Significance of complement components C1q and C4 bound to circulating immune complexes in juvenile idiopathic arthritis: support for classical complement pathway activation

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Abstract

Objective

Immune complexes (ICs) from sera of juvenile idiopathic arthritis (JIA) patients show increased complement opsonization; however, a definitive role for involvement of the classical or alternative pathway is not entirely clear. To delineate the role of these pathways, we measured activated complement products bound to circulating IC (CICs) in the sera of JIA patients.

Methods

Sera from 100 JIA patients and 22 healthy children were collected. C1q, C4, C3, C3d, and membrane attack complex (MAC) bound to CICs were measured by enzyme-linked immunosorbent assay. Data was compared to IgM rheumatoid factor (RF), IgG anti-cyclic citrullinated peptide (CCP) antibodies, C-reactive protein (CRP), and erythrocyte sedimentation rate (ESR) levels.

Results

Mean levels of C1q, C4, and MAC bound to CICs were significantly elevated in JIA patients compared to healthy children. C1q correlated significantly with C4 and MAC bound to CICs and C4 and MAC also demonstrated significant correlation.

No significant differences were noted in complement components bound to CICs when evaluating IgM RF, anti-CCP antibody, and CRP positivity. A significant correlation was noted between MAC bound to CICs and ESR. C1q and MAC bound to CICs mean levels were significantly higher in patients with an elevated ESR compared to those with a normal ESR level.

Conclusion

JIA patients have elevated levels of complement components bound to CICs, particularly from the classical pathway. Moreover, classical pathway components were associated with ESR, a marker of disease activity. MAC bound to CICs also correlated significantly with ESR, further supporting the notion of complement-mediated tissue injury that is triggered by IC-mediated classical pathway activation.

Key words

juvenile idiopathic arthritis, complement system, immune complexes
Introduction

Juvenile idiopathic arthritis (JIA) is a systemic disease characterised by synovial inflammation before 16 years of age (1, 2). JIA is subdivided into seven subtypes based on clinical presentation (1, 2). The presence of circulating immune complexes (CICs) has been previously observed in JIA (3-6).

The complement system is part of the innate immune system and acts as a first line of defense against microbial and environmental threats. In addition to disposing of CICs, it is also a key mediator of inflammatory injury (7). Complement activation can occur through three pathways, including the classical, alternative and the mannose-binding lectin (MBL) pathways. These pathways converge in terminal complement activation resulting in the formation of the membrane attack complex (MAC). Each of the complement pathways has different plasma complement proteins that triggers its activation; the classical pathway is initiated by ICs, the alternative pathway by bacteria and viruses, and MBL by microbes with terminal mannose groups (7).

When functioning appropriately, the complement system finely balances activation and deposition of complement. When this delicate balance is disrupted, the complement system can aggravate the disease process. Defective clearance of CICs leads to accumulation and perpetuation of synovial inflammation (7, 8). Complement activation and levels of CICs have been shown to correlate with disease activity in JIA (3, 9, 10). Previous studies measuring complement activation products have implicated both the classical and alternative pathways in JIA (8, 11-13).

In order to delineate which complement pathway is involved in the pathogenesis of JIA and determine the contribution of these pathways in subtypes of JIA, we measured complement bound to CICs in sera from JIA patients. This study is unique in its ability to measure the total complement activation products bound to CICs, thus providing a direct measure of the complement systems activity that is actually involved in the immune pathological process (14, 15). The overall goal of this study was to better define which pathway of the complement system is critical in the pathogenesis of JIA utilising advanced methods of complement activation measurement.

