C1q-Containing Immune Complexes Purified from Sera of Juvenile Rheumatoid Arthritis Patients Mediate IL-8 Production by Human Synoviocytes: Role of C1q Receptors

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C1q-Containing Immune Complexes Purified from Sera of Juvenile Rheumatoid Arthritis Patients Mediate IL-8 Production by Human Synoviocytes: Role of C1q Receptors

Zhila Khalkhali-Ellis,2* Gary A. Bulla,† Larry S. Schlesinger,‡ Dawn A. Kirschmann,* Terry L. Moore,§ and Mary J. C. Hendrix*  

Immune complexes that vary in size and composition are present in the sera and synovial fluid of juvenile rheumatoid arthritis (JRA) patients. They are believed to be potent inducers of the ongoing inflammatory process in JRA. However, the precise composition and role of these complexes in the pathophysiology of JRA remain unclear. We hypothesized that circulating ICs have the potential to interact with resident joint synovial fibroblasts (synoviocytes) and induce the expression of inflammatory cytokines. To test this hypothesis, cultures of synoviocytes from healthy individuals were treated with ICs isolated from the sera of JRA patients. Studies reported in this work demonstrate that IgM affinity-purified ICs from the sera of JRA patients contain IgM, C1q, IgG, and C3 to a variable extent. These ICs induce IL-8 mRNA and protein production in normal synoviocytes. Our data indicate that C1q in these ICs mediates, in part, IL-8 induction in synoviocytes. This is based on our findings of C1q-binding proteins for collagen stalks (cC1qR) and globular heads (gC1q-binding protein) of C1q in synoviocytes. In addition, collagen stalk and to some extent globular head fragments of C1q inhibit IC-mediated IL-8 induction in synoviocytes. Together, these findings provide evidence for a novel mechanism of IL-8 production by synoviocytes, which could play a key role in inflammation by recruiting leukocytes to synovial tissue and fluid—and subsequently contributing to joint disease. The Journal of Immunology, 1999, 163: 4612–4620.

A prominent feature of juvenile (JRA) and adult forms of rheumatoid arthritis (RA) is the accumulation of polymorphonuclear leukocytes (PMNs) in the inflamed synovium. This develops at least in part through excessive production of PMN chemotactic factors such as IL-8 and macrophage-inflammatory protein-1 in the synovial environment (1–3). Production of these inflammatory mediators in the JRA/RA synovial tissue could occur through diverse mechanisms. Cross-linking FCγRs on monocytes/macrophages, or exposure of macrophages to cytokines such as IL-15, is associated with up-regulation of IL-8 mRNA, and release of bioactive IL-8 protein (4, 5). In addition, bacterial cell wall products such as LPS and IgG-containing immune complexes (ICs) also induce up-regulation of IL-8 message and protein in macrophages (4). Recent studies have identified synovial fibroblasts (synoviocytes) as potent producers of chemokines such as IL-8, macrophage chemoattractant protein-1, and RANTES (6). It has been observed that exposure to cytokines such as IL-1β and TNF-α, or the combination of the two, is associated with the release of IL-8 by synoviocytes (7, 8). Ligation of MHC class II molecules on synoviocytes by superantigens staphylococcal enterotoxin A, Mycoplasma arthritidis-derived superantigen, or anti-class II Ab also results in the induction of chemokine gene expression and protein synthesis (9).

JRA is associated with excessive production and deposition of ICs in serum, synovial fluid (SF), and inflamed joints (10–12). The precise role of these complexes in the pathogenesis of JRA remains enigmatic. However, it has been suggested that ICs contribute to the pathophysiology of specific JRA subtypes (11, 12). Recent studies by Jarvis et al. have indicated that SF ICs from JRA patients are capable of inducing proinflammatory cytokine production in the human myeloid cell line U937 (13). These findings are suggestive of a role for ICs in triggering and/or sustaining the ongoing inflammatory process in the joint.

We have purified and partially characterized the ICs from sera of JRA patients (10). Our studies have identified the presence of IgM rheumatoid factor (IgM RF), and hidden IgM RF (i.e., IgM RF detected by hemolytic analysis in the IgM-containing fraction after separation of serum by acid gel filtration) in these ICs (11). The studies reported in this work were designed to further define the composition of these IgM-containing ICs. In addition, these studies examine the unique ability of ICs to induce the chemokine IL-8 in normal synoviocytes, and to elucidate the mechanism(s) by which this occurs.

