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The First Subcomponent of Complement, C1q, Triggers the Production of IL-8, IL-6, and Monocyte Chemoattractant Peptide-1 by Human Umbilical Vein Endothelial Cells

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We and others have demonstrated previously the occurrence of C1q-containing immune complexes bind to endothelial cells (EC) via a specific receptor for C1q (C1qR). Next to EC (1–3), C1q has also been shown to interact with a large number of other cell types, such as fibroblasts (4), epithelial cells (5), platelets (6), B cells (7), eosinophils (8), neutrophils (9), and monocytes (9). Binding of C1q to EC receptors on these different cell types elicits differential cellular responses: for example, enhancement of phagocytosis by macrophages (10–12), enhanced secretion of Igs by B cells (13), and stimulation of the oxidative metabolism in neutrophils (14). To our knowledge, the effect of C1q binding to endothelium is limited to only two reports describing the enhanced binding to and phagocytosis of Salmonella minnesota (15) and up-regulation of ELAM, ICAM-1, and VCAM-1 (16). Since the initial demonstration of the binding of C1q to EC, three specific receptors for C1q have been described: a receptor for the globular domain of C1q (gC1qR) (17, 18), a receptor for the collagen-like stalks of C1q that has high homology with calreticulin (cC1qR/ CaR) (3, 6, 16, 19–22), and a receptor that enhances phagocytosis by monocytes (C1qRp) (23) (16, 24, 25). While others have suggested that gC1qR is present on the membrane of various cells (17), increasing evidence strongly suggests that gC1qR is a mitochondrial protein (26–28). The other two receptors, namely C1qRCaR and C1qRp, both have been shown to be present on the membrane of EC in vitro.

For host defense, EC are indispensable because they are involved in binding and phagocytosis of pathogens, attraction of inflammatory cells, activation of the coagulant system and the complement system, and local increment of blood vessel permeability. Most of these effects are mediated and regulated by cytokines, chemokines, and adhesion molecules.

Stimulation of EC with IL-1 and TNF, for example, results in direct (ICAM-1, ELAM, VCAM-1) and indirect (via IL-6) up-regulation of adhesion molecules, but will also induce the production of molecules such as RANTES, IL-8, and MCP-1, that are able to attract lymphocytes, neutrophils, and monocytes to the site of inflammation.

In the present study we investigated the ability of EC to produce cytokines and chemokines in response to C1q stimulation. These factors are of importance because they are involved in the attraction of neutrophils and monocytes/lymphocytes (IL-8, MCP-1) to the site of an inflammation.

Materials and Methods
Isolation of C1q and C1q fragments

C1q was isolated from human serum as previously described (29) with some modifications. C1q was precipitated from normal human serum with polyethylene glycol 6000 (3%, w/v) and dissolved in veronal-buffered saline. After adjustment of the conductivity to 12 mS, EDTA was added to a final concentration of 2 mM. The C1q-containing solution was applied to
a human IgG Sepharose column that was incubated overnight with excess rabbit anti-human IgG. After extensive washing with PBS containing 2 mM EDTA, C1q was eluted with the same buffer now containing 1 M NaCl. C1q activity in the fractions was measured by a hemolytic assay (30); positive fractions were pooled, concentrated, and filtered on a Superdex 200 gel filtration column. Finally, to remove contaminating IgG, C1q-containing fractions were pooled and subsequently applied to a protein G column (Pharmacia, Roosendaal, The Netherlands). Again, C1q-containing fractions were pooled and stored on ice until use. The purified C1q was hemolytically active (30) and was shown to be devoid of contaminants as judged by SDS-PAGE (31). Also, approximately 8% of the C1q in the C1q preparation was shown to exist as aggregates (31).

Collagen-like stalks of C1q were prepared by pepsin digestion as described previously (31, 32). To remove noncleaved C1q, the isolated protein was applied to a human IgG Bio-Gel A5 column (Bio-Rad, Richmond, Ca). The fallthrough fractions containing the C1q stalks were then freeze-dried and stored in PBS containing 1% glycerol on ice. With a C1q hemolytic assay, no residual hemolytic activity could be detected.

C1q globular heads were prepared by collagenase treatment as described previously (33). Digested C1q was filtered on a TSK 3000 SW gel filtration column (Pharmacia, Uppsala, Sweden); the peak, which had an apparent molecular mass of 30 kDa and was reactive with polyclonal anti-C1s Abs, was pooled, concentrated, and analyzed on SDS-PAGE. This preparation of globular heads of C1q was devoid of residual C1q hemolytic activity.

