Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 8378–8382, August 1995
Medical Sciences

Identification of C1q as the heat-labile serum cofactor required for immune complexes to stimulate endothelial expression of the adhesion molecules E-selectin and intercellular and vascular cell adhesion molecules 1

(leukocyte/endothelium/immune complex/vasculitis)

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Communicated by H. S. Lawrence, New York University Medical Center, New York, NY, April 28, 1995 (received for review January 5, 1995)

ABSTRACT **To examine the role of complement components as regulators of the expression of endothelial adhesive molecules in response to immune complexes (ICs), we determined whether ICs stimulate both endothelial adhesiveness for leukocytes and expression of E-selectin and intercellular and vascular cell adhesion molecules 1 (ICAM-1 and VCAM-1). We found that ICs [bovine serum albumin (BSA)–anti-BSA] stimulated endothelial cell adhesiveness for added leukocytes in the presence of complement-sufficient normal human serum (NHS) but not in the presence of heat-inactivated serum (HIS) or in tissue culture medium alone. Depletion of complement component C3 or C8 from serum did not prevent enhanced endothelial adhesiveness stimulated by ICs. In contrast, depletion of complement component C1q markedly inhibited IC-stimulated endothelial adhesiveness for leukocytes. When the heat-labile complement component C1q was added to HIS, the capacity of ICs to stimulate endothelial adhesiveness for leukocytes was completely restored. Further evidence for the possible role of C1q in mediating the effect of ICs on endothelial cells was the discovery of the presence of the 100- to 126-kDa C1q-binding protein on the surface of endothelial cells (by cytofluorography) and of message for the 33-kDa C1q receptor in resting endothelial cells (by reverse transcription–PCR). Inhibition of protein synthesis by cycloheximide blocked endothelial adhesiveness for leukocytes stimulated by either interleukin 1 or ICs in the presence of NHS. After stimulation with ICs in the presence of NHS, endothelial cells expressed increased numbers of adhesion molecules (E-selectin, ICAM-1, and VCAM-1). Endothelial expression of adhesion molecules mediated, at least in part, endothelial adhesiveness for leukocytes, since leukocyte adhesion was blocked by monoclonal antibodies directed against E-selectin. These studies show that ICs stimulate endothelial cells to express adhesive proteins for leukocytes in the presence of a heat-labile serum factor. That factor appears to be C1q.**

Vascular endothelium plays a central role in inflammation by expressing specific adhesion molecules including P-selectin, E-selectin, and intercellular and vascular cell adhesion molecules 1 (ICAM-1 and VCAM-1) that attract and localize leukocytes to inflamed sites (reviewed in ref. 1). The recent observation that endothelial expression of E-selectin is required for development of inflammatory injury after intravascular injection of immune complexes (ICs) suggests an active role for endothelium in IC-mediated vasculitis (2). However, the stimulus for endothelial expression of E-selectin or other adhesion molecules after exposure to ICs has not been established. Neither cultured macrovascular endothelial cells nor microvascular endothelium at sites of IC-mediated vasculitis express Fc fragment receptors and, therefore, are not likely to be directly activated by ICs (3–6).

Complement and other soluble mediators of inflammation clearly stimulate the inflammatory functions of endothelial cells. Endothelial cells express receptors for the complement activation product C5a, which, when occupied, stimulate expression of P-selectin (7–9). Similarly, activation of the membrane attack complex (C5b-9) on the endothelial surface also stimulates P-selectin expression (10). The endothelium also expresses receptors for C1q (11–13), although the functional significance of these receptors is unknown.

We studied the results of the interaction of ICs with the endothelium and found that ICs stimulate endothelial cells to express adhesion molecules and become adhesive for leukocytes. This stimulation is dependent upon the presence of a heat-labile serum factor, which we have identified as C1q.

MATERIALS AND METHODS

Materials. Tumor necrosis factor α and interleukin 1α (IL-1α) were purchased from R & D Systems. An E-Toxate kit (for the determination of endotoxin contamination), cycloheximide, pertussis toxin, recombinant human C5a, bovine serum albumin (BSA), and thrombin were purchased from Sigma. RPMI 1640 medium was purchased from GIBCO, and fetal bovine serum was purchased from Atlanta Biologicals (Norcross, GA). Fluorescence-activated cell sorting (FACS) lysing solution was purchased from Becton Dickinson, and fluorescein isothiocyanate (FITC)-conjugated polyclonal goat anti-mouse antibody was purchased from Coulter. Complement component C3-deficient serum, C8-deficient serum, and C1q were purchased from Quidel (San Diego). Rabbit anti-BSA (IgG fraction) was purchased from Organon Teknika.

