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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

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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.

from Cappel, Organon Teknika (Durham, NC); human IgM and goat anti-
human IgM were from Accurate Chemicals and Scientific (Westbury, NY)
or Sigma (St. Louis, MO); human complement C1q was either from Sigma
or The Binding Site (Birmingham, U.K.); C1q samples were tested at The
University of Iowa Hybridoma Core Facility for endotoxin content before
use. Samples with endotoxin levels below 0.2 ng/ml were used in the
experiments. mAbs to human C1q and human IgG were from Sigma; mAbs
to FcγRs CD32 and CD64 were from Pharmingen (San Diego, CA); mAb
to human C5α receptor (CD88) was from Serotec (Raleigh, NC); Sepharose
4B, Sepharose 4B-coupled protein A, Sephadex G75 fine, and blue Sepha-
rose were from Pharmacia Biotech (Uppsala, Sweden). The chemolumines-
cence ECL kit was obtained from Amersham Pharmacia Biotech (Arling-
ton Heights, IL); IL-8 ELISA kit was from R&D Systems (Minneapolis,
MN); guanidinium thiocyanate/phenol solution was either from Tel-Test B
Matter (17) procedure. The gels were either stained with Coomassie bril-
liant blue (Coomassie BB) and visualized, or blotted onto a polyvinylidene
difluoride membrane and probed with appropriate Abs, as described below.

Preparation of C1q stalks and globular heads

Globular heads and collagen-like stalks of C1q were prepared by collage-
nase 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 frac-
tions 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 (10⁵ cells/well) in
serum-free supplement. Purified ICs were added to the cultures at 10 µg/ml, unless otherwise stated. The cul-
tures 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 dif-
f erent 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 ma-
terial. ICs of 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.

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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<tr>
<td>1/F</td>
<td>21</td>
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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>
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<td>poly</td>
<td>8.0</td>
<td>NSAIDs</td>
</tr>
<tr>
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<tr>
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<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>
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<td></td>
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<td>16</td>
<td>NSAIDs, Gold</td>
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<td>18</td>
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<td>NSAIDs, MTX, HCLq</td>
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<tr>
<td>15/F</td>
<td>02</td>
<td>pauci</td>
<td>0.5</td>
<td>NSAIDs</td>
</tr>
<tr>
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<td>7.5</td>
<td>pauci</td>
<td>5.5</td>
<td>NSAIDs</td>
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<tr>
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<td>5.5</td>
<td>pauci</td>
<td>4.0</td>
<td>NSAIDs</td>
</tr>
</tbody>
</table>

poly, polyclonal serum positive JRA; poly, polyclonal serum positive JRA;
pauci, pauciarticular JRA.

NSAID, nonsteroidal antiinflammatory drug; MTX, methotrexate; HCLq, hydroxychloroquine.

Patients

Serum samples were collected from 17 JRA patients: 12 polyclonal sero-
positive 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
sample was dissected free of fat, minced into small
pieces, and cultured in flasks in DMEM with 10% FCS and 50 mg/L gen-
tamicin. Adherent synovial fibroblasts that developed within 8–10 days
were added to the cultures at 10⁵ cells/well in
medium, and if required, concentrated using Microcon 10 microconcen-
trator (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 per-
formed under both reduced and nonreduced conditions, according to Laem-
mlm’s (17) procedure. The gels were either stained with Coomassie bril-
liant 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 unbonded material was col-
llected by brief centrifugation;ug the protein concentration was estimated and
stored at −80°C until used.

Induction studies

Synoviocytes were plated into 24-well culture dishes (10⁵ 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 cul-
tures 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 dif-
f erent 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
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terial. ICs of 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 medium 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 the manufacturer’s cDNA obtained from U937 cells treated for 4 h with a combination of IL-1β and TNF-α (150 pg/ml each) and its characteristic electrophoretic mobility under reduced conditions (Mako, Tokyo, Japan) was reeled as one major band (molecular mass of about 69–72 kDa) resolved as one major band (molecular mass of about 69–72 kDa) by SDS-PAGE, Western blot, and immunohistochemical analyses. Further characterization of glycine-HCl-eluted fractions was achieved by SDS-PAGE, Western blot, and immunohistochemical analyses. 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). The 69–72-kDa band mainly contained serum 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 C3, 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-containing ICs.