Isolation and culture of HUVEC
EC were isolated by collagenase digestion of human umbilical cords (34) and were then cultured on gelatin-coated tissue flasks (Greiner, Alphen a/d Rijn, The Netherlands) in medium 199 containing 10% heat-inactivated FCS, 100 U/ml penicillin, 100 µg/ml streptomycin, Earle’s salts (Sigma, St. Louis, MO), 7.5 U/ml heparin (Organon Technika, Boxtel, The Netherlands), or 0.002% (w/v) endothelial growth factor isolated from bovine serum from patients with Walden-Strom’s disease using boric acid, followed by filtration on Bio-Gel A5 (37). To obtain AIgM, IgM was subsequently blocked with PBS containing 0.01% Tween and 2% casein. After 48-h incubation, supernatants were tested in ELISA for IL-6, IL-8, and MCP-1 concentrations. All incubations were performed in medium 199 containing 0.5% FCS.

Effect of cycloheximide on C1q-mediated production of IL-8, IL-6, and MCP-1 by HUVEC
Quiescent HUVEC were preincubated for 4 h with either medium alone (medium 199 containing 0.5% FCS) or medium containing 10 µg/ml cycloheximide (Sigma), washed, and then stimulated with medium containing 100 µg/ml C1q or BSA in the presence of 10 µg/ml cycloheximide. After 48 h of incubation, the supernatants were harvested, and concentrations of IL-6, IL-8, and MCP-1 were determined by ELISA. As a control, the concentration of factor H was also assessed.

Effect of F(ab’)2 anti-caltrectin on C1q-induced IL-8, IL-6, and MCP-1 production by HUVEC
For the preparation of F(ab’)2 anti-caltrectin (Car) fragments, a polyclonal anti-Car was used (provided by Dr. R. B. Sim, Oxford University, Oxford, U.K.), the specificity of which for Car was previously established by Malhotra et al. (19) using Western blot analysis and RAs with lymphocyte cC1q/CaR. Furthermore, the Ab was shown to be able to bind to C1qR/Car on the cell surface of epithelial cells (38) and neutrophils (39). The IgG fractions were prepared by ammonium precipitation, followed by anion exchange chromatography on DEAE-A50 Sephadex. F(ab’)2 anti-Car, and F(ab’)2 anti-SRBC were obtained after pepsin digestion of, respectively, rabbit IgG anti-Car (19) and rabbit IgG anti-SRBC (prepared in our laboratory) as described previously (40). The F(ab’)2 were repassed over a protein A-Sepharose 4B column (Pharmacia) to remove Fe fragments and undigested IgG. Confluent quiescent layers of HUVEC were incubated with increasing concentrations of F(ab’)2 anti-Car, F(ab’)2 anti-SRBC, or BSA. After 48-h incubation, supernatants were tested in ELISA for IL-6, IL-8, and MCP-1 concentrations. All incubations were performed in medium 199 containing 0.5% FCS.

Analysis of mRNA production by HUVEC after stimulation with C1q
Confluent quiescent layers of HUVEC were incubated in T25 flasks (Greiner, Alphen a/d Rijn, The Netherlands) for 24 h with either medium alone (medium 199 containing 0.5% FCS) or with medium containing 50 µg/ml C1q. Cells were then detached by trypsin treatment, and total RNA was isolated as described by Chomczynski (41). By reverse transcription, 1 µg of RNA was transcribed into cDNA by oligo(dT) priming (42). Oligonucleotide primers were constructed from known cDNA sequences of IL-6 (43) (sense, 5’- GTACCCTCCAGGAGAGTTC-3’; antisense, 5’- ATTACGCTTCAGACCTTGA-3’), IL-8 (44) (sense, 5’- GCTTTCTGATGGAAGAGAC-3’; antisense, 5’- TGGTGATCTGGCTGAG-3’), and β-actin (46) (sense, 5’-CTAATGATGGCTCCTGTTG-3’; antisense, 5’- AAGGAGGCTTGGAGTGC-3’). For the PCR reaction, 10 µl of cDNA, 50 pmol of sense primer, 50 pmol of antisense primer, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer/Cetus, Norwalk, CT), and PCR buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 2.0 mM MgCl2, 2 mM dNTP, and 0.25 mM of each dNTP in a total volume of 100 µl were used. The mixture was heated for 5 min at 95°C followed by 33 cycles of 1.5 min at 95°C, 2.5 min at 55°C, 1.5 min at 72°C, and finished by 10 min at 72°C. Ten milliliters of PCR product was separated by electrophoresis on a 1% agarose gel.