Monoclonal Antibodies. IgG1-, IgG2a-, and IgG2b-isotype control antibodies were purchased from Sigma. Anti-ICAM-1 (IgG2a, E1/7), anti-VCAM-1 (IgG1, E1/6), and anti-E-selectin (IgG2a, H18/7 and H4/18) antibodies were the gifts of M. P. Bevilacqua (Howard Hughes Medical Institute, University of California at San Diego, LaJolla, CA), and anti-C1q antibody (IgG2A, R139) was a gift of A. Tenner (University of California

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Abbreviations: ICAM-1, intercellular cell adhesion molecule 1; VCAM-1, vascular cell adhesion molecule 1; IC, immune complex; NHS, normal human serum; HIS, heat-inactivated serum; IL-1α, interleukin 1α.

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at Irvine). Anti-E-selectin (BMA4D10, rat IgG2a) and anti-CD62 were purchased from Accurate Chemicals.

Isolation of Leukocytes. Blood obtained from healthy volunteers prior to each experiment was treated with anticoagulant (EDTA) in Becton Dickinson Vacutainer tubes. After separation of plasma (centrifugation with an IEC Centra-7R centrifuge at 1500 rpm for 15 min), leukocytes and erythrocytes were washed twice, and the erythrocytes were lysed by incubation with FACS lysing solution. Leukocytes were resuspended (1.5×10^6 per ml) in RPMI 1640 medium. This isolation technique was used because it yields "quiescent" cells as determined by expression of neutrophil adhesion molecules (CD11b/CD18 and L-selectin).

Culture of Endothelial Cells. Human umbilical vein endothelial cells were obtained and cultured by modifications of the method of Jaffe *et al.* (14, 15) and used during passages 3–8; no differences in endothelial responses were observed among passages.

Neutrophil Adherence to Endothelial Monolayers (Heterotypic Adherence). Leukocyte adhesion to endothelial monolayers cultured in 96-well plates was determined as described (16, 17). The data are expressed as the stimulated increment in adherent cells, calculated as:

$$(\text{no. of adherent cells per } \times 400 \text{ field})_{\text{stimulated}} - (\text{no. of adherent cells per } \times 400 \text{ field})_{\text{resting}}.$$

Leukocyte adhesion to resting endothelial cells did not vary whether the endothelial cells were incubated in tissue culture medium [RPMI 1640 medium/fetal bovine serum, 9:1 (vol/vol)] containing 50% (vol/vol) normal human serum (NHS) or 50% (vol/vol) heat-inactivated serum (HIS) or in tissue culture medium alone (8 ± 1, 7 ± 3, or 5 ± 1 cells per $\times 400$ field, respectively; $n = 3$). More than 90% of the adherent cells were polymorphonuclear leukocytes, as determined by differential counts of adherent leukocytes after cytochemical staining for specific esterase (naphthol AS-D chloroacetate esterase, Sigma).

Expression of Adhesive Molecules Assayed by Cytofluorography. Endothelial cells were grown to confluence in six-well tissue culture plates and exposed to various stimuli and agents, as above. After incubation for various time periods (10 min, 4 hr, 18 hr, or 48 hr), the endothelial cells were harvested, labeled with fluorescein-linked antibodies, and analyzed as described (16).

Preparation of ICs. ICs were prepared as described (18, 19) by mixing BSA with rabbit polyclonal anti-BSA IgG antibody in an antigen/antibody ratio of 1:5 (wt/wt).

Endotoxin Contamination. Preparations of ICs, NHS, HIS, and C1q contained <0.06 unit/ml (<0.1 ng/ml) of endotoxin.

Preparation of C1q-Depleted Serum. In preliminary experiments C1q-depleted serum from two different commercial sources proved to be toxic to endothelial cells. Therefore, we prepared C1q-depleted serum by the method of Yonemasu and Stroud (20). Fresh whole serum (100 ml) was dialyzed against 1 liter of 0.026 M EGTA (pH 7.5) for 15 hr at 4°C. The dialysate was collected at this point, and Ca^{2+} was replaced by the addition of Ca^{2+} to a final concentration of 30 mM.