Materials and methods

Serum samples

Sera were collected from the Pediatric Rheumatology outpatient clinics of the Saint Louis University Medical Center and Cardinal Glennon Children’s Medical Center. Sera were frozen in 50μl aliquots and stored at -80°C until analysed. A total of 100 individual JIA samples (87 female, 13 male) were collected, including 68 polyarthritis (41 IgM rheumatoid factor (RF)-negative and 27 IgM RF-positive) and 32 oligoarthritis patients. All patients in this study fulfilled the International League of Associations for Rheumatology (ILAR) criteria for JIA (1, 2). The mean ± standard deviation (SD) age of the JIA patient population was 11.0±5.5 years and the mean ±SD disease duration was 4.1±4.5 years (Table I). Sixty-three JIA patients had active disease and 37 patients were in medical remission at the time of sample collection. JIA patients were categorised based on disease activity as either asymptomatic/low disease activity or moderate/high disease activity (16). The mean ±SD joint count for the JIA population was 7.2±5.5 joints (range 0–28 joints). Sera from 22 healthy children (17 female, 5 male) were also analysed. The mean ±SD age for the healthy population was 9.9±5.1 years. The study was approved by the Institutional Review Board of the Saint Louis University Medical Center.

Measurement of complement components bound to CICs

C1q, C3, C3d, C4 and MAC bound to CICs were measured by the Proceptor™ Fixed Complement ELISA (ProGen Biologics, Wildwood, MO) according to manufacturer’s instructions and as previously described (15). To establish the binding of CIC to the receptor preparation (Proceptor™, ProGen Biologics), we tested the binding of aggregated human γ-globulin (AHG), an in vitro formed CIC from tetanus toxoid (TT)-anti-TT, and observed a linear binding. The spiking and recovery experiments provided CICs were within 80%. The Proceptor ELISA proved to be sensitive to CIC concentrations between 10 μg/ml and 1 μg/ml.

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Competing interests: A.K. Chauhan has a financial interest in Progen Biologics; the other co-authors have declared no competing interests.
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Table I. Demographic and Laboratory features of JIA patients (n=100).

<table>
<thead>
<tr>
<th>JIA (n=100)</th>
<th>IgM RF-positive polyarthritis (n=26)</th>
<th>IgM RF-negative polyarthritis (n=41)</th>
<th>Oligoarthritis (n=33)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>11.0 ± 5.5</td>
<td>13.3 ± 5.8</td>
<td>11.3 ± 5.4</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>4.1 ± 4.5</td>
<td>3.8 ± 3.8</td>
<td>4.3 ± 5.0</td>
</tr>
<tr>
<td>IgG anti-CCP antibodies (U)</td>
<td>26 ± 64</td>
<td>82 ± 98</td>
<td>11 ± 41</td>
</tr>
<tr>
<td>IgM RF (U)</td>
<td>24 ± 44</td>
<td>64 ± 68</td>
<td>14 ± 21</td>
</tr>
<tr>
<td>CRP (mg/dL)</td>
<td>1.9 ± 5.3</td>
<td>1.2 ± 0.99</td>
<td>3.0 ± 8.2</td>
</tr>
<tr>
<td>ESR (mm/hr)</td>
<td>23.1 ± 23.0</td>
<td>21.8 ± 21.5</td>
<td>23.5 ± 24.0</td>
</tr>
<tr>
<td>Moderate/High Disease Activity (n/%)</td>
<td>78 (78%)</td>
<td>23 (88.5%)</td>
<td>37 (90.2%)</td>
</tr>
</tbody>
</table>

Cut-off values for a positive result: IgM RF (≥6U), IgG anti-CCP antibodies (≥20U), CRP (≥0.8mg/dL), ESR (≥20mm/hr). Anti-CCP antibodies: anti-cyclic citrullinated peptide antibodies; CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; mg/dL: milligram/deciliter; mm/hr: millimeter/hour; n: number; RF: rheumatoid factor; U: units.

Fig. 1. Levels of (A) C1q bound to CICs and (B) C4 bound to CICs in JIA subtypes (n=100) and healthy children (n=22) measured by ELISA. Bars indicate the median levels of complement components bound to CICs for each subtype and controls. Dashed line indicates cut-off for a positive result for each assay. P-values were considered statistically significant at p<0.05. CIC: circulating immune complexes, RF: rheumatoid factor.