MCP-1, IL-6, and IL-8 sandwich ELISAs
Ninety-six-well microtitr plates (MaxiSorb F96, Nunc (Roskilde, Denmark) or Greiner) were coated with monoclonal anti-human MCP-1 Ab (R&D Systems, Abington, U.K.), anti-human IL-6 (5E1, provided by Dr. W. D. Uman, University Hospital Maastricht, Maastricht, The Netherlands), or anti-IL-8 mAb (CLB, Amsterdam, The Netherlands) and subsequently blocked with PBS containing 0.01% Tween and 2% casein. After washing, appropriate dilutions of samples were added, incubated for 1 h at 37°C, washed, and then incubated with a rabbit polyclonal anti-MCP-1 (created in our laboratory by immunization of a rabbit with recombinant human MCP-1 (PeproTech, Rocky Hill, NJ)) (47), anti-IL-6 (created in our laboratory by immunization of a rabbit with recombinant human IL-6 (San, Donov, Hanover, NJ), or anti-IL-8 (created in our laboratory using rIL-8 (PeproTech) for 1 h at 37°C. Finally, the wells were incubated with horse-radish peroxidase (HRP)-conjugated polyclonal IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). After addition of the substrate for HRP, 2,2’-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (Sigma), the OD was measured at 415 nm, and the cytokine concentration was calculated relative to a MCP-1, IL-6, or IL-8 standard.
in the presence of C1q (l) or BSA (n) at different doses is shown (B). Supernatants were tested with specific ELISAs for the presence of IL-6, IL-8, and MCP-1. Because the response was comparable for all peptides under study, only data for IL-8 production are shown in Fig. 2. Addition of AlgM enhanced the production of all peptides compared with incubation with C1q alone. Whereas C1q alone resulted in a maximal production of 501 ± 112 pg/ml of IL-6, 1019 ± 52 pg/ml of IL-8, and 2113 ± 104 pg/ml of MCP-1. No significant production was detected after incubation with BSA. As an example of the response, the data for IL-8 production are shown in Fig. 2. Since C1q is known to interact with IgG- and IgM-containing immune complexes (40, 50), we assessed the effect of AlgM on HUVEC preloaded with C1q. HUVEC were preincubated with a suboptimal concentration of C1q and after washing were incubated with increasing concentrations of humanAlgM. After culture, supernatants were tested for IL-6, IL-8, and MCP-1. Because the response was comparable for all peptides under study, only data for IL-8 production are shown (Fig. 3). Addition of AlgM enhanced the production of all peptides compared with stimulation with C1q alone. Whereas C1q alone resulted in a maximal production of 395 ± 33 pg/ml of IL-6, 500 ± 180 pg/ml of IL-8, and 1702 ± 447 pg/ml of MCP-1, addition of AlgM enhanced the maximal production to 997 ± 21 pg/ml of IL-6, 2500 ± 422 pg/ml of IL-8, and 5343 ± 302 pg/ml of MCP-1. Incubation of HUVEC with AlgM alone did not affect the production of IL-6, IL-8, or MCP-1.

To determine whether the effect of C1q was mediated via its collagen-like stalks or globular heads, HUVEC were incubated with C1q in the presence or the absence of the C1q fragments or BSA. Incubation with intact C1q alone resulted in the production of 290 ± 21 pg/ml of IL-8, 111 ± 11 pg/ml of IL-6, and 714 ± 31 pg/ml of MCP-1. Since the inhibitions of peptides due to coincubation with the different C1q fragments were very similar, Fig. 4 only depicts the percent inhibition of IL-8 production. It was found that the presence of the collagen-like stalks of C1q resulted in 69 ± 7% inhibition of IL-8 production. Globular heads of C1q were only able to inhibit C1q-mediated production to a limited extent (13 ± 11%), whereas BSA had no significant effect (3 ± 2%).
To determine whether the observed production of IL-6, IL-8, and MCP-1 was de novo, HUVEC were incubated with C1q in the presence or the absence of cycloheximide. The concentrations of IL-6, IL-8, and MCP-1 were determined and are depicted in Fig. 5. Cycloheximide was able to significantly inhibit C1q-induced production of all peptides. The production of factor H was studied as a control and was shown to be reduced nonsignificantly in the presence of cycloheximide.