Reverse Transcription–PCR of cDNA Encoding 33-kDa C1q Binding Protein. First-strand cDNA from endothelial cells and a B-cell line was generated from 1–2 μg of total RNA [isolated by a modification of the method of Chirgwin *et al.* (21, 22)] in 50 μl containing 3–6 μg of oligo(dT), 0.5 mM dithiothreitol, 40 units of RNasin (Promega), and 8 units of avian myeloblastosis virus reverse transcriptase (Life Sciences, St. Petersburg, FL). The reaction mixture was incubated at 41°C for 2 hr. From the first-strand synthesis reaction, 10 μl was amplified in 50 μl containing 1 μg of the specific primers [sense, 5′-AACCCTCGCAAGGGCAGAA; antisense, 5′-GTAGGATTTGTTCACTGGCCA (23)], 2.5 units of *Taq* polymerase (Perkin–Elmer/Cetus), 0.01% gelatin, 50 mM KCl, and 0.25 mM each dNTP. DNA was amplified by using an initial 4-min 94°C denaturation followed by 30 cycles of denaturation (30 sec at 94°C), annealing (30 sec at 53°C), and extension (45 sec at 72°C). Products were separated by electrophoresis through a 2% (wt/vol) agarose gel (23).

Statistical Analysis. The significance of differences among and between groups was determined by means of analysis of variance and separate post hoc variances.

RESULTS

We studied the effect of ICs on endothelial cell adhesiveness for leukocytes. When we incubated endothelial cells with ICs in tissue culture medium (RPMI 1640 medium containing 10% fetal bovine serum) there was no increase in endothelial adhesiveness for leukocytes. However, incubation of endothelial cells with ICs in the presence of NHS induced a marked increase in endothelial adhesiveness for leukocytes (Fig. 1). In contrast, heat treatment of the serum (30 min at 56°C) abolished its capacity to act as a cofactor for IC-stimulated endothelial adhesiveness (Fig. 1). HIS did not inhibit endothelial adhesiveness because adherence for leukocytes stimulated by IL-1 was unchanged in the presence of HIS (Fig. 1). Maximal endothelial adhesiveness for leukocytes was observed at a concentration of ICs of 0.48 mg/ml and did not increase further with twice the concentration of ICs (data not shown). Maximal adhesiveness after exposure to ICs was observed after 3 hr of incubation and persisted for up to 24 hr (data not shown). Similar results were obtained with polyethylene glycol-precipitated ICs from patients with human immunodeficiency virus-related thrombocytopenia (S. Karpatkin, personal communication).

Since NHS but not HIS supports IC stimulation of endothelial cells, we tested the hypothesis that ICs activated endo-

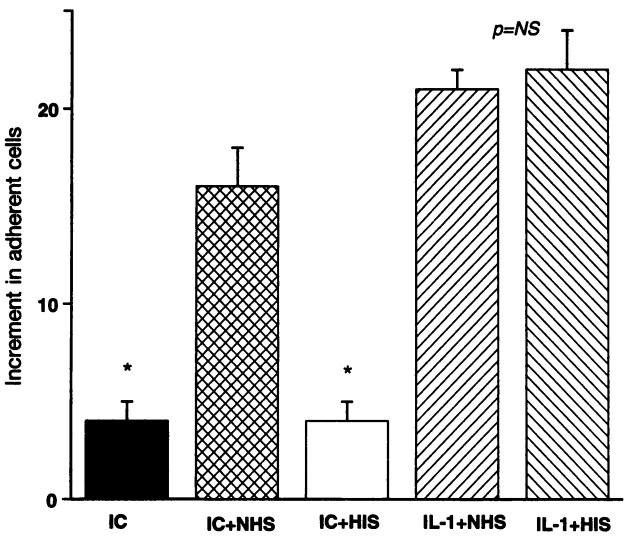

FIG. 1. Effects of ICs and IL-1α on endothelial adhesiveness for leukocytes. Endothelial cells were incubated either with ICs (0.48 mg/ml) in tissue culture medium (90% RPMI 1640 medium/10% fetal bovine serum) alone (■) or containing 50% NHS (▩) or 50% HIS (□) or with IL-1α (10 units/ml) in tissue culture medium containing 50% NHS (▨) or 50% HIS (▧) for 3 hr at 37°C before washing, incubation with leukocytes, and quantitation of adherent leukocytes. The mean increments ± SEM in adhesion, expressed as cells per ×400 field, were: IC plus tissue culture medium, 4 ± 1 ($n = 3$); IC plus NHS, 16 ± 2 ($n = 15$); IC plus HIS, 4 ± 1 ($n = 6$); IL-1 plus NHS, 21 ± 1 ($n = 11$); and IL-1 plus HIS, 22 ± 2 ($n = 6$). ICs increased leukocyte adhesion only in the presence of NHS (★, $P < 0.003$; one-way ANOVA and Student's t test with Bonferroni correction).

