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Platelet-Activating Factor and Kinin-Dependent Vascular Leakage as a Novel Functional Activity of the Soluble Terminal Complement Complex

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The infrequent occurrence of septic shock in patients with inherited deficiencies of the terminal complement components experiencing meningococcal disease led us to suspect that the terminal complement complex is involved in vascular leakage. To this end, the permeabilizing effect of the cytolytically inactive soluble terminal complement complex (SC5b-9) was tested in a Transwell system measuring the amount of fluorescein-labeled BSA (FITC-BSA) leaked through a monolayer of endothelial cells. The complex caused increased permeability to FITC-BSA after 15 min as opposed to the prompt response to bradykinin (BK). The effect of SC5b-9 was partially reduced by HOE-140 or CV-3988, two selective antagonists of BK B2 and platelet-activating factor receptors, respectively, and was completely neutralized by the mixture of the two antagonists. Also, DX-88, a specific inhibitor of kallikrein, partially inhibited the activity of SC5b-9. The permeabilizing factor(s) released after 30 min of incubation of endothelial cells with SC5b-9 caused a prompt leakage of albumin like BK. Intravital microscopy confirmed both the extravasation of circulating FITC-BSA across mesenteric microvessels 15 min after topical application of SC5b-9 and the complete neutralization by the mixture of HOE-140 and CV-3988. SC5b-9 induced opening of interendothelial junctions in mesenteric endothelium documented by transmission electron microscopy. *The Journal of Immunology,* **2004, 173: 6921–6927.**

The endothelium is a potential target of biologically active products of the complement system released in the circulation and in the extravascular fluids in several clinical conditions associated with marked C activation. One consequence of endothelial cell stimulation is the promotion of leukocyte traffic resulting in the initiation and progression of the inflammatory process, but additional biological effects induced by C activation on endothelial cells include triggering of coagulation and regulation of the vascular tone (1–3).

The membrane attack complex (MAC)[3] formed by the late C components may be assembled on the surface of endothelial cells as a result of C activation by cell-bound Abs. Cytolysis of target cells has been considered for years the best known function of this complex following its insertion into the phospholipid bilayer of the cell membrane. Data accumulated during the last 10–15 years have elucidated a number of noncytolytic effects induced by sublytic MAC on endothelial cells including mobilization of P-selectin (4), release of chemokines (5), growth factors (6), and platelet-activating factor (PAF) (7), and increased expression of E-selectin and ICAM-1 in cooperation with TNF-α (8). Stimulation of prothrombinase activity (9) and tissue factor expression (10) and promotion of PGI_2 release (11) leading to modulation of the vascular tone represent additional effects of sublytic MAC. Most of these activities are induced through the action of IL-1α produced by endothelial cells stimulated by the C complex (2).

The terminal C complex (TCC) that assembles in the fluid phase and fails to insert into the cell membrane rapidly decays becoming cytolytically inactive. This complex known as SC5b-9, where S stands for S protein or vitronectin, is detected in the circulation and in the extravascular fluid and is commonly regarded as an irrelevant byproduct of C activation (12), although a possible effect of SC5b-9 was postulated by Ishikawa et al. (13) in pulmonary hydraulic conductivity. We have previously shown that a cytolytically inactive complex (iTCC), prepared by incubating purified late components and differing from SC5b-9 for the lack of vitronectin and clusterin, despite the inability to lyse target cells, was still able to bind to endothelial cells and to stimulate these cells to express adhesion molecules and tissue factor (14). In subsequent studies, evidence was also provided indicating that iTCC applied on the abluminal side of the endothelial cells promoted transendothelial migration of polymorphonuclear leukocytes (PMN) in a Transwell system and leukocyte traffic across rat mesenteric venules monitored in vivo by intravital microscopy (15). More recently, iTCC injected into the lateral ventricle of the CNS in rats was found to recruit intravascular PMN into the tissue and the cerebrospinal fluid and SC5b-9 purified from human serum proved to be as effective as iTCC (16). SC5b-9 is the inactive complex detected in measurable amounts in patients with septic shock (17, 18), immune complex diseases (19),

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[3] Abbreviations used in this paper: MAC, membrane attack complex; SC5b-9, soluble terminal complement complex; iTCC, cytolytically inactive terminal complement complex; BK, bradykinin; PAF, platelet-activating factor; PMN, polymorphonuclear leukocytes; TCC, terminal C complex; iTCC, cytolytically inactive TCC; FI, fluorescent intensity; vFI, venule FI; iFI, interstitial FI.

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severe traumata (20), cardiopulmonary bypass, and other therapeutic procedure including extracorporeal circulation of blood (21).