In brief, serum samples were diluted (1:10–1:100) in phosphate buffered saline (PBS)/0.05% Tween-20. Standards were already coated on each of the 96-well plates in duplicate and were left covered until secondary antibodies were added. The remaining wells were washed with 200 μl of PBS/Tween-20 and diluted sera were loaded in duplicate. The microtiter plates incubated at room temperature (RT) with gentle agitation for 90 minutes, followed by four wash steps. Another wash step was followed by the addition of 100 μl of horse radish peroxidase (HRP)-conjugated secondary antibody to each well and incubation at RT for 60 minutes. The microtiter plates were washed again and 100 μl of tetramethylbenzidine was added until the desired optical density (OD) was reached, followed by the addition of 50μl of 0.5N sulfuric acid to stop the reaction. The OD was measured at 450nm and the concentration was calculated after subtraction of the blank standard.

Cut-off values were established as the mean ±2SD of the healthy individuals’ results. The cut-off value was 77 ng/ml for C1q bound to CICs, 3016 ng/ml for C3 bound to CICs, 146 ng/ml for C3d bound to CICs, 1884 ng/ml for C4 bound to CICs, and 759 ng/ml for MAC bound to CICs.

Laboratory and clinical evaluation

The QUANTA Lite RF ELISA for detection of IgM RF and the QUANTA Lite CCP3 IgG ELISA (INOV A Diagnostics, Inc., San Diego, CA) were used according to manufacturer’s instructions. The cut-off value for IgM RF was 6 U and 20 U for anti-CCP antibodies. Erythrocyte sedimentation rate (ESR) was determined according
to modified Westergren and C-reactive protein (CRP) by electroimmunoassay, with normal values considered at <20 mm/hr and <0.8 mg/dL, respectively.

Statistical analysis
Statistical analyses were performed using the Statistical Package Social Science (SPSS) program version 17.0 (Chicago, IL). Correlations were analysed using Spearman’s rho correlation coefficient. Patient groups were compared using Independent samples t-test, χ² test for proportions, and one-way ANOVA followed by Bonferroni post hoc analysis. p≤0.05 was considered statistically significant in all analyses.

Results
C1q and C4 bound to CICs – classical pathway components
Levels of C1q bound to CICs were positive in 39/100 JIA patients, including 8 with IgM RF-positive polyarthritis, 18 with IgM RF-negative polyarthritis and 13 with oligoarthritis (Fig. 1A). One healthy child also exhibited elevated levels of C1q bound to CICs at 89.2 ng/ml. We observed significant differences in classical complement components bound to CICs in JIA subtypes and healthy children (Fig. 1). Mean levels of C1q bound to CICs were significantly higher in JIA patients (80.9 ng/ml) compared to healthy children (41.8 ng/ml, p=0.012). Positive results for C1q bound to CICs ranged between 80.5 ng/ml-293.8 ng/ml, with the highest being found in a patient with IgM RF-positive polyarthritis. C1q correlated significantly with C4 bound to CICs (r=0.77, p<0.001) and MAC bound to CICs (r=0.50, p<0.001) (Fig. 2A, 2B).

A positive result for C4 bound to CICs was found in 36/100 JIA patients, including 9 with IgM RF-positive polyarthritis, 16 with IgM RF-negative polyarthritis, and 11 with oligoarthritis (Fig. 1B). No healthy children were positive for C4 bound to CICs. C4 bound to CICs also correlated with MAC bound to CICs (r=0.47, p<0.001) (Fig. 2C). Mean levels of C4 bound to CICs were significantly elevated in JIA patients (2055.2 ng/ml) compared to healthy children (1072.3 ng/ml, p=0.025). Bonferroni post hoc analy-
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Table II. Significant variations in complement components bound to CICs in JIA subtypes and healthy and SLE control groups.