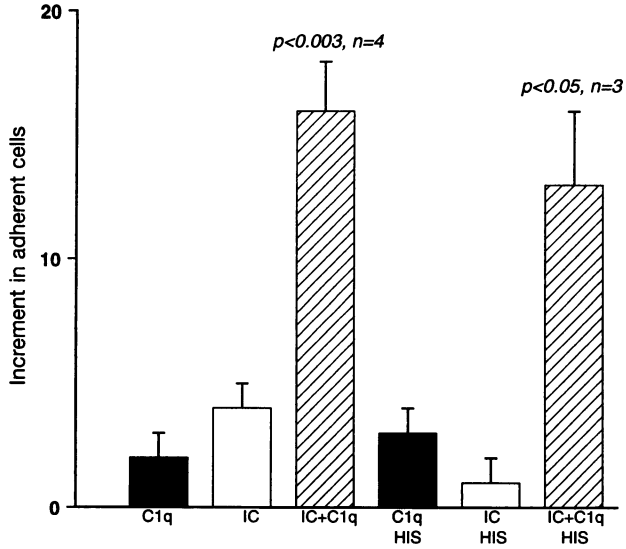

FIG. 2. Effects of C1q, ICs, or their combination on endothelial adhesiveness for leukocytes in the absence and presence of human serum. Endothelial cells were incubated with ICs (0.48 mg/ml) (□), C1q (50 μg/ml) (■), or their combination (▨) in the presence of tissue culture medium (90% RPMI 1640/10% fetal bovine serum) without or with 50% HIS for 3 hr before adherence to leukocytes was quantitated. Shown are the results of the mean increments in adhesion (±SEM), expressed as cells per ×400 field, of four experiments performed in quadruplicate.

thelial cells by a complement-dependent mechanism. ICs stimulated endothelial adhesiveness in the presence of C3- and C8-depleted serum as well as in the presence of NHS (90 ± 6% and 110 ± 6% of the adhesion in the presence of NHS, respectively; $n = 3$, P = not significant). In contrast, ICs did not stimulate endothelial adhesiveness for leukocytes in the presence of serum depleted of C1q (HIS) (25 ± 10% of the adhesion in the presence of NHS; $n = 4$, $P < 0.008$).

Since it has been reported that endothelial cells express receptors for C1q on their surface (11–13) and depletion of C1q diminishes the ability of ICs to stimulate endothelial cells, we next asked whether C1q was responsible for endothelial stimulation by ICs. In the presence of either tissue culture medium with or without 50% (vol/vol) HIS, neither ICs nor purified C1q alone stimulated adhesiveness for leukocytes

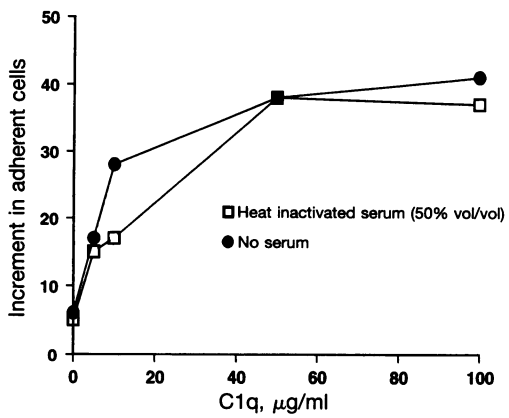

FIG. 3. Effects of C1q concentration on IC-stimulated endothelial adhesiveness for leukocytes. Endothelial cells were incubated with various concentrations of C1q and ICs (0.48 mg/ml) in the presence of tissue culture medium without (●) or with 50% HIS (□) for 3 hr before adherence to leukocytes was quantitated. Shown are the results of a single experiment of two similar experiments performed in quadruplicate. The increment in adhesion is expressed as cells per ×400 field.