Also, de novo production of IL-6, IL-8, and MCP-1 was analyzed by detection of specific mRNA products. Therefore, total RNA, isolated from HUVEC that were incubated with either medium or C1q, was reverse transcribed into cDNA by random priming. Using specific primers for IL-6, IL-8, MCP-1, and β-actin, cDNA was amplified by PCR, an aliquot was electrophoresed on an agarose gel, and the specific bands with correct base pair lengths were analyzed. Fig. 6 demonstrates a basal level of specific mRNA for IL-6, IL-8, and MCP-1. Significant up-regulation of specific mRNA was found after stimulation with C1q for all peptides. As a control for equal loading the intensity of β-actin is shown.

To determine the involvement of cC1qR/CaR in the production of IL-8, IL-6, and MCP-1, inhibition experiments were performed in which HUVEC were preincubated with F(ab′)2 anti-CaR or an irrelevant F(ab′)2 Ab followed by incubation with C1q. Next, the concentrations of IL-8 were determined, and the inhibitory effect of F(ab′)2 anti-CaR was calculated (Fig. 7). Stimulation of HUVEC with 50 μg/ml of C1q alone resulted in the production of 320 ± 62 pg/ml IL-8. Preincubation of HUVEC with increasing concentrations of F(ab′)2 anti-CaR demonstrated a dose-dependent inhibition of IL-8 production with a maximum of 70 ± 8%. Irrelevant F(ab′)2 Ab only induced a maximum of 13% inhibition.

**Discussion**

The present study, to our knowledge for the first time, demonstrates that interaction of C1q with EC in culture results in enhanced, de novo production of IL-8, IL-6, and MCP-1 and not of factor H. This effect was both time and dose dependent.

As we have shown previously, binding of monomeric C1q to cell surface C1qR is far less efficient than binding of multimeric C1q. Therefore, the described results are probably mediated via binding of aggregated C1q, present in the C1q preparation, to
measured and expressed as the percent inhibition of IL-8 production.

FIGURE 7. Inhibition of C1q-induced IL-8 production by preincubation of HUVEC with F(ab')2 anti-calreticulin. Quiescent layers of HUVEC were incubated for 1 h with increasing concentrations of either rabbit F(ab')2 anti-calreticulin or F(ab')2 anti-SRBC. After washing, the cells were incubated in medium containing 50 μg/ml C1q or BSA for another 48 h. Subsequently, the concentrations of IL-8 in the supernatants were measured and expressed as the percent inhibition of IL-8 production.

cC1qR/CaR (31, 51). The observed cytokine and chemokine production might be effected by cross-linking of cC1qR/CaR by these multimeric C1q molecules. This hypothesis is supported by the observation that enhanced cross-linking of the receptor by subsequent incubation with aggregated IgM resulted in further up-regulation of all factors studied.

Cross-linking of cC1qR/CaR by C1q seems to be a prerequisite for the different effects that are described to be mediated via C1q binding. However, until now no clear consensus exists concerning the underlying second messenger system. One study indicated that the concentrations of the second messenger inositol 1,4,5-trisphosphate were clearly up-regulated after stimulation of cC1qR/CaR on platelets by C1q (52), whereas a recent study by Leigh et al. (53) demonstrated the involvement of G protein-coupled signal transduction mechanisms in C1q-mediated chemotaxis of neutrophils.

The results indicate that the effect of C1q on EC is exerted via the collagen-like stalk of C1q. EC have been described to express three types of C1q binding proteins, namely gC1qR, cC1qR/CaR, and C1qRp. gC1qR, a 33-kDa glycoprotein present on EC and on some other cells, was initially described as a membrane receptor interacting with C1q globular heads, but also with vitronectin and kininogen (54, 55). More recently, however, our own studies and those of others have demonstrated that gC1qR is a mitochondrial protein (26–28, 56). The absence of a membrane-spanning domain is consistent with this finding. Even when gC1qR is present on EC membranes, the inhibition experiments with the C1q collagen-like stalks and C1q globular heads in this study suggest that a minimal contribution of the globular heads mediated cytokine and chemokine production.