The finding that iTCC interacts with endothelial cells, stimulating the expression of adhesion molecules and transendothelial migration, led us to investigate the ability of this complex to induce increased permeability of the endothelium. This function is usually associated with C3a and C5a, but the clinical observation that meningococcal disease occurring in patients with inherited deficiencies of the late C components is less frequently complicated by purpura, hypotension, and septic shock (22, 23) than in patients with normal C activity suggests that TCC may also be implicated in the induction of the vascular leakage. Because these clinical complications are associated with structural and functional changes of the endothelium resulting in increased permeability and tissue edema (24), it is conceivable that C-deficient patients are protected from vascular leakage because of their failure to form TCC. In addition, the trimolecular complex C5b-7 inserted into cell membrane as a result of Ab-dependent C activation was shown by Saadi and Platt (25) to induce transient gap formation.

On the basis of these findings, we decided to investigate whether the inactive TCC was able to increase vascular permeability using in vitro and in vivo models. Data are presented indicating that iTCC and SC5b-9 are both effective in inducing vascular leakage and that this effect is mediated by bradykinin (BK) and PAF.

Materials and Methods

C components, antisera, and other reagents

Purified late C components C7, C8, and C9 were purchased from Quidel (San Diego, CA). The mAb aE11 recognizing a C9 neoepitope, and rabbit polyclonal Abs to C9 and to vitronectin were reported in previous publications (14, 26, 27). Goat antiserum to human C5 was purchased from Quidel; alkaline phosphatase-labeled affinity-purified Abs from goat to mouse IgG were obtained from Sigma-Aldrich (Milan, Italy). The IgG were purified from the antisera by affinity chromatography on a protein G-Sepharose column (Pharmacia Biotech, Milan, Italy). FITC-conjugated BSA was purchased from Sigma-Aldrich, and the PAF receptor antagonist CV-3988 was from Biomol (Plymouth Meeting, PA). BK and BK B2 receptor antagonist HOE-140 were kindly provided by Dr. W. Neugebauer (Sherbrooke University, Quebec, Canada), and DX-88 was obtained through the courtesy of Dr. Tony Williams (Dyax, Cambridge, MA).

Preparation of TCC

C5b6 and iTCC were prepared as previously reported (14). Briefly, the C5b6 complex was purified from yeast-activated C7-depleted serum, and iTCC was obtained by mixing C5b6 and the remaining late C components C7, C8, and C9 at optimal ratio. SC5b-9 was prepared as published in detail by Casarsa et al. (16) incubating C5b6 complex and C5-deficient human serum as a source of the late C component and the fluid phase C regulators vitronectin and clusterin. The mixture of C5b6 and C7-deficient human serum, which was unable to support assembly of SC5b-9, served as a negative control. All the assembled C complexes were then purified by fast protein liquid chromatography on a Superose 12 column (Pharmacia Biotech, Milan. Italy) and quantitated by ELISA using mAb aE11 reacting with a neoepitope exposed on activated C9 as capture Ab and goat IgG anti-human C5 as detecting Ab (14).

Endothelial cell leakage

HUVECs were isolated from three to five normal umbilical cords by collagenase digestion and grown in tissue culture plates (Costar, Cambridge, MA) coated with 2% endotoxin-free gelatin (14). The cells (2×10^4) were seeded onto polycarbonate inserts of a 24-well Transwell system (6.5-mm diameter, 3-μm-diameter pores; Costar) coated with 2% gelatin and used 5 days after plating (15). Each Transwell was checked for the formation of intact monolayer on the insert by adding FITC-BSA (1 mg/ml) to the upper chamber and measuring the amount of labeled BSA passed into the lower chamber by a Fluostar (SLT Labinstruments, Grödig, Austria). The Transwells were used only when the intensity of fluorescence in the lower chamber was negligible; in this case, the stimuli were added to the upper chamber together with FITC-BSA, and the fluorescence was evaluated in the lower chamber at various time intervals.