Materials and Methods

Chemicals

mAbs to complement receptors (CRs) CR1 (CD35), CR3 (CD11b/CD18), and CR4 (CD11c/CD18) were from AMAC (Westbrook, ME); mAb to FcyR CD16 and IgG subtype controls were from Becton Dickinson (San Jose, CA); polyclonal goat anti-human C3 and goat anti-mouse IgG were...
from Sigma, or Bio-Rad (Hercules, CA). Biochemical (Lakewood, NJ). The remaining chemicals were obtained from Collaborative Research (Bedford, MA); DMEM was from Life Technologies (Grand Island, NY); IL-8 ELISA kit was from R&D Systems (Minneapolis, MN); chemoluminescence kit (ECL). Positive and negative controls (as described in the figure legend) were included in all experiments.

The blots were blocked in blocking buffer (0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl, 0.05% Tween-20, and 3% nonfat dry milk (TBS)) for 1 h at room temperature. The membranes that were thoroughly washed, treated with HRP-conjugated goat anti-mouse IgG, and developed using a chemiluminescence kit (ECL). Positive and negative controls (as described in the figure legend) were included in all experiments. In some experiments, ICs were subjected to further purification by dye affinity chromatography using microcolumns of blue Sepharose to remove serum albumin present in the IC fractions. The unbound material was collected by brief centrifugation; the protein concentration was estimated and stored at −80°C until used.

**Preparation of C1q stalks and globular heads**

Globular heads and collagen-like stalks of C1q were prepared by collagenease and pepsin digestion of C1q, as described previously (18, 19). The undigested C1q was removed from the gC1q and cC1q by chromatography on Sephadex G-75 and Sepharose 4B columns, respectively (18). The fractions containing the gC1q and/or cC1q were concentrated by freeze drying and stored at −80°C until used. The purity of the fractions was tested by SDS-PAGE and hemolytic assay (20).

**Induction studies**

Synoviocytes were plated into 24-well culture dishes (10^5 cells/well) in serum-free supplement. Purified ICs were added to the cultures at 10 μg/ml, unless otherwise stated. The cultures were maintained for 24–48 h at 37°C in 5% CO2. For time-course studies, cultures were treated with ICs or other inducers (see below) from 0–24 h. The culture supernatants were collected, centrifuged to remove cell debris, and assayed for IL-8 protein by ELISA. The cells were washed several times with PBS and were stored at −80°C until used for RNA extraction. All experiments were performed in duplicates.

Induction studies were also performed using C1q alone, or C1q in different combinations with IgM, IgG aggregates (agg-IgG), or both (referred to as in vitro produced C1q-containing ICs). These in vitro produced ICs were prepared as follows: IgG solutions were heated at 65°C for 20 min to yield agg-IgG (21). The solution was centrifuged to remove insoluble material. Different variable combinations were prepared by adding IgM and C1q to agg-IgG and incubating the mixture at 37°C for 20 min. These in vitro produced ICs were then utilized for induction studies, as described for purified JRA ICs.

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**Table 1. Patient characteristics**

<table>
<thead>
<tr>
<th>Patient/Sex</th>
<th>Age</th>
<th>Onset</th>
<th>Disease Duration (years)</th>
<th>Drugs</th>
</tr>
</thead>
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<td>9.0</td>
<td>NSAIDs</td>
</tr>
<tr>
<td>2/F</td>
<td>18</td>
<td>poly</td>
<td>15.0</td>
<td>NSAIDs</td>
</tr>
<tr>
<td>3/F</td>
<td>16</td>
<td>poly</td>
<td>8.0</td>
<td>NSAIDs</td>
</tr>
<tr>
<td>4/F</td>
<td>15</td>
<td>poly</td>
<td>12.0</td>
<td>NSAIDs, Gold</td>
</tr>
<tr>
<td>5/M</td>
<td>14</td>
<td>poly</td>
<td>0.5</td>
<td>NSAIDs, MTX, HClq</td>
</tr>
<tr>
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<td>poly</td>
<td>0.75</td>
<td>NSAIDs, MTX, HClq</td>
</tr>
<tr>
<td>7/M</td>
<td>14</td>
<td>systemic</td>
<td>2.0</td>
<td>NSAIDs, MTX, HClq</td>
</tr>
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<td>4.0</td>
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</tr>
<tr>
<td>9/F</td>
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<td></td>
<td>10.0</td>
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<tr>
<td>17/F</td>
<td>5.5</td>
<td>pauci</td>
<td>4.0</td>
<td>NSAIDs</td>
</tr>
</tbody>
</table>

