The Cytolytically Inactive Terminal Complement Complex Activates Endothelial Cells to Express Adhesion Molecules and Tissue Factor Procoagulant Activity

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Summary

The membrane attack complex of complement (C) in sublytic concentrations stimulates endothelial cells (EC) to express adhesion molecules and to release biologically active products. We have examined the ability of a cytolytically inactive form of this complex, which is incapable of inserting into the cell membrane, to upregulate the expression of adhesion molecules and of tissue factor (TF) procoagulant activity. The inactive terminal C complex (iTCC) was prepared by mixing C5b6, C7, C8, and C9 and was purified by fast protein liquid chromatography on a Superose 12 column. Binding of this complex to EC was found to be dose dependent and was inhibited by anti-C9 antibodies, as assessed both by ELISA using an mAb anti-C9 neoantigen and by measuring cell-bound ^{125}I-labeled iTCC. Exposure of EC to iTCC resulted in a dose- and time-dependent expression of endothelial leukocyte adhesion molecule 1, intercellular adhesion molecule 1, and vascular cell adhesion molecule 1 accompanied by increased levels of the corresponding mRNA, but not in the rapid expression of P-selectin. Inactive TCC also induced increased TF activity evaluated by a chromogenic assay that measures the formation of factor Xa. These effects were inhibited by anti-C9 antibodies. The data support the conclusion that iTCC may induce proinflammatory and procoagulant activities on EC.

Endothelial cells (EC)[1] are continuously exposed to the action of biologically active products of the complement (C) system, which are readily available in the circulation after C activation by several stimuli, such as immune complexes or endotoxin. The major biological effects of these products on EC is to favor their interaction with circulating leukocytes and to promote migration of these cells into the inflamed tissue (1). The in vivo relevance of these C biological activities is supported by the finding that C is required for the accumulation of neutrophils into the tissue in a rat model of lung injury caused by intrapulmonary deposition of immune complexes or systemic C activation by cobra venom factor (2).

Various C activation products interact with EC and stimulate the expression of adhesion molecules as well as the secretion of proinflammatory factors. Thus, C1q linked to immune complexes binds to C1q receptor on EC stimulating adhesion of leukocytes and expression of E-selectin, intercellular adhesion molecule (ICAM)-1, and vascular cell adhesion molecule (VCAM)-1 (3). Similarly, C5a induces expression of P-selectin and increased adhesion of neutrophils after interaction with the C5a receptor expressed on EC (4). Also, iC3b molecules deposited on the membrane of EC bind to their ligand CD11b/CD18 on PMN and cause rapid neutrophil–endothelial adhesion (5).

In the last few years, various groups have reported data indicating that the membrane attack complex (MAC) of C, formed by the assembly of the five terminal C components, exhibits several biologic effects on EC. These include the release of prostacyclin PGI_2 (6), von Willebrand factor (7), basic fibroblastic growth factor, platelet-activating factor (8), IL-8, and monocyte chemotactic protein 1 (9). MAC has also been shown to stimulate the expression of P-selectin (7) and to potentiate the upregulation of E-selectin and ICAM-1 induced by TNF-α (10). These are noncytotoxic

[1]*Abbreviations used in this paper:* C, complement; EC, endothelial cells; ELAM, endothelial leukocyte adhesion molecule; HUVEC, human umbilical vein endothelial cells; ICAM, intercellular adhesion molecule; iTCC, inactive terminal C complex; MAC, membrane attack complex; RT, room temperature; RT-PCR, reverse transcriptase PCR; SFM, serum-free medium; TF, tissue factor; VCAM, vascular cell adhesion molecule.

A preliminary report of this work was presented in an abstract form at the XVI International Complement Workshop, Boston, USA, June 16–21, 1996.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/97/05/1619/09 $2.00
Volume 185, Number 9, May 5, 1997 1619–1627

effects elicited by sublytic amounts of MAC that inserts into the plasma membranes of EC without producing overt cytolysis.

The assembly of MAC on EC initiates with the binding of the C5b-7 complex formed in the fluid phase to the phospholipid bilayer of the cell membrane followed by activation of C8 and C9. However, the insertion of the C5b-9 complex into the cell membrane is prevented by the action of two serum inhibitors, vitronectin or S protein (11) and clusterin (12), and also by the rapid decay of the metastable binding site transiently exposed in the C7 component of the complex, although the decayed C5b-7 still binds C8 and C9 forming an inactive terminal C complex (iTCC; 13). The fate of these inactive complexes and their biological effects on bystander cells are, to a large extent, unknown. Wang et al. (14) have recently shown that iC5b67 stimulates PMN chemotaxis and Ca^{2+} fluxes, thus extending an original observation made several years ago by Lachmann et al. (15). These findings suggest that the inactive complexes may not be functionally irrelevant.

The purpose of the present study is to examine iTCC for its ability to interact with EC and to induce biological effects. Data will be presented indicating that iTCC not only binds to these cells, but also upregulates the expression of adhesion molecules and induces tissue factor activity.

Materials and Methods

Reagents and C Components. Recombinant human IL-1α (10^7 U/mg) and TNF-α (2.0×10^7 U/mg) were purchased from Boehringer Mannheim (Milan, Italy) and Bachem Biochemica GmbH (Heidelberg, Germany), respectively. LPS from *Escherichia coli* O55:B5, human coagulation factor VII, bovine factor X, BSA, histamine, and *p*-nitrophenyl phosphate were obtained from Sigma Chemical Co. (Milan, Italy). Thromboplastin and S-2765, the chromogenic substrate for factor Xa, were provided by Chromogenix (Mölndal, Sweden), and the human C components from C7 to C9 were from Quidel (San Diego, CA).