Assessment of vascular permeability by intravital microscopy

All the in vivo experiments were performed on male Wistar Kyoto rats weighing 250–270 g and anesthetized i.p. with sodium thiobarbital (100 mg/kg). A procedure previously described in detail was followed for the surgical preparation of rats (15). FITC-BSA diluted in sterile saline was slowly infused into the femoral vein at a concentration of 15 mg/kg 30 min before the start of the experiment. After the incision of the abdominal wall, a portion of the ileal mesentery was gently draped over a Plexiglas transparent pedestal placed on an adjustable stage of an upright microscope (model BX50WI; Olympus Optical, Tokyo, Japan). Two hundred microliters of sterile saline containing 4 μg of either SC5–9 or BK to the final concentration of 10^{-7} was topically applied to the exposed mesentery for 5 min followed by superfusion (1 ml/min flow rate) with sterile buffered saline at 37°C throughout the experiment. The microvascular areas were transilluminated with a 12-V, 100-W direct current-stabilized light source and viewed through a ×10 saltwater dipping objective (Olympus Optical) fitted for epifluorescence. The images of the distribution of FITC-BSA were recorded by a charge-coupled device camera (SensiCam PCO, Kelheim, Germany) connected through a PCI-interface board to a computer device, and analyzed off-line using a dedicated imaging software (Analytica Lite, Milan, Italy). The leakage of FITC-BSA across mesenteric venules was analyzed in 20- × 50-μm windows positioned within the venule (vFI) and in the perivenular interstitium (iFI), 50 μm away from the vessel wall. At least two fields of observations, each including distinct unbranched postcapillary venules (35–40 μm in diameter and 100 μm long), were selected for the analysis. The fluorescent intensity (FI) of extravasated FITC-BSA was assessed automatically using the imaging software analysis and calculated as mean value of gray levels ranging between 0 (black) and 255 (white). The albumin leakage index was expressed as the ratio iFI:vFI.

Transmission electron microscopy

Biopsy specimens obtained from the ileal mesentery after perfusion with different stimuli were washed twice with PBS and once with 0.1 M cacodylate buffer and then minced in 1-mm^2 pieces. After fixation with 3% glutaraldehyde for 3 h at room temperature, the pieces were washed and treated with OsO_4 for 1 h. After two washings with 0.1 M cacodylate buffer (pH 7.4), they were serially dehydrated in ethanol, infiltrated with propylene oxide, and embedded in DERR 332 (Electron Microscopy Sciences, Hatfield, PA). Tissue sections were cut with an ultramicrotome (Leika Ultracut UCT, Vienna, Austria) and collected on 200-mesh copper grid. The grids were stained with uranyl acetate and lead citrate and viewed under a Philips EM 208 transmission electron microscope (Philips, Eindhoven, The Netherlands).

Statistical analysis

Results were expressed as mean ± SD. Data were compared by ANOVA using post hoc analysis for paired multiple comparisons with Fisher's corrected t test. p values of <0.05 were considered statistically significant.

Results

Terminal complement complexes induce endothelial permeabilization

In the first series of experiments, we tested the ability of iTCC and SC5b-9 to cause leakage of FITC-BSA through a confluent monolayer of HUVECs using a concentration of the complexes (5 μg/ml) that in our previous study proved to be effective in promoting transendothelial migration of PMN (15). The effect of these two cytolytically inactive complexes were compared with that of the known permeabilizing polypeptide BK, used as a positive control, and was evaluated at various time intervals after their addition to HUVECs (Fig. 1*A*). As expected, BK produced a rapid effect that began to appear after 5 min and increased thereafter up to 30 min. The percentage of FITC-BSA that leaked into the lower chamber of the Transwell at 30 min was calculated to be ~5%. By contrast, iTCC was ineffective for the first 15 min and reached the maximal permeabilizing effect at 30 min resulting in ~4.8% leakage of FITC-BSA. The results obtained with SC5b-9 were essentially similar to those seen with iTCC. Because the degree of permeability to BSA remained unchanged from 30 min to up to 90 min of observation (data not shown), we decided to stop the analysis of the leakage 30 min after the addition of the reagents. We also

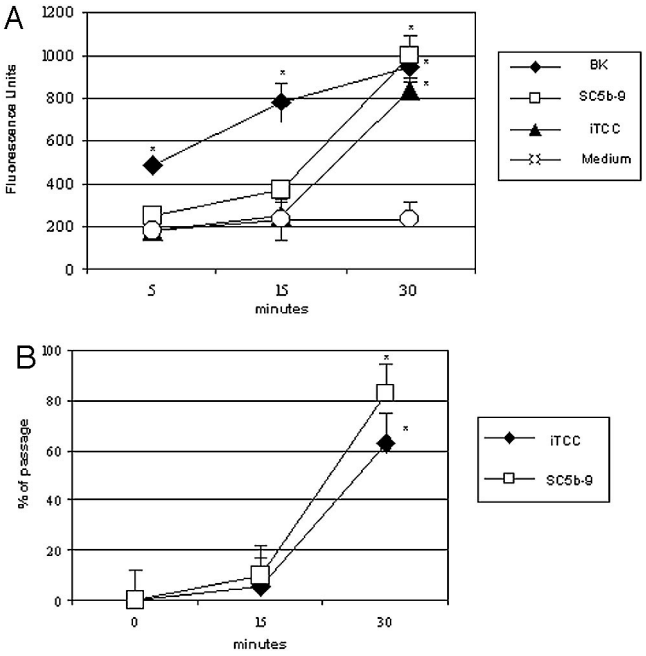

FIGURE 1. Kinetic analysis of BSA and TCC leakage through HUVECs. The cells were grown to confluence onto the inserts of Transwells and exposed to BK (10^{-6} M), iTCC (5 μg/ml), or SC5b-9 (5 μg/ml) added to the upper chamber. The passage of BSA (*A*) into the lower chamber at various time intervals was evaluated by Fluostar and that of iTCC or SC5b-9 (*B*) was measured by ELISA. Values as means ± SD of duplicate determinations of four separate experiments. *, $p < 0.01$ vs medium.

measured the amounts of the two C complexes that had passed through the HUVEC monolayer at various time intervals and found detectable levels of these complexes in the lower chamber of the Transwell only after 30 min (Fig. 1*B*).