*poly*, polyanular seronegative JRA; *poly*, polyanular seropositive JRA; *pauci*, pauciarticular JRA.

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**Synoviocyte culture**

Synovial tissue was obtained from individuals with sport-related injuries who did not have clinical evidence of any inflammatory disorder. This tissue was used to generate synovial fibroblasts, as previously described (15). Briefly, synovial tissue was dissected free of fat, minced into small pieces, and cultured in flasks in DMEM with 10% FCS and 50 μg/L gentamicin. Adherent synovial fibroblasts that developed within 8–10 days were passaged by trypsinization. Cultures of synovial fibroblasts at passages 2–8, which were devoid of contaminating macrophages, T cells, and B cells (as determined by the absence of appropriate markers), were utilized in the experiments.

**Isolation and characterization of ICs**

ICs were isolated from serum samples, as previously described (16). Briefly, 1 ml of sera was applied to a column of F(ab’)_2 fragments of rabbit IgG anti-human IgM coupled to Sepharose 4B. The column was washed and the bound ICs were eluted first with ammonia (1 M, adjusted to 0.01 M glycine-HCl, pH 3). Fractions were neutralized, dialyzed against DMEM medium, and if required, concentrated using Microcon 10 microconcentrator (Amicon, Beverly, MA). The IgM and IgG content of the glycine-HCl-eluted IC fractions were estimated by ELISA using F(ab’)_2 fragments of goat anti-IgM and mouse anti-IgG.

Glycine-HCl-eluted IC fractions were subjected to SDS-PAGE analysis on a 10% resolving and 3.5% stacking gel. Gel electrophoresis was performed under both reduced and nonreduced conditions, according to Laemmli’s (17) procedure. The gels were either stained with Coomassie brilliant blue (Coomassie BB) and visualized, or blotted onto a polyvinylidene difluoride membrane and probed with appropriate Abs, as described below.

In some experiments, ICs were subjected to further purification by dye affinity chromatography using microcolumns of blue Sepharose to remove serum albumin present in the IC fractions. The unbound material was collected by brief centrifugation; the protein concentration was estimated and stored at −80°C until used.

**Induction studies**

Synoviocytes were plated into 24-well culture dishes (10^5 cells/well) in DMEM medium containing Mito^+ serum-free supplement. Purified ICs were added to the cultures at 10 μg/ml, unless otherwise stated. The cultures were maintained for 24–48 h at 37°C in 5% CO2. For time-course studies, cultures were treated with ICs or other inducers (see below) from 0–24 h. The culture supernatants were collected, centrifuged to remove cell debris, and assayed for IL-8 protein by ELISA. The cells were washed several times with PBS and were stored at −80°C until used for RNA extraction. All experiments were performed in duplicates.

Induction studies were also performed using C1q alone, or C1q in different combinations with IgM, IgG aggregates (agg-IgG), or both (referred to as in vitro produced C1q-containing ICs). These in vitro produced ICs were prepared as follows: IgG solutions were heated at 65°C for 20 min to yield agg-IgG (21). The solution was centrifuged to remove insoluble material. Different variable combinations were prepared by adding IgM and C1q to agg-IgG and incubating the mixture at 37°C for 20 min. These in vitro produced ICs were then utilized for induction studies, as described for purified JRA ICs.
C1q fragments (gC1q and/or cC1q), either individually or in combination, were tested for their ability to inhibit IC-mediated IL-8 production in synoviocytes. Quiescent synoviocytes in Mito-supplemented serum-free media were treated with gC1q, cC1q, or a combination of the two (25 μg/mL) in the presence of the ICs (or the in vitro produced ICs) for 48 h. The conditioned media were collected and tested for their IL-8 content by ELISA.