Antibodies. Goat antisera to C5, C6, and C9 were purchased from Quidel, and goat IgG directed against C8 was obtained from Atlantic Antibodies (Scarborough, ME). Antisera to C7 and C9 were raised in goat and rabbit, respectively, by repeated subcutaneous injections of the purified components and their specificity was checked by ELISA and Western blotting. The IgG were purified from the antisera by affinity chromatography on protein G–Sepharose column (Pharmacia Biotech, Milan, Italy). The mAb aE11 recognizing a neoantigen of poly (C9) (16) was a gift from T.E. Mollnes (Bodo, Norway). The following mAb to adhesion molecules were used: anti–endothelial leukocyte adhesion molecule (ELAM)-1 and anti-P selectin were from Cymbus Bioscience Ltd. (Southampton, U.K.), and RR1/1 anti–ICAM-1 (17) and 4B9 anti–VCAM-1 (18) were obtained through the courtesy of R. Rothlein (Boehringer Ingelheim, Ridgefield, CT) and J. Harlan (University of Washington, Seattle, WA), respectively. An mAb that recognizes human tissue factor and neutralizes its procoagulant activity was provided by American Diagnostica Inc. (Greenwich, CT). Alkaline phosphatase-labeled affinity-purified antibodies from goat to mouse IgG were purchased from Sigma Chemical Co.

Preparation of C5b6. C5b6 was purified from 300 ml of fresh frozen human plasma depleted of C7 by immunoaffinity chromatography using cyanogen bromide–activated Sepharose 4B (Pharmacia Biotech) coupled with IgG anti-C7. The fractions eluted from the column were checked for the presence of C5, C6, and C7 by ELISA after a procedure previously described in detail (19) with a sensitivity limit of ~1–3 ng/ml. The fractions containing C5 and C6 at levels comparable to those of the original plasma and undetectable C7 were pooled and recalcified by the addition of 20 mM $CaCl_2$. The C5b6 complex was purified from yeast-activated C7-depleted serum essentially as described by Harrison and Lachmann (20) with some modifications that included fractionation of the active material obtained from euglobulin precipitation and DEAE-Sephacel chromatography through Ultrogel AcA 34 (IBF-LKB, Milan, Italy) and final purification by gel filtration on Superose 12 using the fast protein liquid chromatography system (Pharmacia Biotech). The purified C5b6 had a protein concentration of 800 μg/ml and a hemolytic titer of 250,000 U/ml, where one unit corresponds to the volume of C5b6 that lyses 2×10^7 guinea pig erythrocytes in 30 min at 37°C in the presence of 2 μl EDTA human serum to a final volume of 250 μl.

Preparation of iTCC. The inactive terminal C complex was obtained by mixing C5b6 (3 μg), C7 (1 μg), C8 (1.5 μg), and C9 (4 μg) in PBS to a final volume of 50 μl. The concentration of C7 was chosen to be in slight excess of the amount required to fully inactivate the hemolytic activity of C5b6 when mixed with this complex for 10 min at 37°C before the addition of guinea pig erythrocytes and EDTA serum (20). C8 was present in the mixture in an equimolar ratio to C7, and C9 was added in large excess to favor the polymerization of this component during the assembly of iTCC. The formation of complex was followed by ELISA using solid phase–bound goat IgG anti-C5 (1/1,000) that was incubated first with purified C5b6 (300 ng) for 1 h at 37°C, and, after washing, with a mixture of C7 (100 ng), C8 (150 ng) and C9 (400 ng) in PBS for 30 min further at 37°C. The amount of bound TCC was evaluated by its reaction with 1/1,000 aE11 for 1 h at room temperature (RT) followed by 1/4,000 alkaline phosphatase-conjugated affinity-purified goat anti–mouse antibodies (Sigma Chemical Co.) for 1 h at RT. After addition of *p*-nitrophenyl phosphate (1 mg/ml) in 0.1 M glycin buffer, pH 10.4, containing 0.1 mM $MgCl_2$ and 0.1 mM $ZnCl_2$, the enzymatic reaction was developed at 37°C and read kinetically at 405 nm using a Titertek Multiskan ELISA reader (Flow Laboratories, Milan, Italy). The complexes were allowed to form for 30 min at 37°C, for an additional 2 h at RT, and then purified by fast protein liquid chromatography on a Superose 12 column. The iTCC was also obtained by affinity chromatography on mAb aE11 bound to Protein G–agarose (Sigma Chemical Co.) and cross-linked with dimethyl pimelimidate dihydrochloride according to Schneider et al. (21). The bound iTCC was eluted with 4 M guanidine-HCl in 0.1 M sodium phosphate, pH 7, dialyzed first against 1 mM HCl, and subsequently against PBS. Protein G–agarose column with bound IgG anti-C5 was used in some experiments to absorb purified iTCC. Free C9 multimers were measured by ELISA using solid phase–bound mAb aE11 and goat IgG anti-C9 labeled with biotin (19) as revealing reagent.

Radiolabeling of C7. The Iodogen reagent (Pierce, Rockford, IL) was used to label C7 with ^{125}I following the instructions of the manufacturer, and the unbound iodine was removed by gel filtration through Sephadex G25 (Pharmacia Biotech) column. The labeled C7 was still capable of interacting with C5b6 and its specific activity was estimated to be 2×10^6 cpm/μg. ^{125}I-C7 was incorporated into iTCC by incubating C5b6 first with the labeled C component for 5 min at 37°C, and subsequently with 10-fold excess cold C7, C8, and C9 following the protocol re-

ported above for the preparation of C5b-9. The labeled complex was purified through Superose 12 to remove unbound ^{125}I-C7 and the other free terminal components.