The other two C1q binding proteins, cC1qR/CaR and C1qRp, are more likely to be involved in C1q-mediated triggering of EC, because both these receptors are present on EC. Moreover, the interaction between cC1qR/Ca and C1q has been demonstrated, whereas such an interaction between C1qRp and C1q is suggested by experiments demonstrating that Abs against C1qRp are able to block C1q-mediated enhancement of phagocytosis by monocytes. CC1qR/CaR, a 60-kDa glycoprotein, is present on almost every cell type studied, including EC (3, 19, 24, 57–64). It binds to the collagen-like stalks of C1q and has been shown to mediate, for example, C1q-induced platelet aggregation (65). The recently cloned and sequenced 126-kDa C1qRp, with high levels of expression on myeloid and EC, has been shown to be involved in C1q-mediated enhancement of monocyte phagocytosis (16, 23, 25).

In the present study we were able to show that F(ab')2 anti-CaR inhibits IL-8 production by 70% after stimulation of HUVEC with C1q. This experiment indicates the involvement of cC1qR/CaR in the above described effects. However, because production of IL-8 was not fully inhibitable by F(ab')2 anti-CaR, other C1q binding molecules in the membrane may be involved.

Until now, only two functions were described for the different C1q receptors on EC. First, it was shown earlier that binding of C1q to cC1qR/CaR results in a reduction of C1q hemolytic activity (22, 66). Second, binding of C1q to cC1qR/CaR mediates binding of immune complexes, which may lead to increased adhesion of leukocytes or bacteria (16, 51, 67).

As we have shown in the present study, binding of immune complexes might also enhance the C1q-mediated triggering of EC and production of cytokines and chemokines to a great extent. Therefore, we hypothesize that in vivo, binding of immune complexes to EC can result in vascular damage in different ways. First, C1q-containing immune complexes may activate the complement system that might injure autologous cells in the vicinity of the immune complex deposits by formation of a membrane attack complex. Also, chemotactic cytokines, formed during the complement cascade, may attract inflammatory cells. Second, C1q-containing immune complexes may induce cross-linking of C1qR/CaR on the cell surface. As shown in this study, this may greatly enhance the production of IL-6, IL-8, and MCP-1. In general, IL-6 will act as a proinflammatory molecule because it is able to stimulate the growth and differentiation of B cells and T cells (68, 69); it is capable of inducing synthesis of acute phase proteins by hepatocytes (70, 71), and it enhances leukocyte adherence to EC (72). However, IL-6 can also be viewed as an anti-inflammatory cytokine, since it inhibits TNF production by monocytes (73, 74) and induces the release of IL-1R antagonist and soluble TNF receptor in the liver, which are inhibitors of IL-1 and TNF, respectively (75, 76).

Attraction of inflammatory cells, on the other hand, is predominantly mediated by IL-8 and MCP-1, which belong to different subfamilies of structurally homologous cytokines, entitled the α, β, and C subfamilies of chemokines (77, 78). Members of the α subfamily, such as IL-8, are chemotactic for neutrophils (79), whereas members of the β subfamily, such as MCP-1, macrophage inflammatory protein-1α, and RANTES, are chemotactic for monocytes and lymphocytes (80).

Next to chemotraction, IL-8 may also enhance adherence of neutrophils to endothelium by increasing β2 integrin expression and regulation of trans-endothelial migration of neutrophils (81).

In addition to the C1q stimulus, IL-6, IL-8, and MCP-1 are known to be produced in response to autocrine stimulation of EC with IL-1 and TNF. Stimulation with IL-1 and TNF, however, results in a number of responses, including enhancement of permeability (82); expression of adhesion molecules such as ICAM-1, ELAM, and VCAM-1 (83, 84); production of hemopoietic growth factors, IL-1, IL-6, leukemia inhibitory factor, IL-8, platelet-derived growth factor, MCP-1, RANTES, platelet-activating factor, and the PGs PGE2 and PGH2 (84–87). Many of these factors can subsequently activate other cells or have an effect on EC itself. Therefore, we hypothesize that the reaction of EC to stimulation with C1q is probably not limited to IL-6, IL-8, and MCP-1, but may also involve, for example, adhesion molecules or molecules with procoagulant activity. Subsequently, lymphocytes, neutrophils, and monocytes may be attracted by IL-8 and MCP-1, respectively, whereas IL-6 and IL-8 may enhance the adherence of these cells to EC (72, 81). These mechanisms therefore may amplify an ongoing inflammatory response, leading to tissue injury.
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