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**References**
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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,1* 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 FcγR 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); FCS and gentamicin were purchased from Ambion (Austin, TX); MitoClear (Trizol reagent; Gaithersburg, MD); RETROscript and Quantum RNA kits (RNA STAT-60 reagent; Friendswood, TX) or from Life Technologies; IL-8 ELISA kit was from R&D Systems (Minneapolis, MN); 4B, Sepharose 4B-coupled protein A, Sephadex G75 fine, and blue Sepharose were from Pharmacia Biotec (Uppsala, Sweden). The chemoluminescence kit (ECL). Positive and negative controls (as described in the figure legend) were included in all experiments. 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.

Patients

Serum samples were collected from 17 JRA patients: 12 polyarticular (7 seropositive with a mean disease duration of 7 yr, and 5 seronegative with a mean disease duration of 6 yr), 4 pauciarticular (mean disease duration of 5 yr), and 1 systemic onset (Table I). All patients met American College of Rheumatology criteria for the diagnosis of JRA (14).

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 (18). The undigested Clq 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 (10^4 cells/well) in DMEM medium containing MitoClear 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 Clq alone, or Clq 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. ICs of variable combinations were prepared by adding IgM and Clq 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 MgCl$_2$; 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 the manufacturer’s cDNA. cDNA obtained from U937 cells treated for 4 h with a combination of IL-1β and TNF-α (150 pg/ml each) was used as positive control. PCR reactions were run in a 20 μl and contained 0.125 mM of each dATP, dCTP, dGTP, and dTTP; 1.5 mM MgCl$_2$; 50 mM KCl; 10 mM Tris-HCl, pH 8.3; 3 ng 32P end-labeled IL-8 and 18S oligonucleotides; 0.08 μM 18S primers; 0.33 μM 18S competitor; and 100 ng of IL-8 primers (22): 5'-ATGACCTTCAAGCTGGCGCGTG-3' and 5'-TTATGATTCATCGACGCTCCTAACAAATCTC-3'.

The number of amplification cycles used was empirically determined to ensure a linear relationship between input cDNA and PCR product yield for both 18S and IL-8. The products were analyzed by polyacrylamide gel electrophoresis, and the bands were quantified using a Molecular Dynamics (Sunnyvale, CA) STORM 860 PhosphorImager and analyzed with Imagequant software.

For the detection of receptors for C1q (C1qRp and gC1qR), total RNA was extracted from synoviocytes using a monophase solution of phenol and guanidine isothiocyanate (Trizol reagent). Total RNA (1 μg) was reverse-transcribed using an oligo(dT) primer and superscript reverse transcriptase in a final volume of 20 μl (50 μl at 42°C). The reaction was stopped by heating at 95°C for 5 min. The resulting cDNA was amplified by PCR in buffer containing 500 mM KCl, 100 mM Tris-HCl, 1% Triton X-100, 1.5 mM MgCl$_2$, 20 pmol gene-specific 3' and 5' primers, and 0.5 μl Taq DNA polymerase in a total volume of 25 μl. The reaction mixture was overlaid with mineral oil, hot started at 94°C, and subjected to 35 cycles (60°C annealing temperature) in Robocycler, Stratagene (La Jolla, CA). The number of cycles was previously determined to lie in the linear part of the amplification curve. Total RNA from skin fibroblasts and PMNs were used as positive controls. The primer sequence used for detection of 33gC1qR and C1qRp was: 33gC1qR, 5'-CTGCACACCTGACGGAGACAAAGC-3' and 5'-TCTGTCTGCTCTACTGGCTCTT-3'; C1qRp, 5'-CTTCCTCTTTAAGGCCCTTGTTG-3' and 5'-TCTTGGCATTGCGAGGATGTC-3'.

The PCR products were analyzed by electrophoresis on a 1% agarose gel containing ethidium bromide.