RNA isolation, cDNA synthesis, and PCR analysis
Untreated and IC-treated synoviocytes were subjected to RNA isolation using Tel-Test B guanidinium thiocyanate/phenol solution and according to the manufacturer’s direction. cDNA was generated from approximately 300 ng of RNA using the RETROscript kit. The reverse-transcription solution contained 0.4 mM dATP, dCTP, dGTP, and dTTP; 4 μM random decamers; 50 mM KCl; 1.5 mM MgCl2; 10 mM Tris-HCl, pH 8.3; 10 U placental RNase inhibitor; and 100 U Moloney murine leukemia virus reverse transcriptase. Reaction mixtures were incubated at 42°C for 1 h and heat inactivated at 92°C for 10 min.

IL-8 mRNA levels in the control and the IC-treated synoviocytes were analyzed by quantitative relative RT-PCR using the Quantum RNA Kit and according to the manufacturer’s instructions. As a control for PCR amplification, levels of 18S RNA were measured using a 2:8 ratio of 18S competitor, according to manufacturer’s cDNA obtained from U937 cells treated for 4 h with a combination of IL-1 and TNF-α (150 pg/mL each). In all, positive control reactions were performed in triplicate and run on the same gel. A dilution series was prepared from 20 μl and contained 0.125 mM of each dATP, dCTP, dGTP, and dTTP; 1.5 mM MgCl2; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 3 ng 32P end-labeled U937 cells treated for 4 h with a combination of IL-1 and TNF-α according to the manufacturer’s instructions. cDNA obtained from fibroblasts and cultured cells were extracted using Tel-Test B guanidinium thiocyanate/phenol solution and according to the manufacturer’s instructions. The PCR products were then analyzed by ethidium bromide.

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Interaction of ICs with primary cultures of human synoviocytes

Active disease state in JRA/RA is associated with the influx of PMNs in the SF and synovial tissue. Abundance of inflammatory mediators and their interactions with synovial macrophages and synoviocytes results in the production of PMN chemotactic factors such as IL-8. Thus, we postulated that synoviocytes could interact with the circulating ICs and produce inflammatory cytokines. To test this prediction, we examined whether interaction of ICs with synoviocytes leads to IL-8 production. ELISA analysis of conditioned medium from synoviocyte cultures (without prior treatment with the ICs) indicated a low basal IL-8 level. Upon treatment with JRA ICs, an increase in IL-8, both at the mRNA and protein level, was observed in these cultures (Fig. 2). We selected an IC concentration of 10 μg/ml for induction assays, based on a limited study depicting this concentration near saturation (data not shown). The induction of IL-8 message in response to ICs was time dependent, reaching a maximum at about 4 h and declining thereafter. This coincided with the concomitant release of IL-8 protein into the culture medium (Fig. 2). ICs from polyarticular seropositive JRA patients induced higher IL-8 levels when compared with that of seronegative JRA cases (Fig. 3A). No major differences in IL-8 production were observed between the ICs from seronegative polyarticular cases and that of pauciarticular JRA. The IC from the systemic onset JRA patient induced the lowest IL-8 protein levels (Fig. 3B).

These studies demonstrated the ability of JRA ICs to interact with normal synoviocytes and induce IL-8 mRNA and protein production. In addition, ICs from seropositive polyarticular JRA exhibited greater IL-8 induction when compared with seronegative polyarticular or pauciarticular JRA patients. Due to the lack of multiple samples for systemic onset JRA, statistical analysis could not be achieved; however, IC from this patient resulted in the lowest IL-8 induction in synoviocytes (Fig. 3B).