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>JIA subtype</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>C4 bound to CICs</td>
<td>Healthy IgM RF-negative polyarthritis</td>
<td>0.041</td>
</tr>
<tr>
<td>C3 bound to CICs</td>
<td>Healthy IgM RF-negative polyarthritis</td>
<td>0.018</td>
</tr>
<tr>
<td>MAC bound to CICs</td>
<td>Healthy Oligoarthritis</td>
<td>0.017</td>
</tr>
</tbody>
</table>

Bonferroni post hoc analysis of association between complement components bound to CICs in JIA subtypes and control groups. Significant differences at p<0.05. CIC: circulating immune complexes; JIA: juvenile idiopathic arthritis; MAC: membrane attack complex; RF: rheumatoid factor.

C3 and C3d bound to CICs – alternative pathway components/amplification loop

Thirty-seven of 100 JIA patients were positive for C3 bound to CICs. This included 7 with IgM RF-positive polyarthritis, 18 with IgM RF-negative polyarthritis, and 12 with oligoarthritis (Fig. 3A). No healthy children were positive for C3 bound to CICs. The mean level of C3 bound to CICs was significantly higher in JIA patients (2362.3 ng/ml) compared to healthy children (1599.8 ng/ml, p=0.036). However, the mean level in JIA patients was below the cutoff for a positive result. Positive values in JIA ranged from 3041.2–7765.7 ng/ml, with the highest levels found in a patient with IgM RF-negative polyarthritis. Bonferroni post hoc analysis of complement components in JIA subtypes and control groups revealed a significant variation in levels of C3 bound to CICs, with elevated levels found in IgM RF-negative polyarthritis patients compared to healthy children and IgM RF-positive polyarthritis patients (Table II). C3 bound to CICs correlated significantly with C3d bound to CICs (r=0.43, p<0.001).

C3d bound to CICs was positive in 5/100 JIA patients, including 4 with IgM RF-negative polyarthritis and one with oligoarthritis (Fig. 3B). There was no significant difference in mean levels of C3d bound to CICs in JIA patients (89.0 ng/ml) compared to healthy children (80.0 ng/ml, p=0.26). Three of the five (60.0%) JIA patients positive for C3 bound to CICs were also positive for C3d bound to CICs.

Membrane attack complex bound to CICs

Thirty-nine JIA patients were positive for MAC bound to CICs, including 7 with IgM RF-positive polyarthritis, 18 with IgM RF-negative polyarthritis, and
14 with oligoarthritis (Fig. 4). None of the healthy children were positive for MAC bound to CICs. Mean levels of MAC bound to CICs were significantly higher in JIA patients (983.5 ng/ml) compared to healthy children (336.4 ng/ml, \( p = 0.004 \)). As previously noted, MAC bound to CICs correlated significantly with C1q and C4 bound to CICs (Fig. 2). Interestingly, levels of MAC bound to CIC showed a significant variation in oligoarthritis patients compared to healthy children (Table II).

Of the 39 JIA patients positive for MAC bound to CICs, 31 (81.6%) were also positive for C1q bound to CICs, C4 bound to CICs, or both. Conversely, only 18 (46.2%) of JIA patients positive for MAC bound to CICs were also positive for C3 bound to CICs, C3d bound to CICs, or both. Table III shows the positivity of MAC bound to CICs and its relationship with positivity of classical (C1q/C4) and alternative (C3/C3d) pathway complement components.

**Complement components bound to CICs and their relationship with laboratory parameters and disease activity**

The polyarticular patients were initially subdivided by nephelometry results, not by the ELISA method used in this study. Forty-three JIA patients were positive for IgM RF measured by ELISA, including 20 IgM RF-positive polyarthritis patients, 17 IgM RF-negative polyarthritis patients, and 6 with oligoarthritis. Twenty of 43 (46.5%) patients positive for IgM RF were also positive for C1q bound to CICs, C4 bound to CICs, or both (Table IV). Mean IgM RF levels trended higher in patients positive for C1q and C4 bound to CICs compared to patients positive for C3 and C3d bound to CICs, but no statistically significant differences were noted.