To examine the mRNA expression of c1qr1 in synoviocytes, total RNA (10 μg) was separated by gel electrophoresis in 1% agarose containing 2.2 M formaldehyde. The gel was capillary blotted onto GeneScreen Plus nylon membrane (NEN/DuPont, Boston, MA), baked, and UV cross-linked. The membrane was then probed with radiolabeled cDNA probe for calreticulin (cC1qR) (a generous gift from Drs. J. D. Capra, Oklahoma Medical Research Foundation, Oklahoma City, OK, and Yih-Sheng Yang, South West Medical Center, Dallas, TX). Images of the autoradiograms were digitally captured using a CCD video camera and Snappy Video Snapshot system.

**ELISA**

Commercially available ELISA kits were used for the quantification of IL-8 protein from the cell-free supernatant and according to the manufacturer’s instruction. The assay sensitivity was less than 10 pg/ml. The presence of FcγRs was assayed by ELISA using an anti-μG, anti-λG, anti-C1q, and anti-C3 were summarized in Table II and indicate the presence of anti-C1q-, anti-μG-, and anti-λG antibodies. Synoviocytes were incubated in six-well culture dishes (3 x 10$^5$ cells/well). They were then treated with or without ICs from different JRA patients for 24 h. The supernatants were collected for ELISA, and the cells were washed several times with PBS and harvested by trypsin (0.01%, 2–3 min). A total of 5 x 10$^6$ cells was suspended in 100 μl PBS (containing 1% FCS) and allowed to react with appropriate fluorochrome-conjugated mAbs or subotypic control Abs for 30 min at 4°C. The cells were gently washed, fixed in 1% formaldehyde in PBS, and analyzed using the FACScan or FACSVerifier flow cytometer (Becton Dickinson). Compensation settings were determined using unlabeled Abs. Analyses of flow-cytometric data were performed using the CellQuest or WinList software (Verity). IgG subtype controls were run with all samples and used to set the positive gates.

The 937 cell line (American Type Culture Collection, Manassas, VA) and PBMCs from a healthy individual were used as positive controls.

Recovery of C1q receptor(s) from synoviocytes

Cell lysates prepared from 5 x 10$^6$ synoviocytes were preclared on Sepharose 4B column. The unbound fraction was incubated with cyanogen bromide-activated Sepharose 4B coupled to a mixture of C1q stalk and C1q globular fragments (prepared from digestion of C1q) overnight at 4°C. The unbound material was removed by centrifugation and the matrix was washed repeatedly with fresh buffer containing protease inhibitor mixture. The bound receptor protein(s) was eluted with a solution of 0.8 M NaCl and desalted, and their protein content was quantified using BCA reagent.

The eluate was concentrated by freeze drying and analyzed by SDS-PAGE. The protein bands were visualized by silver staining of the gel.

**Results**

Characterization of the components of serum-isolated ICs

Previously, we had reported isolation of ICs from the sera of JRA patients using IgM affinity purification procedures (23). We decided to further define the composition of these ICs to determine whether a significant relationship exists between JRA disease state and IC composition. Based on the ELISA analysis of the glycine-HCl-eluted fractions, the difference between the ICs from seropositive and seronegative polyarticular onset JRA was mostly in the relative amount of IgM and IgG present in the ICs, with an IgM: IgG ratio being greater in the ICs from seropositive polyarticular JRA cases (0.72 ± 0.12 (n = 5) and 0.34 ± 0.08 (n = 5), respectively). ELISA analysis for the C1q content of the IC fractions also revealed higher C1q levels in seropositive compared with seronegative polyarticular or pauciarticular JRA ICs (data not shown).