Endothelial Cell Culture. Human umbilical vein endothelial cells (HUVEC) were prepared according to Jaffe et al. (22) and cultured in wells of tissue culture plates coated with 2% endotoxin-free gelatin as previously described (23). The cells were kept in culture either in endotoxin-free medium 199 (Sigma Chemical Co.) supplemented with 20% newborn calf serum (Hyclone Labs., Logan, UT) or in human endothelial serum-free medium (SFM; GIBCO BRL, Gaithersburg, MD) with fibroblast growth factor (20 ng/ml) and epidermal growth factor (10 ng/ml).

ELISA on HUVEC. Binding of iTCC and expression of adhesion molecules on HUVEC were evaluated on cells of the first passage grown to confluence in 96-well tissue culture plates (Nunc, Mascia Brunelli, Milan, Italy). After three washings with HBSS (Sigma Chemical Co.), the cells were exposed to iTCC in HBSS containing 1% BSA to a final volume of 200 μl for 1 h at 37°C and the bound complex was quantified as reported above. The expression of the adhesion molecules on HUVEC was assessed on cells incubated with 100 μl of mAb 4B9 to VCAM-1 (5 μg/ml), mAb RR1 to ICAM-1 (5 μg/ml), mAb 1.2B6 to ELAM-1 (1 μg/ml), and AK-6 to P-selectin (1 μg/ml) for 1 h at RT. For the detection of P-selectin, the cells were fixed with 1% paraformaldehyde in PBS for 15 min at RT in the dark before exposure to the relevant mAb.

Binding Assay for ^{125}I-iTCC. The procedure followed to measure the binding of ^{125}I-iTCC to HUVEC was similar to that described for the immunoenzymatic assay except that the cells were incubated with the labeled complex (~30,000 cpm) in HBSS containing 1% BSA for 1 h at 37°C. After careful removal of the supernatants, the cells were washed three times and finally dissolved in 200 μl of 1 M NaOH. Both unbound and cell-bound radioactivities were counted in a gamma counter (Cobra B5005; Packard Instruments Company, Meriden, CT) and the results were expressed as bound ^{125}I-iTCC fractions of the total amounts of complex added.

Evaluation of Tissue Factor Activity. A chromogenic assay based essentially on the method published by Mulder et al. (24) was used to measure the expression of tissue factor (TF) by endothelial cells. In brief, HUVEC grown to confluence were stimulated with iTCC or cytokines for 4–6 h and washed three times with medium 199. The cells were then treated with 0.2 mg/ml collagenase (Worthington Biochemical Corp., Freehold, NJ) in PBS (~30 μl/well) for 15 min at 37°C. After addition of 250 μl of 10 mM Hepes solution, pH 7.45, supplemented with 137 mM NaCl, 4 mM KCl, 11 mM glucose, 2.5 mM $CaCl_2$, and 0.5% BSA to stop the enzymatic reaction, the plates were centrifuged at 250 *g* for 10 min and the cell supernatant was gently removed. The cells sedimented in each well were then incubated with 25 μl of 50 mM Tris buffer, pH 7.4, containing 0.2% BSA and 0.3 U/ml of factor VII for 3 min at 37°C followed by 25 μl/well of the same buffer supplemented with 13 mM $CaCl_2$ and 0.5 U/ml of factor X for additional 7 min at 37°C. The formation of factor Xa was evaluated kinetically at 405 nm with the Titertek Multiskan ELISA reader after the addition of 25 μl of the chromogenic substrate S-2765 (1.87 mM in H_2O). Optimal readings were generally obtained after an incubation of 60–90 min at 37°C.

SDS-PAGE and Western Blotting. TCC was analyzed by SDS-PAGE and immunoblotting following a procedure previously described (19) with some modifications that included boiling of the complex for 5 min in the sample buffer containing 6% SDS, an electrophoretic run on a 6–10% gradient gel under nonreducing conditions and the use of the semidry SemiPhor transfer unit (Heifer Scientific Instruments, San Francisco, CA) for blotting.

Detection of mRNA for Adhesion Molecules. The procedures for RNA extraction with phenol-chloroform from cells lysed in a guanidine thyocianate solution, the reverse transcription with random examers in the presence of Mo-MLV reverse transcriptase (Perkin-Elmer, Cetus, Norwalk, CT), and the PCR reaction performed to obtain a semiquantitative PCR analysis have previously been described (25). The oligonucleotides were purchased from Duotech (Milan, Italy). The following primers were used to amplify the VCAM-1 gene: forward 5′ CAAGTCTACATATCACCCAAGA 3′, from position 758 to position 779, and back 5′ GGAACCTTGCAGCTTACAGTGACAGAGCTCCC 3′, from position 1081 to position 1112, according to the published cDNA (26). The primers employed to amplify the ELAM-1 gene were the following: forward 5′ GGTAGGAACCCAGAAACCTCTGA 3′ from position 365 to position 387 and back 5′ TTCCCAGATGAGGTACACTGAAG 3′, from position 985 to position 967 according to the published cDNA (27).

The amplified products were run through a 1% agarose gel containing ethidium bromide and transferred onto a nylon filter (Zeta-Probe; Bio Rad Labs., Hercules, CA). Hybridization was carried out according to standard protocols (28) with oligonucleotides that had been labeled in a 25-μl reaction mixture containing 50 ng oligo in kinase buffer with ^{32}PγATP (6,000 Ci/mmol; DuPont New England Nuclear, Boston, MA) in the presence of T4 kinase (Boehringer Mannheim GmbH, Mannheim, Germany) and then passed through Quick Spin Columns (Boehringer Mannheim, GmbH).