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
and Fig. 1). C3 reactivity was variable among the samples tested, with the activity observed in the area in which 26 kDa was detected (Table II). 40 and 35 kDa were reactive with anti-IgM, while the 58 and 26 kDa protein band exhibited a strong reactivity with anti-IgM and anti-IgG-reactive proteins in the majority of ICs tested. The 92-kDa band with anti-C1q suggests the possibility of a stable complex between one of the three C1q chains (or a C1q fragment) and IgM. The concurrent reactivity of this band with no distinct pattern emerging.

The presence of both anti-C1q- and anti-IgM-reactive components in the 92-kDa band raised the question of whether this band contained more than one protein. To explore this further, this 92-kDa protein band was excised from the gel and subjected to further SDS-PAGE analysis using a 7.5% acrylamide gel. Prolonged electrophoresis revealed one single band corresponding to 92 kDa (data not shown), indicating the absence of different proteins with similar electrophoretic mobility. The concurrent reactivity of this band with anti-C1q suggests the possibility of a stable complex between one of the three C1q chains (or a C1q fragment) and IgM. This association is resistant to reducing conditions used for electrophoresis sample preparation.

FIGURE 1. A, SDS-PAGE analysis of IgM affinity-purified ICs from the sera of seropositive (lane 1) and seronegative (lane 2) polyarticular JRA patients. For Western blot analysis, these ICs were resolved on identical independent gels and transblotted onto polyvinylidene difluoride membranes. The blots were blocked, and probed with Abs to human IgM, IgG, C1q, and C3. B, Western blot analysis of the seropositive JRA IC (same as lane 1A). The 92-kDa band (indicated by the arrow) revealed strong reactivity with anti-IgM (lane 1B) and anti-C1q (lane 2B), minimal reactivity with anti-IgG (lane 3B), and no reactivity with anti-C3 (not shown). C, A sample of C1q-IgG was subjected to SDS-PAGE and Western blot analysis, and probed with anti-C1q (under identical conditions as above). The membrane was stripped and subsequently probed with anti-IgG to ensure no cross-reactivity of anti-C1q with IgG.

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&lt;sup&gt;a&lt;/sup&gt;</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>++</td>
</tr>
<tr>
<td>57</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
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</tr>
<tr>
<td>35</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>26</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

<sup>a</sup> 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.

<sup>b</sup> Anti-C3 reactivity varied from patient to patient, and no direct correlation was observed between the level of C3 and IL-8 production.

<sup>c</sup> Reactivity with anti-C1q was much more pronounced (++++) in seropositive polyarticular JRA ICs.

<sup>d</sup> This band was later identified as serum albumin.

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

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).

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 immunoadsorption chromatography of synoviocyte cell lysates. These receptor proteins had apparent molecular masses of ~33 and 60

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**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 (105 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 zero time mRNA level which was arbitrarily set at a value of 1.
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

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.

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

<table>
<thead>
<tr>
<th>ICs</th>
<th>Agg-IgG (μg/ml)</th>
<th>C1q (μg/ml)</th>
<th>IgM (μg/ml)</th>
<th>IL-8 (pg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>40 ± 11</td>
</tr>
<tr>
<td>50</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>79 ± 18</td>
</tr>
<tr>
<td>–</td>
<td>150</td>
<td>–</td>
<td>–</td>
<td>109 ± 20</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>50</td>
<td>–</td>
<td>123 ± 18</td>
</tr>
<tr>
<td>–</td>
<td>–</td>
<td>50</td>
<td>50</td>
<td>255 ± 26</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>50</td>
<td>–</td>
<td>359 ± 43</td>
</tr>
<tr>
<td>50</td>
<td>50</td>
<td>100</td>
<td>–</td>
<td>453 ± 50</td>
</tr>
<tr>
<td>100</td>
<td>50</td>
<td>100</td>
<td>100</td>
<td>499 ± 56</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>789 ± 71</td>
</tr>
</tbody>
</table>

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 C1qR on synoviocytes, which further confirms the unique expression of this receptor on cells of myeloid origin, platelets, and endothelial cells.
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 kninogen-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 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 patients 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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