IgG anti-CCP antibodies were also elevated in JIA patients negative for C1q and C4 bound to CICs.

**CRP levels** were available for 87 JIA patients, of which 30 (34.4%) were positive (≥0.8 mg/dL). This included 9 with IgM RF-positive polyarthritis, 13 with IgM RF-negative polyarthritis, and 8 with oligoarthritis. Mean CRP levels were significantly elevated in JIA patients with moderate/high disease activity (2.2 mg/dL) compared to those who were asymptomatic/low disease activity (13.2 mm/hr, \( p = 0.002 \)).

**ESR levels** were significantly higher in JIA patients with moderate/high disease activity (13.2 mm/hr, \( p = 0.002 \)). Table IV shows the mean CRP values for each of the studied complement groups, with no significant differences noted. While no significant differences were found between the classical and alternative pathway components, a significant association was noted between MAC bound to CICs and ESR (r=0.26, \( p = 0.009 \)). JIA patients with high ESR levels demonstrated significantly elevated levels of C1q bound to CICs (92.6 ng/ml) and MAC bound to CICs (1211.8 ng/ml) compared to JIA patients with normal ESR levels (62.9 ng/ml and 701.8 ng/ml, respectively) (\( p = 0.033 \) and \( p = 0.014 \), respectively).
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Table IV. Concentration and positivity of laboratory parameters based on the presence of classical and alternative complement pathway components in JIA patients (n=100).

<table>
<thead>
<tr>
<th></th>
<th>C1q/C4 negative</th>
<th>C4 positive</th>
<th>C1q positive</th>
<th>C1q/C4 positive</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n=57</td>
<td>n=4</td>
<td>n=7</td>
<td>n=32</td>
</tr>
<tr>
<td><strong>IgM RF Conc.</strong></td>
<td></td>
<td></td>
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<tr>
<td>(n/% positive)</td>
<td>18 U (23/40.4%)</td>
<td>42 U (3/75.0%)</td>
<td>6 U (2/28.6%)</td>
<td>34 U (15/46.9%)</td>
</tr>
<tr>
<td><strong>IgG anti-CCP Conc.</strong></td>
<td></td>
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<tr>
<td>(n/% positive)</td>
<td>27 U (13/22.8%)</td>
<td>71 U (1/25.0%)</td>
<td>8 U (0/0.0%)</td>
<td>23 U (8/25.0%)</td>
</tr>
<tr>
<td><strong>CRP Conc.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(n/% positive)</td>
<td>2.5 mg/dl (16/34.0%)</td>
<td>2.4 mg/dl (1/25.0%)</td>
<td>0.51 mg/dl (1/14.3%)</td>
<td>1.3 mg/dl (10/40.0%)</td>
</tr>
<tr>
<td><strong>ESR Conc.</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>(n/% positive)</td>
<td>21.2 mm/hr (21/37.5%)</td>
<td>35.0 mm/hr (2/50.0%)</td>
<td>16.2 mm/hr (3/50.0%)</td>
<td>25.2 mm/hr (13/40.6%)</td>
</tr>
<tr>
<td><strong>C3/C3d negative</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>n=61</td>
<td>19 U (30/49.2%)</td>
<td>5 U (1/50.0%)</td>
<td>9 U (1/33.3%)</td>
<td></td>
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<tr>
<td><strong>C3 positive</strong></td>
<td></td>
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<tr>
<td>n=34</td>
<td>15 U (11/32.4%)</td>
<td>1 U (0/0.0%)</td>
<td>2 U (0/0.0%)</td>
<td></td>
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<tr>
<td><strong>C3d positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=2</td>
<td>3.2 mg/dl (9/36.5%)</td>
<td>0.55 mg/dl (1/50.0%)</td>
<td>2.6 mg/dl (1/50.0%)</td>
<td></td>
</tr>
<tr>
<td><strong>C3/C3d positive</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>n=3</td>
<td>31.3 mm/hr (25/41.7%)</td>
<td>23.5 mm/hr (18/54.5%)</td>
<td>17.7 mm/hr (1/33.3%)</td>
<td></td>
</tr>
</tbody>
</table>