The "internal" oligonucleotide to detect VCAM-1 has the following sequence: 5′ TTCTGTGAATCCATCCACAAAGC 3′, from position 789 to position 811 of the published cDNA sequence (26). The sequence of the internal oligonucleotide to reveal ELAM-1 was as follows: 5′ GAGCATGGAAGCCTGGTTTGCAG 3′, from position 675 to position 697 of the published cDNA (27).

The human β-actin gene was amplified from 5 μl of the same reverse transcribed mixture using the following primers: forward 5′ AAGATGACCCAGATCATGTTTGAG 3′ and back 5′ GGAGCAATGATCTTGATCTTC 3′ as previously reported (25, 28).

Statistical Analysis. Data are reported as mean ± SEM. The Student's *t* test was used to compare two groups of data.

Results

Characterization of iTCC. Mixing C5b6 with the remaining late components in the proportion reported in Materials and Methods resulted in the formation of TCC that rapidly lost hemolytic activity. The results presented in Fig. 1 clearly show that lysis induced by the complex dropped to ~25% of the control value in about 30 s from initiation of the complex formation, and was negligible within 1 min of incubation. By contrast, the time required for the stabilization of the complex was somewhat longer. Thus, the expression of C9 neoantigen, followed as a marker of TCC formation, though already apparent 1 min after mixing the C reagents, progressively increased with time reaching its highest value after 30 min of incubation (Fig. 1). As expected, all the terminal C components, including the C9 dimer, were detected when iTCC was examined by SDS-PAGE and Western blotting (Fig. 2). Free C9 multimers

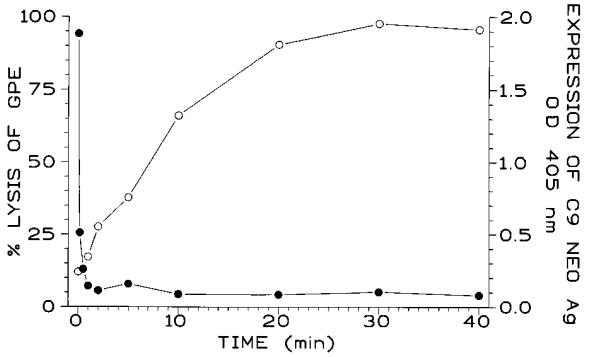

Figure 1. Kinetic of assembly and functional decay of iTCC. 50 μl aliquots of a 1-ml mixture containing C5b6 (3 μg), C7 (1 μg), C8 (1.5 μg) and C9 (4 μg) in PBS were tested hemolytically at various time intervals on guinea pig erythrocytes (●). The assembly of iTCC was measured kinetically by ELISA (○). Details of the assays are given in Materials and Methods. GPE, guinea pig erythrocytes.

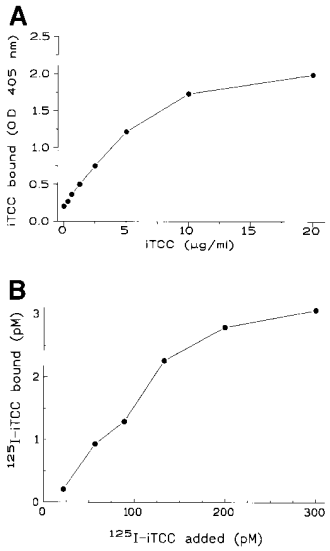

Figure 3. Binding of iTCC to HUVEC. HUVEC monolayers were incubated with increasing concentrations of either cold iTCC (*A*) or ^{125}I-iTCC (*B*) in HBSS containing 1% BSA to a final volume of 200 μl. After 1 h of incubation at 37°C, the amount of bound iTCC was measured by ELISA using mAb aE11 as revealing reagent or by counting the cell-bound radioactivity. Binding of ^{125}I-iTCC was inhibited when mixed with 10 M–fold excess of cold iTCC.

could not be detected in the unretained fraction of iTCC (1 μg) adsorbed onto IgG anti-C5 bound to protein G–agarose when tested by ELISA using solid phase bound mAb aE11 and biotin-labeled goat anti-C9 as revealing reagent with a sensitivity limit of 1–2 ng/ml.

Binding of iTCC to HUVEC. The interaction of iTCC with the endothelial cells was analyzed with mAb aE11 which recognizes a C9 neoantigen expressed by the complex. The data presented in Fig. 3 *A* indicate that the amount of iTCC bound to HUVEC was related to the dose of iTCC offered up to 5 μg/ml and showed the tendency to reach a plateau with higher concentrations of the complex. The specificity of these findings was confirmed by the negative results obtained when C5b6 was added to HUVEC cultured in TC199 supplemented with 10% C7-depleted serum or when the cells were exposed to the same amount of purified C9 used for the preparation of iTCC (data not shown). The experiments performed with ^{125}I-iTCC essentially confirmed the results obtained with ELISA and, moreover, showed that the bound complex represents ~1% of the total radioligand added (Fig 3 *B*).

To evaluate the kinetic of binding of the inactive complex to HUVEC, the cells were incubated with iTCC and washed at various time intervals to measure the amount of bound complex. The data presented in Fig. 4 indicate that approximately half of the total amount of bound complex could be detected on the surface of EC after 15 min of incubation.