Effect of C1q and in vitro produced ICs on synoviocytes

To identify the component(s) of the ICs accountable for IL-8 induction, IgG, C1q, and IgM were examined either individually or in different combinations for their ability to induce IL-8 in cultured synoviocytes. These studies demonstrated that IgG (either monomeric or as agg-Ig) was a poor inducer of IL-8 (Table III). C1q alone tested at concentrations of 0–150 μg/ml had minimal IL-8-inducing capacity. However, higher concentrations stimulated the release of IL-8 (in a concentration-dependent manner) from synoviocytes. IgM could also stimulate IL-8 induction in synoviocytes;

Table II. The extent of reactivity of the IC protein components with antibodies to IgG, IgM, C3, and C1q

<table>
<thead>
<tr>
<th>Molecular Mass (kDa)</th>
<th>Anti-IgG</th>
<th>Anti-IgM</th>
<th>Anti-C3</th>
<th>Anti-C1q</th>
</tr>
</thead>
<tbody>
<tr>
<td>90–92</td>
<td>+</td>
<td>+++++</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>70–72</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>57</td>
<td>+++++</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>40</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>35</td>
<td>–</td>
<td>+</td>
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<tr>
<td>26</td>
<td>+++++</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

* IgM affinity-purified ICs from a seronegative JRA patient were resolved on SDS-PAGE, then blotted onto a polyvinylidene difluoride membrane for probing with mAbs to IgM, C1q, C3, and IgG. The blots were then treated with HRP-labeled secondary Ab and visualized using an enhanced chemiluminescent kit. The scoring was based on the extent of reactivity with the mAbs determined by densitometric scanning of the film.

** Anti-C3 reactivity varied from patient to patient, and no direct correlation was observed between the level of C3 and IL-8 production.

++ Anti-C1q was much more pronounced (+++++) in seropositive polyarticular JRA ICs.

*This band was later identified as serum albumin.
FIGURE 2. IL-8 induction in synoviocytes in response to treatment with the ICs isolated from a seronegative JRA serum. A, ELISA analysis for IL-8 protein content of conditioned media collected from synoviocyte cultures (10^4 cells/well of a 24-well culture dish) treated with the IC (5 μg/ml) for the specified time intervals. B, mRNA was isolated from synoviocyte monolayers treated as in A and used as a template for RT-PCR. Products were separated on a 4% polyacrylamide gel, and the gel was dried and exposed to x-ray film overnight. mRNA from U937 cells treated with IL-1β and TNF-α was included as a positive control. A 2-fold serial dilution of cDNA from induced U937 was included to ensure that the PCR amplification produced a linear response. C, Quantification of IL-8 message was performed by PhosphorImage analysis. Each point represents an average of two independent experiments; each normalized to the 18S signal, with the exception of a single experiment for the 10-h time point. The zero time mRNA level was arbitrarily set at a value of 1.

By utilizing a similar approach, we assayed for the presence of CRs: CR₁, CR₃, CR₄, and C5a R (CD88) on synoviocytes. None of these receptors could be detected on synoviocytes (quiescent or IC-induced). PBMCs from a healthy control individual were positive for some of the receptors tested (data not shown).

The absence of FCγRs and CRs on synoviocytes was further confirmed by immunofluorescence microscopy. Adherent monolayer cultures of synoviocytes grown on coverslips and fixed in cold acetone were negative for the above receptors (data not shown).

Presence of C1q-binding protein(s) in synoviocytes

Cellular interactions with ICs involve FCγ or complement receptors. However, our flow cytometry and immunohistochemistry analyses demonstrated the absence of these receptors on synoviocytes. Thus, we hypothesized that the interaction of synoviocytes with these C1q-bearing ICs could occur via C1q receptors and/or C1q-binding proteins identified on a variety of cell types. Currently, C1q receptor designated C1qRp, cC1qR, and gC1q-binding protein have been identified and their function extensively investigated. By utilizing RT-PCR analysis, we were able to demonstrate the presence of transcript for gC1q-binding protein (640 bp) in synoviocytes (Fig. 5A). C1qRp transcript was not detected in synoviocytes (with or without the IC induction). RNA from PMNCs used as a positive control revealed the transcripts for C1qRp (Fig. 5A). DNA sequence analysis of the 640 and 450-bp bands confirmed the amplification of gC1q-binding protein and C1qRp transcripts (data not shown). Northern blot analysis also indicated the presence of mRNA for cC1qR (calreticulin) in synoviocytes (Fig. 5B).