To evaluate the dose-dependent effect of the complexes, increasing concentrations of both iTCC and SC5b-9 were tested for their permeabilizing activity. As shown in Fig. 2, iTCC began to induce the effect at 2.5 μg/ml, reaching the plateau at 5 μg/ml with no

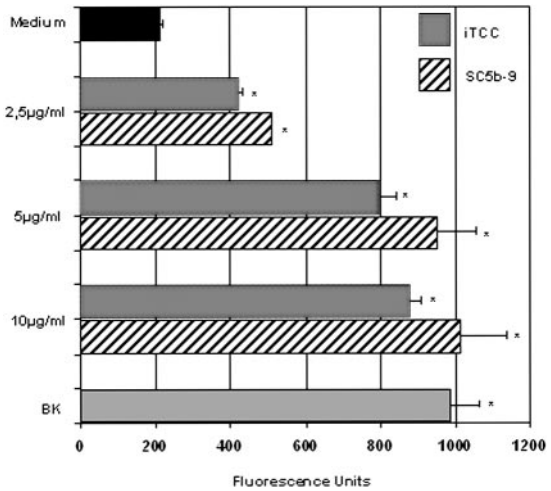

FIGURE 2. Effect of increasing doses of iTCC or SC5b-9 on the permeability of HUVEC. The cells grown to confluence onto the insert of the Transwell were incubated for 30 min with increasing concentrations of either iTCC or SC5b-9 and with BK (10^{-6} M), as a positive control, and the FITC-BSA passed through the monolayer was measured using Fluostar. Values are means ± SD of duplicate determinations of 3 different experiments. *, $p < 0.01$ vs medium.

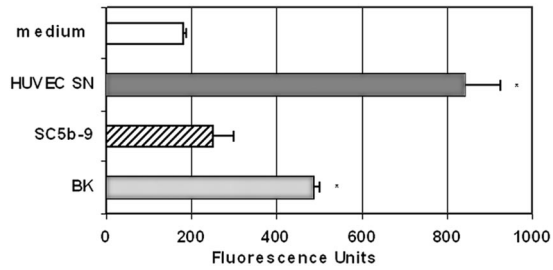

FIGURE 3. Contribution of HUVEC to the release of the permeabilizing factors induced by the C complex. The HUVEC monolayer was incubated with SC5b-9 (5 μg/ml) for 30 min, and the supernatant (SN) was collected and incubated with fresh HUVEC for 5 min. SC5b-9 (5 μg/ml), or BK (10^{-6} M) were tested separately on HUVEC for the same length of time. The amount of FITC-BSA passed through the monolayer of HUVEC into the lower chamber was evaluated by Fluostar. Values are mean fluorescence U ± SD of duplicate determinations of three different experiments. *, $p < 0.01$ vs medium.

further increase at 10 μg/ml. SC5b-9 was as active as iTCC at the same concentrations. The concentration range of the active complex is compatible with the levels of SC5–9 found in many pathological conditions (18, 28, 29). On the basis of these results and given the fact that SC5b-9 is usually detected in the circulation and in the extravascular fluids, we decided to use only this complex in all the other experiments at the concentration of 5 μg/ml. The effect obtained with this amount of iTCC or SC5b-9 roughly corresponds to that obtained with BK used at a concentration of 10^{-6} M. The activity of iTCC was inhibited by two different anti-C9 Abs, whereas the effect of SC5b-9 was neutralized both by anti-C9 and anti-vitronectin Abs (data not shown).