Analysis of iTCC binding to HUVEC in the presence of polyclonal IgG antibodies directed against the terminal components revealed a marked inhibitory effect of the anti-C9 antibodies, whereas the anti-C6 and the anti-C7 had only a marginal inhibitory activity and the other antibodies were ineffective (Fig. 5). The inhibitory effect induced by goat anti-C9 could also be obtained using rabbit IgG anti-C9.

Expression of Adhesion Molecules on HUVEC Induced by iTCC. Exposure of HUVEC to iTCC resulted in upregulation of the cell expression of VCAM-1, ICAM-1, and ELAM-1 (Fig. 6). This effect was dose related and was evident in cells stimulated with 5 μg/ml of iTCC, though the complex was already active in lower amounts. The complex purified by affinity chromatography with mAb aE11 proved to be more effective than iTCC obtained by gel filtration on Superose 12 inasmuch as it induced a similar expression of adhesion molecules at half the concentration (data

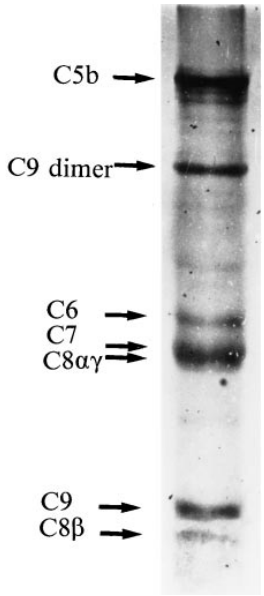

Figure 2. SDS-PAGE analysis of purified iTCC. A total amount of 2 μg of iTCC was applied to the gel and run under nonreducing conditions in a 6–10% gradient gel. The position of the bands and their reference to the individual components were checked in parallel by Western blot using specific antisera.

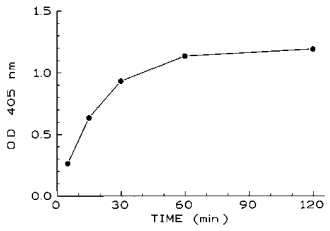

Figure 4. Kinetic of iTCC binding to HUVEC. EC monolayers were incubated at 37°C with 5 μg/ml of iTCC for various lengths of time and the amount of bound complex was measured by ELISA as indicated in Fig. 3.

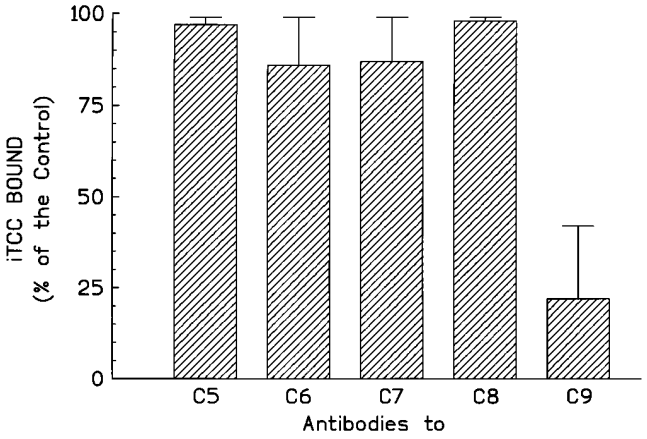

Figure 5. Inhibition of iTCC binding to HUVEC by antibodies to the terminal C components. EC were incubated with ^{125}I-iTCC (300 pM) in the presence of goat antibodies to the individual late components (100 μg). After 1 h incubation at 37°C, the cells were washed and the radioactivity was counted. The results are presented as mean ± SEM of percent values of the control obtained with normal goat IgG in three different experiments.

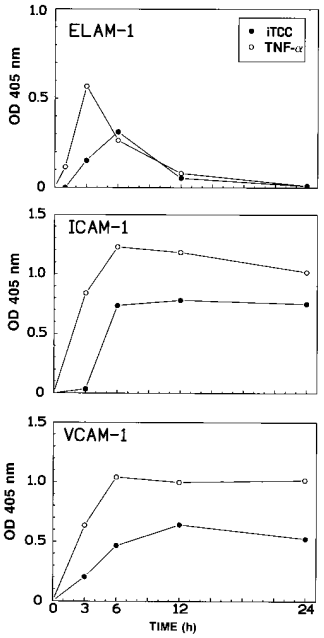

Figure 7. Time courses of induction of ELAM-1, ICAM-1, and VCAM-1 expression by HUVEC exposed to iTCC (5 μg/ml) or TNF-α (200 U/ml) in complete SFM containing 1% BSA. Cells were harvested at the indicated times and examined for adhesion molecule expression by ELISA.

not shown). Kinetic experiments performed with an optimal amount of iTCC (5 μg/ml) showed that the expression of the three adhesion molecules on the surface of endothelial cells was maximal 6 h after addition of the complex and persisted up to 24 h in the case of ICAM-1 and VCAM-1, whereas the expression of ELAM-1 decreased to background level after 12 h of incubation (Fig. 7). Exposure of HUVEC to the C complex for 1 h followed by washing was sufficient to induce the expression of the adhesion molecules after 6 h of incubation, but the amount of detectable molecules was threefold higher in the continuous presence of the stimulus (data not shown). The upregulation of adhesion molecules obtained with iTCC was significantly inhibited by the addition of goat IgG anti-C9 to the incubation mixture, but could not be reproduced with C9 alone (5 μg/ml), and the extent of the increased expression varied between one third and one half that induced by other well-known stimulating agents, such as IL-1α, TNF-α, and LPS (Fig. 8). The stimulating activity of the complex was unaffected by the presence of polymyxin B (5 μg/ml) and was totally lost when iTCC was boiled for 10 min (data not shown).