The presence of C1q receptor proteins was confirmed by immunofluorescence microscopy on synoviocyte cell lysates. These receptor proteins had apparent molecular masses of ~33 and 60 combinations of IgM and C1q appeared to have an additive effect. Among different C1q containing in vitro produced ICs tested, those containing IgM were most effective. In fact, suboptimal concentrations of C1q (i.e., below 100 μg/ml) could enhance IgM-induced IL-8 production considerably. In addition, combination of agg- or monomeric IgG with C1q or C1q-IgM had a marginal effect on the induction of IL-8 in synoviocytes (Table III).

Effect of C1q fragments on IC-mediated IL-8 induction in synoviocytes

To determine the role of collagen stalks and globular heads of C1q in mediating IL-8 induction in synoviocytes, cultures of synoviocyte were treated with the ICs in the presence or absence of C1q fragments. Collagen-like stalks caused the inhibition of IC-mediated IL-8 induction by 71.2 ± 5.9%, while globular heads of C1q inhibited the IL-8 production by 23.9 ± 3.5%. A combination of agg- or monomeric IgG with C1q or C1q-IgM had a marginal effect (5.9%), while globular heads of C1q had 3.5% inhibition of IC-induced IL-8 production (Fig. 4). Similar results were obtained when in vitro produced ICs were used (data not shown).

Flow-cytometric and immunohistochemical analysis for the presence of FCγ and complement receptors on synoviocytes

Several lines of evidence have indicated that ICs mediate their effect via FCγ or complement receptors present on the responsive cells. To investigate the nature of IC interaction with synoviocytes, we analyzed synoviocytes for the presence of three FCγRs: CD16, CD32, and CD64, on synoviocytes before and after treatment with the ICs from seropositive polyarticular JRA serum. These studies were performed on synoviocytes harvested from monolayers by brief exposure to a dilute trypsin solution to minimize the cleavage of the receptors from the cell surface. FCγRs were not detected on quiescent or the IC-induced synoviocytes from five different cultures. Control U937 cells run in parallel with synoviocytes were positive for CD32 (data not shown).
kDa (Fig. 5C) when subjected to SDS-PAGE under reduced conditions, thus indicating that the interaction of synoviocytes with C1q-containing ICs occurs, at least in part, via C1qRs present in these cells.

Discussion

The present study describes the isolation and characterization of C1q-containing ICs from the sera of JRA patients. These C1q-containing ICs have the ability to interact with synoviocytes and induce the production of IL-8. Our studies provide evidence that the IC-mediated IL-8 induction in synoviocytes occurs, in part, via C1q receptor(s) identified in synoviocytes.

Studies from our laboratory and that of others have revealed the presence of IgM RF-containing ICs in the sera of JRA patients (23, 24). These studies have also indicated a correlation between the level of these ICs and disease activity, thereby demonstrating a connection with a more advanced stage of the disease (23). The present study was undertaken to isolate and characterize IgM affinity-purified ICs from sera of different onset JRA patients, and then to address the interactions between these ICs and primary human synoviocyte cultures.

Evidence is presented that these ICs possess a complex protein profile, as determined by conventional ELISA, SDS-PAGE, and Western blot analysis. They contain components reactive with mAbs to IgM, C1q, and IgG, and in some ICs with C3, with the highest ratio of IgM:IgG found in the seropositive JRA ICs. The seropositive JRA ICs also contained higher levels of C1q when tested by ELISA. The main component of the ICs (as determined by SDS-PAGE), a 90–92-kDa protein under reduced conditions, is

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Table III. IL-8 induction in synoviocytes and in response to treatment with in vitro produced ICs

<table>
<thead>
<tr>
<th>Apg IgG (μg/ml)</th>
<th>C1q (μg/ml)</th>
<th>IgM (μg/ml)</th>
<th>IL-8 (pg/ml)</th>
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</tr>
<tr>
<td>100</td>
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<td>100</td>
<td>789 ± 71</td>
</tr>
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</table>