The permeability of HUVECs induced by SC5b-9 is mediated through the action of kinins and PAF

Because the permeabilizing effect of SC5–9 was delayed compared with the prompt response obtained with BK, the possibility was considered that the C complex acts by inducing the release of intermediate mediators. To address this issue, HUVECs were exposed to SC5b-9 for 30 min, the time at which the complex exhibited the maximal permeabilizing effect, and the supernatant was

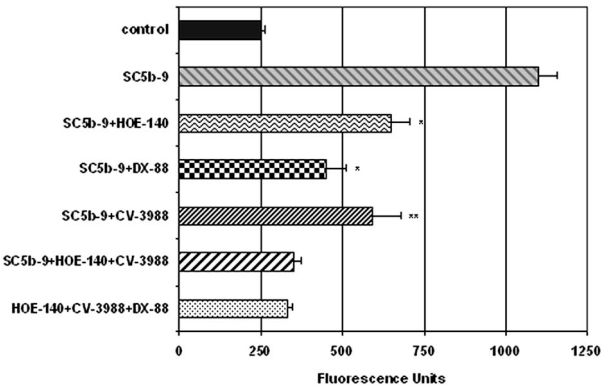

FIGURE 4. Effect of the kallikrein inhibitor DX-88, B2-BK (HOE-140), and PAF (CV-3988) receptor antagonists on SC5b-9-dependent endothelial cell leakage. DX-88 (0,1 mg/ml), HOE-140 (10^{-5}M), CV-3988 (3×10^{-5} M), or the mixture of the molecules were added to the HUVEC monolayer 10 min before incubation with SC5b-9(5 μg/ml). Thirty minutes after the addition of SC5b-9, FITC-BSA added to the upper chamber was collected into the lower chamber and measured by Fluostar. Values are means ± SD of duplicate determinations of three different experiments. *, $p < 0.05$; **, $p < 0.01$ vs SC5b-9.

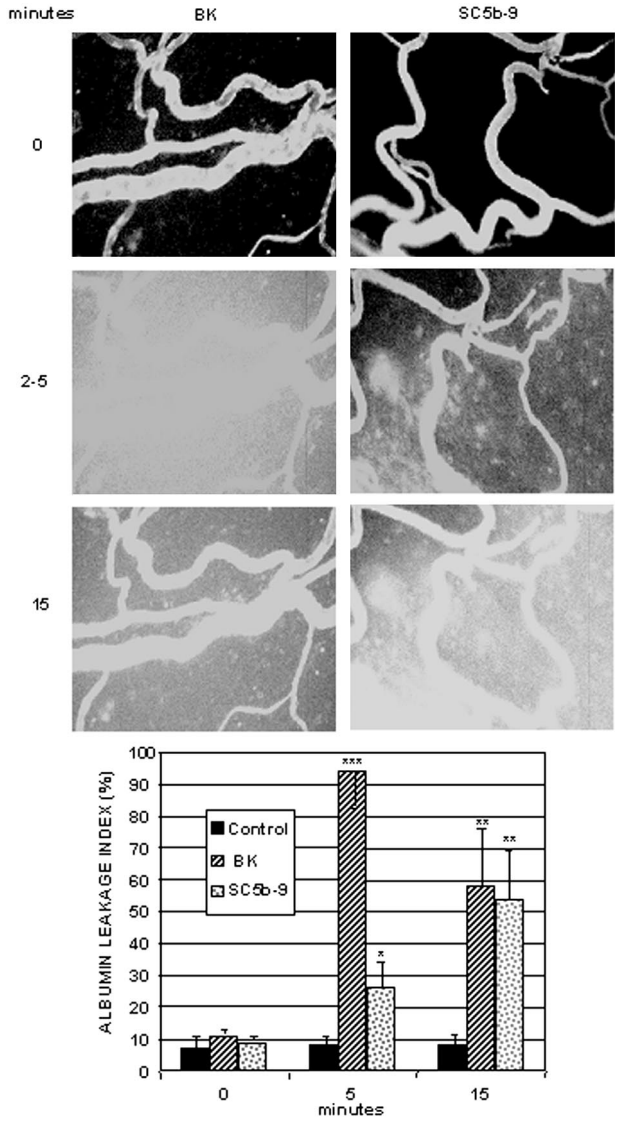

FIGURE 5. In vivo effect of SC5b-9 on the vascular leakage. The ileal mesentery was superfused with BK (10^{-7} M) or SC5b-9 (4 μg/200 μl), and the amount of FITC-BSA extravasated into the perivascular areas was monitored under basal conditions and 2–5 and 15 min after the topical application of the stimuli. Values are means ± SD of the observations made in three animals as specified in *Materials and Methods*. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.005$ vs control.