The complex was also examined for its ability to stimulate the expression of P-selectin previously shown to be rapidly induced by sublytic amounts of MAC (7). Fig. 9 shows that iTCC has a negligible effect on the expression

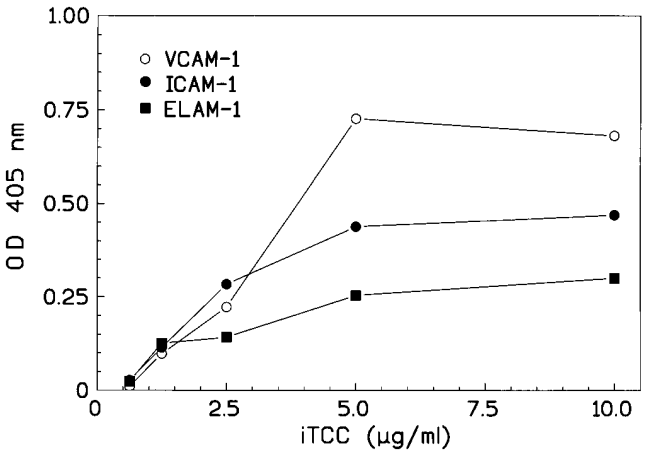

Figure 6. Dose response curve of iTCC-induced upregulation of VCAM-1, ILAM-1, and ELAM-1 molecules on HUVEC. EC were exposed to increasing concentrations of iTCC in 200 μl of complete SFM containing 1% BSA and tested by ELISA for the expression VCAM-1 and ICAM-1 after an overnight incubation and for the expression of ELAM-1 after 5 h of incubation.

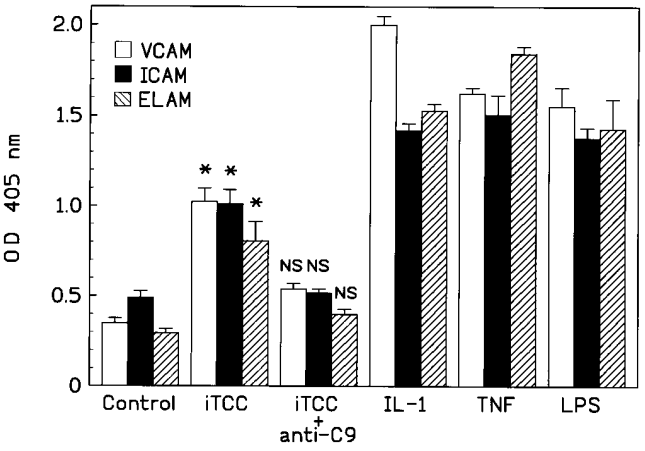

Figure 8. Comparison of the stimulating effect of iTCC (5 μg/ml), IL-1α (10 U/ml), TNF-α (200 U/ml), and LPS (1 mg/ml) in 200 μl of complete SFM containing 1% BSA. The experimental conditions are similar to those used for Fig. 6. Data showing inhibition of the stimulatory effect of iTCC by anti-C9 and the effect of C9 are also reported. The results are presented as mean ± SEM of eight different experiments. *P <0.05 versus control; NS, not significant.

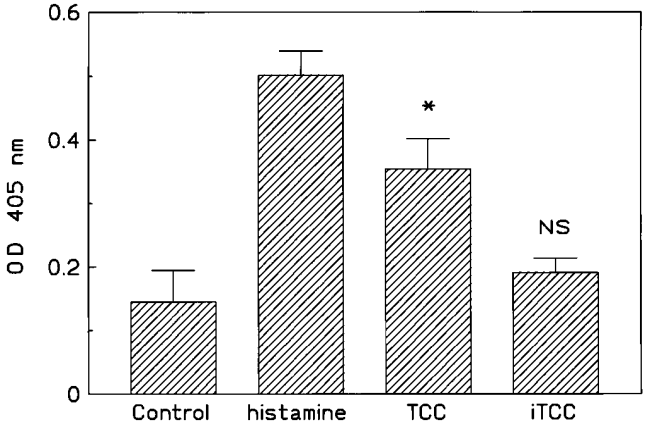

Figure 9. Expression of P-selectin on HUVEC stimulated with active and inactive TCC and with histamine. The active and inactive TCC were prepared using the same concentrations of C components (see Materials and Methods) and differed only because the C components were mixed in the presence of HUVEC to form the active complex and were preincubated in their absence to obtain iTCC. Histamine was added in concentration of 250 mM. The cells were incubated with the various reagents for 10 min at 37°C and the amount of P-selectin expressed on HUVEC was measured by ELISA after fixing the cells with paraformaldehyde. The results represent the mean ± SEM of four different experiments. *P <0.05 versus control; NS, not significant.

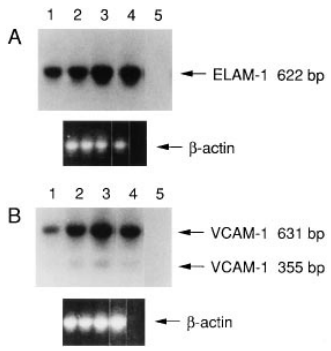

Figure 10. Semiquantitative RT-PCR analysis of the ELAM-1 (*A*) and VCAM-1 (*B*) genes and, for comparison, of the β-actin gene. EC (6×10^4) incubated under the various experimental conditions for 2 h at 37°C were lysed in 300 μl of guanidine solution and then processed for RT-PCR analysis. (lane *1*) Control cells; (lane *2*) cells treated with 10 U/ml IL-1α; (lane *3*) 1 mg/ml LPS; and (lane *4*) 5 μg/ml iTCC. Sterile distilled water substituting for RNA was similarly processed as a negative control (lane *5*).

of P-selectin, unlike active TCC and histamine used as positive controls.