*Synoviocytes were plated (in serum-free Mito + supplemented media) at 4 × 10^4 cells/well of a 96-well culture dish. They were then treated with the above concentrations of the in vitro-produced ICs. After 24 h, the media were collected and assayed for IL-8 protein by ELISA. Experiments were done in triplicate, and the values for IL-8 are given as mean ± SEM.
highly reactive with anti-IgM and anti-C1q. The stability of the IgM:C1q complex under the reduced conditions employed in the present study is unusual. Without further structural analysis performed on this particular protein band, we can only speculate that its reactivity with anti-IgM and anti-C1q is indicative of an IgM-C1q cross-link stable under the electrophoretic conditions used. Such a possibility is being investigated by structural analysis of the protein purified from one of the ICs tested. However, it has been demonstrated that C1q has avidity for both IgG and IgM and forms a tight association with IgG, which is irreversible even in the presence of SDS and/or mercaptoethanol (25). To our knowledge, this is the first report of the presence of 92-kDa anti-IgM, anti-C1q-reactive IC in the sera of JRA patients. Furthermore, the relative abundance of this protein band seems to correlate with the disease severity, being the highest in the seropositive polyarticular onset JRA.

Our observation of increased levels of this protein band in seropositive compared with that of seronegative JRA sera could account for the greater ability of the ICs from seropositive polyarticular JRA patients to induce IL-8 production. These C1q-bound IgM ICs are potent inducers of IL-8 (even at concentrations as low as 1 μg/ml) in synoviocytes. Increased IL-8 production could result in the accumulation of PMNs and lymphocytes in the synovial tissue and SF of JRA/RA patients (26). In addition, these C1q-bearing ICs could facilitate leukocyte trafficking by interacting with vascular endothelial cells and stimulating the expression of adhesion molecules critical for leukocyte recruitment to the inflamed joint (27). Indeed, a correlation between the number of PMNs and the level of complement activation has been observed within inflamed RA joints (28).

By utilizing in vitro constructed ICs (without the putative Ag) to simulate in vivo produced ICs, we were able to address the direct involvement of C1q in the IC-mediated synoviocyte production of IL-8. Based on the published information regarding the high avidity of C1q for both IgG and IgM (21), we were confident that these in vitro constructed ICs could simulate their in vivo produced counterparts. These studies indicated that C1q alone could induce IL-8 production in synoviocytes only at concentrations >150 μg/ml. Association of C1q with immune complexes enhances the IL-8 production. ICs composed of IgM and C1q appear to be the most potent inducers of IL-8 production. In addition to induction of inflammatory cytokines, these C1q-ICs could activate the complement system. Our previous studies have indicated the complement-fixing ability of IgM affinity-purified ICs from the sera of JRA patients (10). Interestingly, IgM alone could also induce IL-8 in synoviocytes. The mechanism of IgM-induced IL-8 production is not fully elucidated. However, it has been reported that IgM RFs from RA patients bind MHC class I (29). Therefore, it is possible that the additive effect of IgM on C1q-mediated IL-8 induction could result from the cooperative effect of MHC present on these synoviocytes.

The ability of these C1q-bearing ICs to interact with synoviocytes and trigger IL-8 production represents a novel additional mechanism contributing to the inflammatory process and pathophysiology of JRA. Such observations could have significant implications in the pathophysiology of an IC-mediated disorder such as arthritis. Since JRA is associated with excessive production and deposition of ICs in serum, SF, and synovial tissue, it is conceivable that synoviocytes could have a significant function in the clearance of ICs, specifically C1q-bearing ICs.

Our studies have indicated that synoviocytes (quiescent and IC induced) lack FCγ and complement receptors commonly involved in the IC-mediated cellular responses. Based on data presented in this work, the interaction of C1q-ICs with synoviocytes is mediated via the C1q-binding proteins present on synoviocytes. Of the three C1q receptors known to date, C1qRp (~126 kDa) and C1qR (~60 kDa) bind to the collagen-like domain of C1q, while the 33-kDa gC1q-binding protein shows affinity for the globular head (30–32). Our studies failed to demonstrate the presence of C1qRp on synoviocytes, which further confirms the unique expression of this receptor on cells of myeloid origin, platelets, and endothelial.