Further characterization of glycine-HCl-eluted fractions was achieved by SDS-PAGE, Western blot, and immunohistochemical analysis using Abs specific for IgM, IgG, and complement C1q and C3. Based on electrophoretic data obtained from Coomassie brilliant blue-stained gels, under reduced conditions these ICs were resolved as one major band (molecular mass of about 69–72 kDa) and a second band of 90–92 kDa (Fig. 1A). Minor protein bands of 58, 40, 35, and 26 kDa were also present and could be observed either by increased sample loading or by silver staining the gel (data not shown). The 69–72-kDa band mainly contained serum albumin, as determined by its retention on a blue Sepharose column and its characteristic electrophoretic mobility under reduced and nonreduced conditions. The IC from the pauciarticular JRA patient exhibited levels comparable with seronegative polyarticular JRA, while that of systemic onset JRA had the lowest level (data not shown). The data generated from Western blot analysis using anti-IgM, anti-IgG, anti-C1q, and anti-C3 are summarized in Table II and indicate the presence of anti-C1q-, anti-IgM-, and anti-IgG antibodies. The presence of the above mentioned receptors was also investigated using immunohistochemical analysis of paraformaldehyde or acetone-fixed adherent monolayers. Synoviocytes plated on coverslips were treated with the ICs for the appropriate time interval, then washed and fixed in 1% paraformaldehyde or cold acetone, and blocked. They were then treated with fluorochrome-labeled Abs to FcγRs and/or CRs. The coverslips were washed and mounted for microscopic evaluation.

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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, C3, 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;
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 (10^5 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 the single experiment for the 10-h time point. The zero time mRNA level was arbitrarily set at a value of 1.

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 on the induction of IL-8 in synoviocytes (Table III).

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

By utilizing a similar approach, we assayed for the presence of CRs: CR₁, CR₃, CR₄, and C5α 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 C1qR, 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 of synoviocyte cell lysates. These receptor proteins had apparent molecular masses of ~33 and 60

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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 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

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** Induction of IL-8 protein in synoviocytes, in response to treatment with the purified ICs from sera of different JRA patients. Synoviocytes were plated at 10^5 cells/well of a 24-well culture dish and in DMEM media supplemented with Mito^-^ serum-free supplement. They were then treated with different ICs (5 μg/ml) for 24 h, with each experiment performed in triplicate. The media were collected, centrifuged to remove cell debris, and analyzed by ELISA for IL-8 protein content. Each point represents the mean ± SD of the mean. A. Scattergram demonstrating IL-8 induction in normal synoviocytes and in response to the ICs from different JRA study cases. The + symbol indicates the mean value for each group. B. Bar graph depicting IL-8 induction in synoviocytes and in response to the ICs from representative JRA study cases. Poly^+^ (polyarticular seropositive), poly^-^ (polyarticular seronegative), and pauci (pauciarticular).

![FIGURE 4.](http://www.jimmunol.org/)

**FIGURE 4.** Effect of C1q fragments on the IC-mediated IL-8 induction of synoviocytes. Synoviocytes (5 × 10^4 cells/well of 24-well culture dish) were cultured in serum-free Mito^-^ supplemented DMEM for 24 h; the media were replaced with that containing either the IC alone or in combination with collagen stalk or globular head fragments of C1q. After 48 h, the spent media were collected, centrifuged to remove cell debris, and assayed for IL-8 content by ELISA. Experiments were performed in triplicates.

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^[a] 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 serum 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 cC1qR (~60 kDa) bind to the collagen-like domain of C1q, while the 33-kDa gcC1q-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
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 cC1qR, as collagen stalks of C1q (cC1q) could markedly inhibit the C1q-IC-mediated IL-8 induction in synoviocytes. However, C1q globular heads (gC1q) could also exert some inhibitory activity (up to 25%) on IL-8 production. Whether such an effect is mediated via the gC1q-binding protein needs to be determined. The gC1q-binding protein (also known as kininogen-binding protein (31)) was originally described as a membrane receptor (31, 32), but has recently been located 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 C1q 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 seropositive compared with that of seronegative JRA patients, have the ability to interact with synoviocytes and induce IL-8 expression and protein production. The C1q 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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References