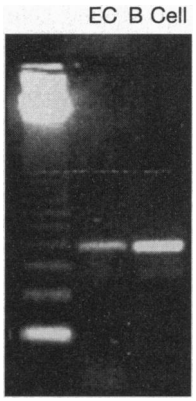

FIG. 4. Endothelial cells express message for the 33-kDa C1q receptor. RNA was isolated from a B-cell line (GM6314) and confluent endothelial cells (EC). After reverse transcription, the resulting DNA was subject to PCR with primers specific for the 33-kDa C1q receptor as described in text. Shown is a representative experiment of two performed with different endothelial sources of RNA. The PCR fragment obtained from endothelial reverse-transcribed RNA calculates to ≈468 bp.

(Fig. 2). In contrast, C1q completely restored the capacity of HIS to support IC-stimulated endothelial adhesiveness for leukocytes and conferred on tissue culture medium lacking HIS the ability to support IC-induced endothelial adhesiveness by leukocytes (Fig. 2). Moreover, the concentration of C1q required to permit maximal stimulation of endothelial cells by ICs was identical in the presence or absence of other serum components and was within the range of C1q found in normal serum (≥50 μg/ml; Fig. 3).

There are at least three different types of C1q receptors (23–25), one of which (the 50- to 76-kDa C1q binding protein) has been isolated from endothelial cells (26). By use of reverse transcription–PCR we found that endothelial cells express a message for the 33-kDa C1q receptor found on platelets and B cells (Fig. 4). Using specific monoclonal antibodies, we have also found that endothelial cells express the 100- to 126-kDa C1q binding protein on their surfaces (Fig. 5).

To determine whether adhesive molecules were expressed by IC-stimulated endothelial cells, we examined the expression of E-selectin, ICAM-1, and VCAM-1 on resting and stimulated endothelial cells. We observed that, like cytokines, ICs stimulated endothelial expression of E-selectin and VCAM-1 and increased expression of ICAM-1. As with endothelial adhesiveness for leukocytes, cytokines stimulated greater expression than ICs did of all three of these adhesive proteins (Fig. 6). In some, but not all, experiments there appeared to be a bimodal expression of E-selectin on the IC-stimulated endothelial cells.

Depending on the stimulus, endothelial cells express either preformed adhesive molecules from intracellular reserves (P-selectin) or newly synthesized adhesive proteins (e.g., E-selectin and VCAM-1) and lipids (platelet-activating factor). To better determine which adhesive molecule(s), if any, mediated IC-stimulated endothelial adhesiveness, we studied the

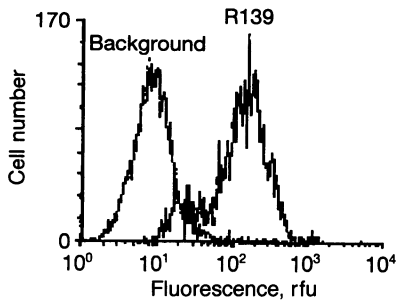

FIG. 5. Endothelial cells express the 100- to 126-kDa C1q receptor. Endothelial cells were harvested and then incubated with saturating concentrations of R139, an antibody against the myeloid C1q receptor, or with an isotype control antibody followed by incubation with fluorescein isothiocyanate-labeled goat anti-mouse IgG. Shown is a representative cytofluorogram of two different experiments. rfu, Relative fluorescence units.

effect of cycloheximide, an agent that blocks protein synthesis, on stimulated endothelial adhesiveness for leukocytes. Cycloheximide almost completely blocked IL-1- and IC-stimulated endothelial adhesiveness for leukocytes (84 ± 3% and 90 ± 6% inhibition, respectively, $n = 3$, $P < 0.005$). This observation suggested that P-selectin was not the primary adhesive molecule involved in endothelial adhesiveness under the conditions studied. Moreover, these results confirmed previous studies that *de novo* synthesis of adhesive molecules was required for cytokine-stimulated endothelial adhesiveness and indicated that similar *de novo* synthesis of adhesive proteins was responsible for IC-stimulated endothelial adhesiveness.

To establish that the adhesive molecules expressed by IC-stimulated endothelial cells mediated adhesiveness, we studied the effect of specific monoclonal antibodies to E-selectin on IC- and cytokine-stimulated endothelial cells. Incubation of cytokine- and IC-stimulated endothelial cells with monoclonal antibodies to E-selectin blocked cytokine- and IC-stimulated endothelial adhesiveness for leukocytes (Table 1).