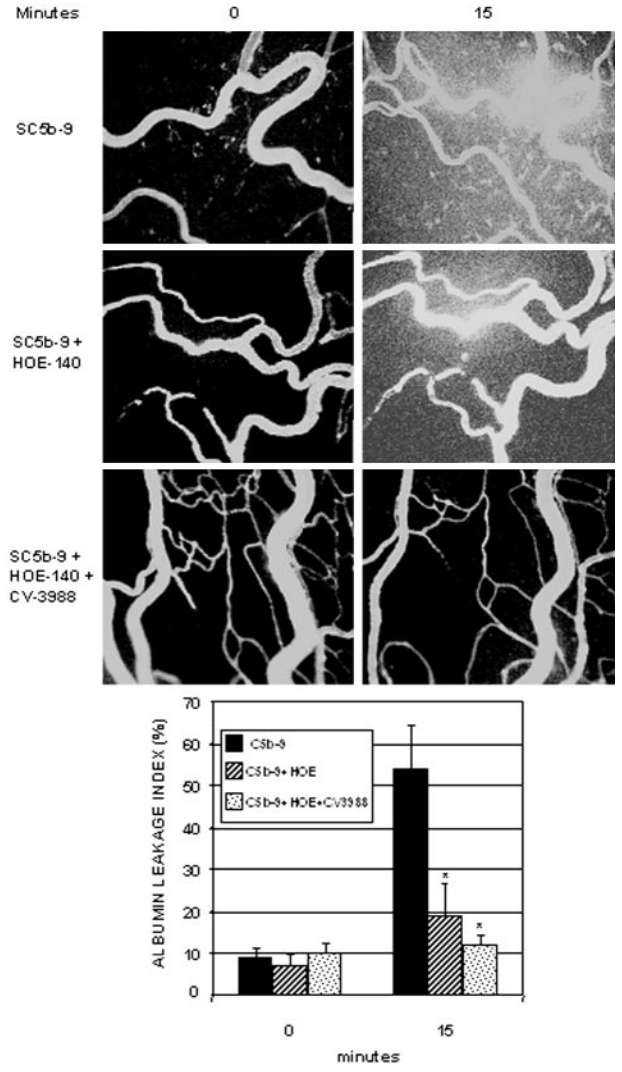

FIGURE 6. Effect of HOE-140 and CV3988 on SC5b-9-induced vascular leakage. The ileal mesentery was superfused with SC5b-9 alone (4 μg/200 μl) or with SC5b-9 mixed with either HOE-140 (10^{-6} M) or the combination of HOE-140 and CV-3988 (3×10^{-5} M), and the leakage of FITC-BSA was assessed under basal conditions and 15 min after the topical application of the reagents. Data are means ± SD of the observations made in three animals as specified in *Materials and Methods*. *, $p < 0.01$ vs SC5b-9.

tested for its ability to induce leakage of BSA on untreated HUVEC after incubation for 5 min. As shown in Fig. 3, the cell supernatant caused a prompt transendothelial passage of an amount of BSA that was higher than that induced by BK, whereas SC5b-9 had only a marginal effect, which was in line with the data presented in Fig. 1*A*. Formation of vasoactive factor(s) with this rapid effect required the presence of HUVECs because no such activity was found in the culture medium incubated with SC5b-9 for 30 min in the absence of HUVECs. On the basis of these results, we reckoned that BK released as a result of HUVEC stimulation by SC5b-9 was a likely candidate to mediate this effect. To this end, HUVECs were treated with HOE-140, a specific BK B2 receptor antagonist, 10 min before addition of BK or SC5b-9. The results presented in Fig. 4 show that the transendothelial passage of BSA was reduced but not completely abolished by the presence of this antagonist, and a partial inhibitory effect was also obtained by the addition of DX-88, a recombinant inhibitor of plasma kallikrein. Because the effect of SC5b-9 was only partially blocked by HOE-140, we investigated the possibility that PAF may contribute to the increase in the permeability of HUVEC induced by SC5b-9 using CV-3988, a specific PAF receptor antagonist. As already seen with HOE-140, CV-3988 incubated with HUVEC for 10 min before the addition of the complex again reduced but did not completely prevent the leakage of BSA, whereas the mixture of the two antagonists HOE-140 and CV-3988 completely inhibited the transendothelial passage of BSA (Fig. 4).

In vivo effect of SC5b-9 on vascular permeability

Having found that SC5b-9 induced increased permeability of HUVECs to BSA, we next examined the ability of the C complex to promote in vivo vascular leakage to BSA across mesenteric venules. As expected, BK caused a prompt extravasation of FITC-BSA that was already visible 5 min after exposure of the ileal mesentery to this peptide, confirming the results obtained in vitro (Fig. 5). The

