td>44</td>
<td>1.33±0.058</td>
<td>0.335</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>Probable rheumatoid arthritis</td>
<td>30-59 (45-9)</td>
<td>17</td>
<td>1.19±0.077</td>
<td>0.290</td>
<td></td>
</tr>
<tr>
<td>Gout</td>
<td>27-71 (40-3)</td>
<td>14</td>
<td>1.527±0.185</td>
<td>0.454</td>
<td>&lt;0.01†</td>
</tr>
<tr>
<td>TS</td>
<td>18-64 (40-1)</td>
<td>28</td>
<td>1.003±0.035</td>
<td>0.183</td>
<td>0.1&lt; &lt;0.2</td>
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<tr>
<td>Osteomyelitis</td>
<td>18-65 (41-9)</td>
<td>7</td>
<td>1.301±0.096</td>
<td>0.253</td>
<td>0.05&lt; &lt;0.1</td>
</tr>
<tr>
<td>Osteosarcoma</td>
<td>12-19 (14-4)</td>
<td>5</td>
<td>1.394±0.057</td>
<td>0.127</td>
<td>&lt;0.05</td>
</tr>
</tbody>
</table>

* Statistically highly significantly deviated from the normal healthy adult level with P<0.001. † Statistically significantly deviated with P<0.01.
Table 4 Concentrations of synovial fluid Clq and its ratios to those of synovial fluid albumin

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Concentration (µg/ml)</th>
<th>P value</th>
<th>Clq (µg/ml)/Albumin (mg/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>17</td>
<td>104.0 ± 10.11</td>
<td>41.67</td>
<td>3.69 ± 0.25</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TS</td>
<td>24</td>
<td>45.8 ± 3.48</td>
<td>17.04</td>
<td>2.03 ± 0.10</td>
<td>0.5&lt;</td>
</tr>
<tr>
<td>DJD</td>
<td>50</td>
<td>38.5 ± 1.61</td>
<td>11.24</td>
<td>1.61 ± 0.05</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

* Statistically highly significantly deviated from the average value in all hydroarthrosis with P<0.001. † Statistically significantly deviated with P<0.01.

SYNOVIAL FLUID C3
We measured the C3 in synovial fluids from the same patients with RA, TS, or DJD whose Clq was quantified. In these patients synovial fluid albumin was also quantified and the C3/albumin ratio was calculated. These results are presented in Table 5. The C3 level in RA was high and significantly deviated from the mean level in other hydroarthrosis, but the level in TS or DJD showed no significant deviation. The value of C3 (µg/ml)/albumin (mg/ml) for RA was high and showed highly significant deviation (P<0.001), while those for TS or DJD showed no significant deviation. In Fig. 2 C3/albumin ratios in these synovial fluids were plotted. The C3/albumin ratio was high only in RA, but its deviation was less conspicuous than that of Clq/albumin in RA (Fig. 1).

CORRELATION OF Clq AND C3 IN SERUM OR IN SYNOVIAL FLUID
Serum Clq and C3 were measured on the same serum specimens (115 cases) of patients with RA,

Table 5 Concentrations of synovial fluid C3 and its ratios to those of synovial fluid albumin

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>No. of cases</th>
<th>Concentration (µg/ml)</th>
<th>P value</th>
<th>C3 (µg/ml)/Albumin (mg/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>RA</td>
<td>17</td>
<td>0.444 ± 0.030</td>
<td>0.944</td>
<td>15.81 ± 0.80</td>
<td>&lt;0.001*</td>
</tr>
<tr>
<td>TS</td>
<td>24</td>
<td>0.312 ± 0.021</td>
<td>0.112</td>
<td>12.37 ± 0.46</td>
<td>0.5&lt;</td>
</tr>
<tr>
<td>DJD</td>
<td>50</td>
<td>0.271 ± 0.013</td>
<td>0.093</td>
<td>0.05 &lt;</td>
<td>&lt;0.001*</td>
</tr>
</tbody>
</table>

* Statistically highly significantly deviated from the average value in all hydroarthrosis with P<0.001. † Statistically significantly deviated with P<0.01.
probable rheumatoid arthritis, gout, TS, osteomyelitis, or osteosarcoma. Synovial fluid Clq and C3 were also quantified on the same synovial fluids (91 cases) of patients with TA, TS, or DJD. In both serum specimens and synovial fluids there were no statistically significant correlations between Clq and C3 levels.

Discussion

The activities and/or protein amounts of C components in various bone and joint diseases, including rheumatoid arthritis, were found to be slightly depressed in rheumatoid arthritis (Ruddy and Austen, 1970; Zvaifler, 1973; Ruddy et al., 1975), and it was suggested that these decreases may be due to a hypersynthesis and hypercatabolism of Clq components in this disease (Ruddy et al., 1975).

Our results show that serum Clq levels increased conspicuously in RA and osteomyelitis, but in levels in TS and osteosarcoma, even in cases of marked inflammation, were within the normal range. In synovial fluids both Clq levels and Clq/albumin ratios were raised only in patients with RA. These raised levels of Clq seem to be contradictory to the findings of Ruddy and Austen (1970). The discrepancy may be attributed to SRID with a higher ionic strength (Agnello et al., 1970) to avoid any binding of Clq to immune complexes or altered immunoglobulins, and to pretreatment of synovial fluids with hyaluronidase to remove any unfavourable effects of hyaluronic acids on diffusion in this study. The finding of no differences in Clq levels between RA patients with detectable rheumatoid factor (RF) and without RF may show that our buffer system for SRID and such pretreatment of synovial fluid may be essential to quantify absolute protein amounts of Clq.

Serum C3 levels measured immunochemically were elevated in RA and gout, but the degree of elevation was less conspicuous than that in Clq. The synovial fluid C3 in RA was also slightly elevated. These results are also contradictory to earlier report of Ruddy and Austen (1970). Possible reasons for this elevation may be a different assay method used by us and/or alternation of antigenicities of C3 by conversion to C3i in rheumatoid arthritis (Zvaifler, 1969).

The elevation of Clq was not contradictory to the earlier suggestion of the hypermetabolism of C in rheumatoid arthritis, as reported by Ruddy et al. (1975). Since Clq is produced and secreted by fibroblasts, as shown by Reid and Solomon (1977) and in a paper by Sano et al., (1979), in which marked increase of Clq level in progressive systemic scleroderma, Behçet's disease, and anaphylactoid purpura was reported, a relatively higher degree of hypersynthesis and lower degree of hypercatabolism of Clq in rheumatoid arthritis may be a more feasible explanation for the elevated level of Clq.

The lack of correlation between amounts of Clq and C3 in serum and in synovial fluid suggested that these two C components fluctuated independently and may have independent pathological roles in various bone and joint diseases.

Except for a few restricted proteins which are synthesised by the synovium in rheumatoid arthritis (Kushner and Somerville, 1971; Maini, 1977b), it has been considered that synovial fluid proteins are mere dialysates of plasma to which hyaluronate has been added (Sandson and Hamerman, 1962). The finding that concentrations of albumin (mg/ml) in synovial fluids were rather constant in all hydroarthroses with various pathogeneses (Table 3) indicates that synovial fluid albumin is indeed a dialysate of plasma. Thus the expression of values of synovial fluid Clq and C3 as ratios to those of albumin in the same specimens appeared to be a more reliable procedure in view of fluctuations in the volume of synovial fluids in various hydroarthroses, the results showing increasing statistical significance (Tables 4 and 5).

Studies to elucidate in more detail the mechanisms of elevation of Clq and C3 and effects of hyaluronic acid on these components in RA are now in progress.

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