Table 1. Antibody inhibition of stimulated increments in adhesion

Stimulant	Antibody inhibition, % ± SEM		*P*
	Control	Anti-E-selectin	
IL-1α	7 ± 3	76 ± 7	<0.0003*
ICs	−7 ± 1	65 ± 12	<0.003*

Endothelial cells were incubated in tissue culture medium containing 50% NHS with or without IL-1 (10 units/ml) or ICs (0.48 mg/ml) for 3 hr. The monolayers were washed extensively then incubated with leukocyte suspensions in the presence of either anti-E-selectin antibodies (H18/7 or BMA4D10, two experiments each; total $n = 4$) or isotype control IgG ($n = 4$) before nonadherent leukocytes were decanted and adherent leukocytes counted, as described. The mean control stimulated increments in adhesion for these experiments were 34 ± 5 cells per ×400 field (IL-1) and 17 ± 4 cells per ×400 field (ICs). Incubation of endothelial cells with H4/18, a monoclonal antibody against E-selectin that does not block E-selectin-mediated adhesion, did not inhibit either IL-1- or IC-stimulated endothelial adhesiveness (data not shown).
*Anti-E-selectin vs. control.

DISCUSSION

Our results show that ICs, in the presence of a serum cofactor, stimulate endothelial cells to express adhesive molecules and become adhesive for leukocytes. Moreover, we provide strong evidence that C1q is the necessary serum cofactor required for ICs to stimulate endothelial cells: (*i*) ICs do not stimulate endothelial cells in the presence of HIS, and C1q is heat labile; (*ii*) ICs do not stimulate endothelium in the presence of C1q-depleted serum; (*iii*) neither alternative pathway complement activation nor late-acting complement components (after C3 in the pathway) are required for ICs to stimulate endothelial cells; (*iv*) C1q restores the capacity of ICs to stimulate endothelium in the presence of HIS; and (*v*) ICs plus C1q alone stimulate endothelial cells to become adhesive for leukocytes and to express E-selectin, ICAM-1, and VCAM-1 in a manner virtually identical to that seen with ICs and NHS.

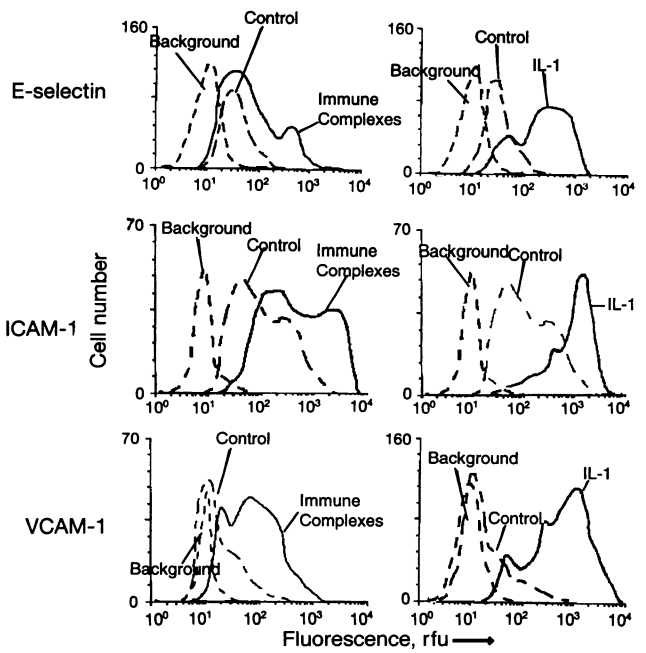

FIG. 6. Effects of ICs on endothelial expression of adhesive molecules. Confluent monolayers of endothelial cells were incubated in tissue culture medium without (background) or with 50% NHS (control) or in the presence of ICs (0.48 mg/ml) (*Left*) or IL-1α (10 units/ml (*Right*) for 3 (E-selectin) or 18 hr (ICAM-1 and VCAM-1) before monolayers were harvested, labeled with specific monoclonal antibodies, and analyzed by cytofluorometer. Shown are representative cytofluorographs of a single representative experiment of five (E-selectin) or three (ICAM-1 and VCAM-1) separate experiments. rfu, Relative fluorescence units.