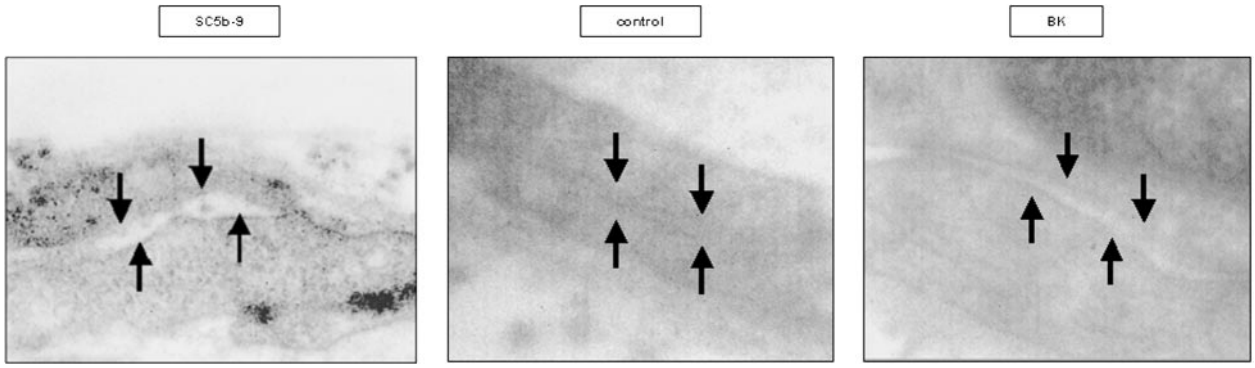

FIGURE 7. Transmission electron microscopic analysis of rat mesenteric microvasculature exposed to SC5b-9 or BK. Biopsy specimens of rat mesenteric ileum superfused with the different stimuli were cut and minced in fixing buffer and processed as described in *Materials and Methods*. ×36,000.

increased vascular permeability induced by BK reached an albumin leakage index value of >90% after 5 min and declined to ~58% after 15 min. A significant increase in BSA leakage was also induced by SC5b-9, although the kinetic was different from that observed with BK (Fig. 5). The mean value of albumin leakage index was ~25% 5 min after the addition of SC5b-9 and progressively increased to ~50% at 15 min. The vascular permeability in response to SC5b-9 was markedly controlled by previous exposure of the ileal mesentery to HOE-140 and completely inhibited by the mixture of HOE-140 and CV-3988 (Fig. 6).

SC5b-9 induces opening of intercellular junctions

Because the increased permeability stimulated by some vasoactive substances has been associated with the formation of interendothelial gaps (30, 31), we decided to investigate the possible association between the loss of the endothelial barrier function, as revealed by the extravasation of albumin, and the appearance of morphological changes of the endothelial cells. To address this issue, the mesentery was superfused with SC5b-9, BK, or saline solution. The animals were sacrificed at 30 min, and a mesenteric biopsy specimen was cut and prepared for transmission electron microscopy analysis. As expected, BK caused ultrastructural changes in the endothelium of rat mesenteric microvasculature characterized by the appearance of openings of intercellular junctions already visible 5 min after treatment with this peptide together with the extravasation of FITC-BSA (Fig. 7). SC5b-9 induced similar morphological changes, although the openings were visible only 30 min after the addition of the complex.

Discussion

C activation in the circulation leads to the release of biologically active products that induce several functional changes of the endothelium including promotion of inflammation and coagulation. Vascular leakage is one such change that results in tissue edema and is commonly associated with the action of C3a and C5a (2, 32). The in vitro and in vivo data presented in this study clearly indicate that the inactive terminal complex is also able to cause vascular permeability.

The finding that iTCC has a direct effect on the permeability of HUVEC monolayer in a Transwell system extends our previous observations that the complex stimulates the expression of adhesion molecules and tissue factor and promotes transendothelial migration of leukocytes, despite its inability to cause cell lysis (14, 15). The permeabilizing effect is not restricted to iTCC but is also seen with SC5–9, a complex detected in the circulation in clinical conditions associated with massive C activation and commonly regarded as an irrelevant byproduct of C activation. However, the report by Brandtzaeg et al. (18) that the level of SC5b-9 is strongly correlated to the levels of endotoxin and to fatality in patients with meningococcal disease might suggest that the complex is implicated in the development of the disease complications including septic shock. We have previously shown that this complex injected into the lateral ventricle of rats promoted inflammation mobilizing leukocytes into the CSF (16). The ability of Abs to C9 to control the albumin leakage induced by both iTCC and SC5b-9 supports the conclusion that the effect of these complexes is specific. In the case of SC5b-9, the neutralizing activity of the Abs to vitronectin suggests that the effect of this complex may also be mediated by its binding to the vitronectin receptor on endothelial cells. The biological relevance of the interaction between SC5b-9 and the vitronectin receptor was emphasized a few years ago by Biesecker (33), who showed that myoblasts adhered to solid phase-bound SC5b-9, implicating the potential role of this complex in cell attachment and cellular response at tissue level. Whether binding of iTCC to HUVEC is receptor mediated cannot be established. Our preliminary data suggest the existence of such a receptor; however, the data are not conclusive and must be further substantiated.