Cut-off values: C1q (>89ng/ml), C4 (>1884ng/ml), C3 (>3016ng/ml), C3d (>146ng/ml), and MAC (>1859ng/ml). IgG anti-CCP antibodies (≥6U), IgG anti-CCP Conc., CRP Conc., ESR Conc., C3/C3d positive, C3/C3d negative, C3/C3d positive.

Discussion

Activation of both classical and alternative complement pathways have been documented in various subtypes of JIA (11-13). Our study examined various complement components bound to CICs in polyarticular and oligoarticular JIA patients to determine their significance in the disease process. Our results suggest that the classical pathway plays a dominant role over the alternative pathway in JIA, regardless of the subtype studied.

While previous studies from the 1990’s have implicated the classical complement pathway in the pathogenesis of JIA, several of these studies were conducted with small JIA patient populations, consisting of 7–26 patients (10, 17-19). It is noted in such studies that due to the small sample size, the statistically associations may be an aberration rather than a physiologically significant finding (10). A recent study using 136 JIA patients found classical pathway activation in systemic-onset JIA patients (20). Our study of 100 polyarticular and oligoarticular JIA patients found the presence of classical complement components, C1q and C4 bound to CICs, along with significant correlation with the terminal complement complex, MAC. This is one of the largest studies demonstrating classical complement activation in JIA.

Few studies in JIA have found evidence of alternative pathway activation (13, 21). Significantly elevated levels of C3 bound to CICs were noted in the JIA population. However, due to the amplification loop, higher levels of alternative pathway proteins in plasma, elevated levels of alternative pathway components are expected. C3d, a long-lived breakdown product of C3b, was found in only a small number of JIA patients. This product can be generated by both the classical and alternative pathways (22) and the presence of C3d bound to CICs may be indicative of prolonged inflammation and accumulation of complement (23). More studies are necessary to determine the significance of C3d bound to CICs in JIA.

The combined presence of classical and alternative pathway components in JIA patients may indicate the initial activation of the classical pathway leading to amplification of the alternative pathway (23).

It is known that IgM and complexed IgG are effective classical complement activators. IgM RF and IgG anti-CCP antibodies were found at higher levels in JIA patients with classical complement components bound to CICs. However, no statistically significant data was found in our study. This is similar to previous reports where no significant correlation was found between complement components and IgM RF (11, 22).

The relationship between C1q bound to CICs and ESR further acknowledges the role of the classical complement pathway in JIA. While a direct correlation was not made between complement components bound to CICs and disease activity, this finding supports the notion of such a relationship.

Excessive deposition of MAC on the cell membrane results in cell lysis, while sublytic deposition of MAC triggers pleiotropic cellular responses, including upregulation of inflammatory mediators (24). The association of MAC with serum parameters of disease activity, such as ESR, further supports the notion of complement-mediated tissue injury that is triggered by IC-mediated classical pathway activation. MAC has received far less attention in past JIA studies. Our study found that 39% of JIA patients demonstrated a positive result for MAC bound to CICs in serum. In addition, the levels of MAC bound to CICs were significantly elevated in JIA patients compared to healthy children and correlated significantly with C1q and C4 bound to CICs in JIA. A role for the anaphylatoxin C5a in the SKG mouse model of arthritis has been suggested (25). Thus, it is not farfetched that C5b, which leads to formation of MAC, may also be critical to JIA disease pathology.

In conclusion, we found evidence of classical complement pathway activation in
a large cohort of JIA patients, with further support for amplification loop initiation. Moreover, MAC bound to CICs was also detected in JIA patients, which significantly correlated with classical pathway complement components.

References