We observed that endothelial cells become adhesive for leukocytes after exposure to aggregates of ICs and C1q. It is possible that neutrophils or other leukocytes bind, via their Fc receptors, to IgG that is bound indirectly to C1q receptors on the surface of endothelial cells. Although we have not rigorously excluded this explanation, we have presented three separate lines of evidence that are most consistent with the hypothesis that endothelial expression of adhesive molecules mediates the increment in adhesiveness. First, IC–C1q aggregates stimulate endothelial expression of E-selectin, ICAM-1, and VCAM-1. Second, we found that endothelial cells must synthesize new proteins in response to stimulation by ICs to become adhesive for leukocytes. Last, treatment of endothelial cells with antibodies that bind to and block E-selectin markedly diminishes IC–C1q-stimulated endothelial adhesiveness. Indeed, the presentation of ICs (by C1q receptors) on the surface of endothelial cells may serve, like platelet-activating factor (27), to prime neutrophils adherent to the vascular endothelium for increased oxidant and lysosomal enzyme release.

At least three different types of C1q receptor have been described: a 33-kDa protein isolated and cloned from Raji cells and present on a variety of other cell types, a 56- to 72-kDa protein isolated from Raji cells (collection receptor), and a 100- to 126-kDa protein present on cells and cell lines of myeloid origin including neutrophils and mononuclear cells (23–25). On myeloid cells C1q receptors enhance the internalization of immunoglobulin-coated particles and production of oxygen metabolites by a Ca^{2+}-dependent, protein kinase C-dependent mechanism (25, 28–30). C1q enhances immunoglobulin production by B cells, association of ICs with mesangial cells, and fibroblast chemotaxis and adhesion (31–41). Previous studies have suggested that C1q must be complexed to immunoglobulin in a lattice to engage its receptor effectively and to stimulate cells (41), which is concordant with our finding that C1q monomers—i.e., C1q in the absence of ICs—did not stimulate endothelial cell adhesiveness.

Recent studies have suggested that late-acting complement components stimulate endothelium to express P-selectin. Both assembly of the membrane attack complex (C5b-9) in the plasma membrane of endothelial cells and fluid-phase C5a stimulate the expression of the adhesion molecule P-selectin (9, 10). Although we could not demonstrate a role for late-acting complement components such as C5a in the induction of endothelial adhesiveness, our studies do not rule out a role for C5a in stimulation of endothelial cells by ICs. We may not have seen the effect of C5a on endothelial expression of

P-selectin because we incubated endothelial cells with ICs and human serum for much longer periods of time than those required for optimal expression of P-selectin and examined endothelial adhesiveness at a time point when P-selectin-mediated endothelial adhesiveness would be expected to have diminished (3 hr; ref. 42). Moreover, the effect of recombinant C5a on endothelial P-selectin expression was previously studied in the absence of NHS, whereas we utilized ICs in the presence of NHS; C5a is rapidly metabolized in human serum to C5a desarg, which is a far less potent agonist at C5a receptors (43) and may have stimulated less endothelial expression of P-selectin in our experiments.

Previous studies have suggested that endothelial expression of adhesive molecules is required for the development of IC-mediated vasculitis, although the stimulus for expression of these adhesive molecules was not defined (2). Our studies demonstrate that once ICs have fixed complement, they can stimulate endothelial cells. Beynon and colleagues (44) reported that IC-stimulated neutrophils caused greater injury (disruption of barrier function) to stimulated endothelial cells than to resting endothelial cells because the neutrophils had to adhere to the endothelial cells first to injure them. This observation suggests that, by inducing endothelial cells to express adhesion molecules, ICs plus C1q promote the interaction between circulating leukocytes and the endothelium and set the stage for vascular injury in immune complex-mediated vasculitis.

We thank Dr. Gerald Weissmann for his discussion and for reviewing this manuscript. This research was performed with the support of grants from the SLE Foundation, Inc.; the Arthritis Foundation, New York Chapter; the American Heart Association, New York Affiliate; a merit grant from the Department of Veterans' Affairs; the Charles C. Culpeper Foundation; the U.S. Public Health Service (AR11949 and HL19721); the General Clinical Research Center (MO1RR00096); and the Kaplan Cancer Center (CA16087). We acknowledge Bellevue Hospital and the Health and Hospitals Corporation of New York City for support.

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