The detection of iTCC and SC5b-9 in the lower chamber of the Transwell following stimulation of HUVECs with the two complexes clearly indicates that the increased permeability of these cells is not selective for BSA. As a result of the increased permeability of the endothelium, these complexes may diffuse out into the subendothelial areas and stimulate perivascular macrophages and other cells to release proinflammatory mediators. The leakage of SC5b-9 through the monolayer of endothelial cells is compatible with the appearance of wide focal dilations in intercellular junctions of HUVECs seen on electron microscopy sections of rat mesenteric vessels of rats exposed in vivo to SC5b-9. C-dependent intercellular gaps were already observed by Saadi and Platt (25) in monolayers of porcine endothelial cells incubated with human xenoreactive natural Abs and human serum as a source of C. However, the formation of gaps in this model was strictly dependent on the insertion of the trimolecular complex C5b-7 into the membrane, and the gaps tended to close when the C5b-9 complex was fully assembled on the cells. Conversely, the loss of barrier function that occurs under our experimental conditions does not require Ab-mediated C activation and is caused by the fully assembled and cytolytically inactive terminal complexes.

The leakage of FITC-BSA through the endothelium of rat mesenteric veins exposed to SC5b-9 proves that the loss of barrier function is not restricted to the in vitro model but can also be induced in vivo, thus extending our previous observation that

iTCC stimulates egression of leukocyte across rat mesenteric postcapillary venules into the perivascular tissue (15). A point to emphasize is that the extravasation of BSA is stimulated by SC5b-9 added at the extravascular sites in the mesenteric ileum, suggesting that the complex is equally effective in promoting vascular permeability on both sides of the vessels. We cannot exclude that the complex induces increased permeability through the intermediate action of mediators released by mast cells or macrophages present in the ileal mesentery and shown to be involved in the promotion of acute peritoneal inflammation (34).

One of the mediators involved in the SC5b-9-induced vascular leakage is likely to be BK, a potent vasodilator (35) with a strong and rapid activity on vascular permeability (36) as indicated by the inhibitory effect of HOE-140, a specific and selective antagonist of the kinin B2 receptor (37, 38), and the neutralizing activity of DX-88, an inhibitor of plasma kallikrein. Evidence that SC5–9 incubated with endothelial cells promotes the release and accumulation of BK-like material in the medium is also supported by the observation that the culture medium collected after a 30-min incubation of HUVECs with SC5b-9 induces a prompt BSA leakage very similar to that of BK. These findings indicate that SC5–9 interacts with endothelial cells and promotes the formation of BK possibly from a complex kininogen-prekallikrein present on the surface of the endothelium (39, 40). A similar mechanism has been proposed by Katada and Majima (41) to explain the release of BK and the arterial vasodilatation observed in isolated and perfused mesenteric artery following the interaction of angiotensin II with its type 2 receptor. The functional relationship that cell bound SC5b-9 establishes with the putative receptors for high molecular mass kininogen on endothelial cells including gC1qR, cytokeratin 1 and u-PAR (42) remains to be elucidated.

The other endogenous agent participating to the BSA leakage induced by SC5b-9 is PAF. In fact, the other half of the effect, which remains in the presence of HOE-140, is blocked by CV-3988, a selective antagonist of the PAF receptor. BK and PAF are the two major mediators because a complete block of the effect of SC5–9 is observed with the combination of HOE-140 and CV-3988. The contribution of PAF to the endothelial cell leakage is not surprising because Kilgore et al. (7) have shown that sublytic MAC assembled on endothelial cells stimulates the release of PAF and in turn promotes neutrophil adhesion, although the complex used in the present study differs from MAC for being cytolytically inactive.

Our finding that PAF activity is detected in the supernatant of HUVEC incubated with SC5b-9 for 30 min may be difficult to reconcile with the short half-life of PAF. This is certainly true if BK and PAF were rapidly released by HUVEC following the addition of SC5b-9 to the culture medium. However, the release of these peptides is unlikely to occur so rapidly because we have previously shown that the binding of iTCC to HUVEC requires $>$15 min to complete (14). Furthermore, the data presented in Fig. 1 show that the SC5b-9-mediated HUVEC permeability is a slow process and begins to appear after 20 min of cell incubation with the complex. This suggests that PAF and BK are released by HUVEC in the last 5–10 min of incubation with SC5b-9.

In conclusion, we have provided evidence that iTCC and its physiological counterpart SC5b-9 promotes the permeability of HUVECs through the release of BK and PAF, besides stimulating transendothelial migration of leukocytes. SC5b-9 should therefore be considered as a possible therapeutic target together with other vasoactive products of the C cascade in clinical situations, such as septic shock, associated with marked C activation. Furthermore, antagonists for PAF and the BK B2 receptors are likely to find promising applications in these conditions.

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