**FIGURE 5.** A, Expression of 33-kDa C1q-binding protein in synoviocytes. Total RNA was isolated from synoviocyte monolayers and used as a template for RT-PCR. After reverse transcription, the resulting DNA was subjected to PCR using the specific primers for gC1q-binding protein and C1qRp, as described in the text. PCR products were separated on a 1% agarose gel containing ethidium bromide. RNA from PBMCs and skin fibroblasts were included as positive controls. PBMCs contained the transcript for C1qRp and skin fibroblasts had the transcript for gC1q-binding protein, while synoviocytes contained the transcripts for gC1q-binding protein alone. The arrows indicate the position of C1qRp and gC1q-binding protein PCR products. B, Northern blot analysis of synoviocyte mRNA for the presence of C1qR (calreticulin). Total RNA (10 μg) from two different synoviocyte cultures was subjected to electrophoresis on a 1% agarose gel containing ethidium bromide. RNA from PBMCs and skin fibroblasts were included as positive controls. PBMCs contained the transcript for C1qRp and skin fibroblasts had the transcript for gC1q-binding protein, while synoviocytes contained the transcripts for gC1q-binding protein alone. The arrows indicate the position of C1qRp and gC1q-binding protein PCR products. C, C1q receptor proteins isolated from cultured synoviocytes. Synoviocytes (5 × 10⁶) were lysed in 1 ml of lysis buffer (50 mM Tris-HCl, pH 7.4, containing 100 mM NaCl, 1% Triton X-100, 2 mM PFMS, 10 μg/ml of each aprotinin, pepstatin, and leupeptin) at 4°C. The lysate was centrifuged to remove debris and nuclei. The supernatant was precleared on Sepharose 4B, and incubated with CNBr-activated Sepharose 4B coupled to a mixture of cC1q and gC1q fragments overnight at 4°C. The unbound material was removed by centrifugation; the matrix was washed repeatedly with PBS containing protease inhibitor mixture. The bound material was eluted with 0.8 M solution of NaCl, desalted, and concentrated by freeze drying. The eluate was analyzed by SDS-PAGE (10% resolving and 3.5% stacking gel). The gel was stained with silver and photographed. Molecular weight markers are indicated on the left-hand portion of the gel.
cells (30). The other two C1q-binding proteins identified in synoviocytes appear on a variety of cell types (27, 33–36). They are believed to mediate a wide range of cellular responses, including platelet aggregation (33), enhanced expression of E-selectin, ICAM-1, and VCAM-1 (27), and inflammatory cytokine production by HUVEC (37).

To our knowledge, the presence of these receptors and their interaction with C1q and the C1q-ICs have not been previously reported in synoviocytes. This observation identifies synoviocytes as important contributors to the inflammatory process ongoing in the arthritic joints, and reflects their significant role in the host defense. Our studies indicate that C1q-ICs mediate their effect mostly through cClqR, as collagen stalks of C1q (cClq) could markedly inhibit the C1q-IC-mediated IL-8 induction in synoviocytes. However, C1q globular heads (gClq) could also exert some inhibitory activity (up to 25%) on IL-8 production. Whether such an effect is mediated via the gClq-binding protein needs to be determined. The gClq-binding protein (also known as kninnogen-binding protein (31)) was originally described as a membrane receptor (31, 32), but has recently been localized in the mitochondria, and, therefore, is considered mostly a C1q-binding protein rather than a membrane receptor (38, 39).

Most probably, the interaction of C1q-ICs with synoviocytes is not limited to IL-8 induction and/or complement fixation. Cross-linking of the Clq receptor could induce the expression of adhesion molecules (as observed by Lozada and colleagues (27) on endothelial cells) on synoviocytes, and, hence, facilitate the ingress of leukocytes and lymphocytes into the inflamed joint. Whether synoviocytes from JRA/RA patients respond similarly (compared with normal synoviocytes) to C1q and/or C1q-bearing ICs remains to be determined.

In conclusion, studies reported in this work indicate the presence of anti-IgM-, anti-C1q-reactive ICs in the sera of JRA patients. These ICs, which are present at higher levels in the sera of seronegative JRA patients, have the ability to interact with synoviocytes and induce IL-8 expression and protein production. The Clq Rs identified on synoviocytes play a key role in C1q-IC/synoviocyte interactions. These findings signify a novel mechanism contributing to the inflammatory process in JRA and could represent a possible function for synoviocytes in IC clearance and general maintenance of the synovial environment.

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