RNA Messages for ELAM-1 and VCAM-1 in iTCC-Treated HUVEC. To confirm the results observed at the protein level, HUVEC activated by iTCC and, for comparison, by IL-1α and LPS or control cells, were examined for the expression of RNA messages for ELAM-1 and VCAM-1 using the semiquantitative reverse transcriptase PCR (RT-PCR) reported in Materials and Methods. The analysis was performed on the same RNA preparations purified from an equal number of cells, that had been stimulated under the same experimental conditions and then lysed in an equal volume of guanidine solution before being processed for the isolation of RNA. The results of ELAM-1 (*A*) and VCAM-1 (*B*) amplification presented in Fig. 10 show a clear increase in the messages for both molecules in cells exposed to iTCC, IL-1α, or LPS when compared to the pattern of the unstimulated cells, despite a comparable amount of β-actin RNA in all samples. In addition, a previously reported alternatively spliced form of VCAM-1 of lower molecular size (355 bp; reference 29) can be recognized in cells stimulated by iTCC, IL-1α, and LPS.

Expression of TF Activity on HUVEC Induced by iTCC. The ability of iTCC to induce endothelial TF activity was investigated by exposing HUVEC to the amount of complex (5 μg/ml) that promotes expression of the adhesion molecules. In a time-course experiment we found that TF activity was hardly detectable 1 h after addition of iTCC, but gradually increased starting after 2 h of incubation reaching the highest level after 6 h (Fig. 11). The stimulating effect of the C complex was approximately one half to one third lower than that obtained with optimal concentrations of LPS, TNF-α, and IL-1α, used as positive controls, and was blocked by the presence of IgG anti-C9 (Fig. 12). Additional experiments showed that TF activity induced by iTCC was associated with the expression of a molecule that was inhibited by cycloheximide (10 μg/ml) and was functionally neutralized by an mAb directed against the TF protein (data not shown).

Discussion

The data presented in this study show that iTCC binds to HUVEC and stimulates the expression of adhesion molecules and TF, though is unable to insert into the cell membrane. In preparing iTCC, we took advantage of the short half-life of less than 1 min required for TCC to interact as MAC with bystander cells (13, 14). For this reason, the inactive complex was prepared by incubating the mixture of C5b6 and the remaining terminal C components for at least 30 min at 37°C and for an additional 2 h at RT. TCC, prepared under these conditions, was unable to lyse guinea pig erythrocytes, which represent a highly susceptible target for lysis induced by human C complex, and had no cytolytic effect on HUVEC allowing normal survival of these cells in culture.

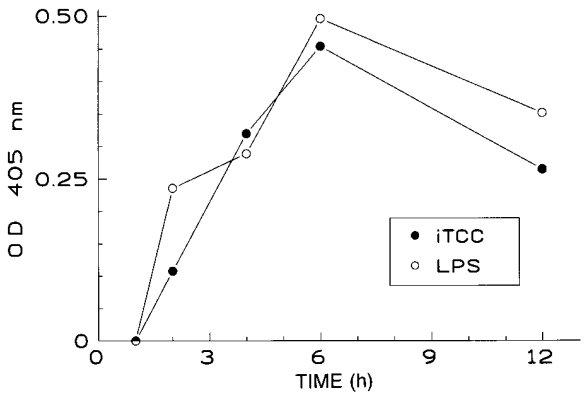

Figure 11. Time courses of induction of TF expression by HUVEC exposed to iTCC (5 μg/ml) or LPS (1 mg/ml). Cells were harvested at the indicated times and examined for TF expression by a chromogenic assay.

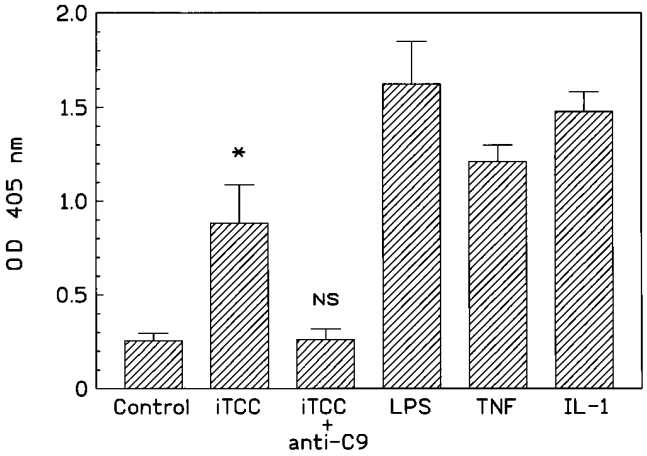

Figure 12. Expression of TF on HUVEC stimulated with iTCC, alone or in the presence of anti-C9, LPS, TNF-α, or IL-1α. The cells were incubated for 5 h with the different reagents used at the same concentrations reported in the legend to Fig. 8 and tested for the amount of TF expressed by a chromogenic assay. The results represent the mean ± SEM of four different experiments. *P <0.05 versus control; NS, not significant.

Binding of iTCC to HUVEC was supported by two sets of data. First, the presence of the complex on the cell surface was revealed by ELISA using mAb aE11, which recognizes only C9 assembled in the TCC, but not the native molecule. The specificity of this finding was confirmed by the observation that aE11 did not react with cells treated with a mixture of C5b6- and C7-depleted serum, a condition that does not allow assembly of the complex. Further evidence was obtained with experiments performed with ^{125}I-labeled complex, that showed binding of ~1% of iTCC to HUVEC.

Based on the results of inhibition experiments with polyclonal antibodies against the terminal C components, we concluded that C9 is the component of the complex that mostly contributes to its interaction with the EC, since the antibody to C9 proved to be the only one to exert a strong inhibitory effect. This finding was not surprising since C9 undergoes a process of polymerization when it is incorporated into the soluble TCC in the absence of vitronectin and clusterin, thus becoming its major constituent with an average number of seven molecules per complex (30). Whether binding of iTCC to endothelial cells is receptor mediated can not be established with the present data and is currently under investigation. However, Wang et al. (14) have recently shown that the inactive form of C5b67 bound to erythrocytes is released from the surface of trypsin-treated cells in higher amounts than the hemolytically active complex suggesting a different type of interaction of the active and inactive complexes with the cell membrane.

The observation that HUVEC exposed to iTCC expressed ELAM-1, ICAM-1, and VCAM-1 clearly indicates that binding of the inactive complex to these cells has important biological consequences, one of which is to promote the inflammatory process. In this respect, iTCC differs from the cytolytically active complex since Kilgore et al. (10) recently failed to induce upregulation of these adhesion molecules on HUVEC with MAC alone, despite the fact that they used a concentration of complex similar to that of iTCC used in this study. The only effect of MAC that they were able to see was an enhancement of TNF-α–induced cell activation.

The expression of ELAM-1, ICAM-1, and VCAM-1 on HUVEC stimulated by iTCC followed a kinetic pattern essentially similar to that observed by other groups with TNF-α and IL-1α (18, 27, 31) and in this study with TNF-α, although the latter had a tendency to induce a more rapid and greater response. The lag phase of 4 to 6 h needed to reveal a clear expression of the three molecules on HUVEC after the addition of iTCC clearly indicates that protein synthesis was required, as has already been shown for other inducers. This is confirmed by the finding that iTCC induced an increase in the corresponding RNA messages that preceded the expression of the molecules on the cell surface.

Our major concern was to exclude the possibility that LPS contaminating the C reagents might be responsible for cell activation, but this was ruled out by the findings that the C complex was still effective in the presence of polymyxin B and totally lost its activity after boiling. Further evidence in favor of the specificity of action of iTCC is provided by the data showing inhibition of the stimulatory activity of the C complex by antibodies to C9. Of course, this observation does not exclude the fact that the effect of iTCC can be mediated by soluble C9 or some forms of C9 multimers present in the iTCC preparation, although our failure to detect C9 polymers by ELISA and to induce cell stimulation with native C9 makes the contribution of free C9 highly unlikely. The inability of iTCC to stimulate the rapid expression of P-selectin on HUVEC contrasts with the appearance of this adhesion molecule on the surface of MAC-treated EC originally reported by Hattori et al. (7) and confirmed in the present study. These data again emphasize the differences in the biological effects elicited by the two complexes on HUVEC.

Exposure of endothelial cells to iTCC was sufficient to initiate the extrinsic pathway of coagulation just like treatment of these cells with LPS and other cytokines. The molecule on HUVEC responsible for this activity is recognized by an mAb that is able to block the TF-dependent formation of factor Xa and the consequent hydrolysis of the chromogenic substrate used in our assay system. The induction of TF in endothelium by iTCC requires protein synthesis since the expression of the molecule on the cell surface can be inhibited by the presence of cycloheximide in the incubation mixture.

Saadi et al. (32) have recently shown that MAC stimulates synthesis of TF in porcine EC exposed to human or rabbit antibodies and human C. This is a model system for xenotransplants and is basically different from our experimental model not only because it evaluates the effect of a cytolytically active complex, though tested in sublytic amounts, but also because the source of MAC is heterologous with respect to the target cells. Under these condi-

tions, MAC operates in the absence of homologous restriction imposed by CD59, which is present on the surface of EC (33). In addition, in their model TF is expressed over a period of 16–42 h and requires the release of IL-1α as an intermediate step, whereas in our system the expression of TF is observed in ~4–6 h. Despite these differences, however, all together these data provide experimental support for the contribution of both cytolytically active and inactive TCC to favor accumulation of fibrin deposits and formation of thrombi in vessels in the course of immune-mediated disorders associated with persistent and sustained C activation.

In conclusion, a novel role has been recognized for iTCC that has long been considered an irrelevant by-product of the C activation cascade. The serum levels of the inactive complex reported in various clinical disorders (34–36) are compatible with the concentrations that were found to be effective in this study. It is quite possible, however, that under conditions of massive C activation, high amounts of iTCC can be formed in vivo, some of which escapes control by vitronectin and clusterin, and that a great proportion of this complex rapidly disappears from the circulation (37) interacting with EC. Such a possibility is suggested by the mild clinical course of meningococcal disease observed in patients with inherited deficiencies of the terminal components, who experience less frequently septic shock, intravascular coagulation, and purpura than C-sufficient patients (38, 39).

The authors wish to thank Mrs. Nadia Polentarutti for excellent technical assistance. This work was performed in the framework of the E.C. Concerted Action (N° BMH4-CT96-1005) and was supported by grants provided to F. Tedesco from Italian Ministero della Università e della Ricerca Scientifica e Tecnologica (40 and 60%) and Consiglio Nazionale delle Recerche (N° 92.02828.CT04).

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Received for publication 15 November 1996 and in revised form 4 March 1997.

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