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NEUTROPHIL ADHESION TO HUMAN ENDOTHELIAL CELLS IS INDUCED BY THE MEMBRANE ATTACK COMPLEX: THE ROLES OF P-SELECTIN AND PLATELET ACTIVATING FACTOR

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Abstract—A variety of inflammatory diseases are accompanied by activation of the complement system. We examined the role of the membrane attack complex (MAC) in mediating neutrophil adhesion to endothelial cells. To assemble the MAC in endothelial cell monolayers, a C5b-like molecule was created through the treatment of purified C5 with the oxidizing agent chloramine-T, followed by addition of the remaining components (C6-C9) that constitute the MAC. Use of this method abrogated potentially confounding effects mediated by other complement components (e.g., C5a). MAC assembly resulted in a rapid (30 min), concentration-dependent increase in neutrophil adherence. A monoclonal antibody directed against P-selectin inhibited MAC-mediated neutrophil adhesion. A whole cell EIA confirmed P-selectin expression after formation of the MAC. Incubation of neutrophils with the platelet-activating factor receptor antagonist, CF 3988, also significantly decreased adhesion, indicating that PAF plays a role in MAC-mediated adhesion. These results suggest that the MAC can promote neutrophil adhesion through P-selectin and PAF-mediated mechanisms.

INTRODUCTION

One of the pivital events required for the recruitment of neutrophils into sites of acute inflammation is the adherence of neutrophils to the vascular endothelium. This interaction involves several distinct processes mediated by adhesion-promoting molecules located on the surfaces of both neutrophils and endothelial cells (1,2). One of the early events associated with neutrophil adherence, rolling of neutrophils along the endothelium, is mediated by P-selectin, a member of the selectin family of adhesion molecules (3,4). P-selectin (GMP-140, PADGEM),

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is stored within cytoplasmic Weibel-Palade bodies. Upon stimulation of the endothelium by agonists such as thrombin or histamine, P-selectin is rapidly (within minutes) translocated to the surface of the endothelial cell (5,6). In turn, P-selectin can interact with its counter-ligand on the neutrophil. The P-selectin counter-ligand is thought to be a sialylated *O*-linked oligosaccharide-containing glycoprotein (e.g., sialyl Lewis^{X}) or other related sulfated glycoproteins and glycolipids (3,7,8). The interaction of P-selectin with its counter-ligand is associated with neutrophil activation characterized by the shedding of L-selectin, shape change, and conformational changes in the CD11b/CD18 $\beta 2$ integrin complex (2).

While the early events of neutrophil adhesion are mediated, in part, by interactions with P-selectin, platelet activating factor (PAF) has also been implicated in early neutrophil-endothelial cell adhesion (9–11). PAF, a biologically active phospholipid, is rapidly synthesized (within minutes) by endothelial cells following stimulation by agonists including thrombin, histamine and leukotriene C4 (5,12). Once synthesized, PAF remains on the endothelial cell membrane, where it may interact with overlying neutrophils (10). The interaction of P-selectin and PAF with the neutrophil is throught to be an integral event in the establishment of the higher affinity adhesive interactions between the β_2-integrins, LFA-1 (CD11a/CD18) and Mac-1 (CD11b/CD18) and endothelial adhesion molecules (2,13,14).

The recruitment and subsequent activation of neutrophils involves several additional soluble mediators including a number of cytokines and derivatives of arachidonic acid metabolism. Products of the activated complement system have also been implicated in the recruitment and activation of neutrophils. The C3 cleavage product, iC3b, has been shown to function directly in neutrophil adherence by acting as a ligand for the CD11b/CD18 integrin complex (15). Furthermore, the formation and subsequent deposition of iC3b upon the endothelium promotes the expression of both interleukin-1 (IL-1) and ICAM-1 (16). The anaphylatoxins C3a and C5a possess a wide range of biological activities (e.g., increased vascular permeability, neutrophil chemotaxis and activation) (17). C5a has recently been shown to directly promote neutrophil adhesion to endothelial cells by increasing the expression of P-selectin (18). While the roles of iC3b and the anaphylatoxins in neutrophil activation and neutrophil-endothelial cell adhesion have been extensively investigated, the pathophysiologic role(s) of the MAC in neutrophil-endothelial adhesive interactions have yet to be fully determined.

Using antibody-sensitized endothelial cells and whole serum, Hattori, et al. (6) reported that formation of the MAC on endothelial cells promotes expression of P-selectin, suggesting that the terminal complement components may directly promote neutrophil adhesion. In the present study we sought to determine whether formation of the MAC, in the absence of other complement-

derived (e.g., iC3b, C3a, C5a), can promote neutrophil adherence to endothelial cells. Purified complement components (C5–C9) were utilized to determine the ability of the MAC to increase the adherence of neutrophils to HUVECs. A C5b-like C5 molecule coupled to C6 was employed to promote MAC assembly, thereby obviating the need for antibody-sensitized target cells or a model system that employs whole serum.

METHODS

Endothelial Cells. Human umbilical vein endothelial cells (HUVECs) were isolated from umbilical veins by treatment with 0.1% collagenase in Dulbecco's modified Eagle's medium. Cells were plated at 5×10^4 cells/well in gelatin-coated 96 well plates and incubated at 37°C, 5% CO_2. Cells were grown in M199 medium supplemented with 20% heat-inactivated fetal calf serum, L-glutamine (4 mM), penicillin (100 U/ml), streptomycin (100 μg/ml), 25 μg/ml endothelial cell growth supplement (Collaborative Research, Bedford, Massachusetts) and 15 U/ml bovine heparin. Cells were characterized by a cobblestone appearance and utilized between the first and third passages.

Non-Enzymatic Formation of C5b-Like C5C6 Complex (C5C6).* Purified components of the membrane attack complex (C5–C9) were purchased from Calbiochem (La Jolla, California) and resuspended in serum-free medium. Formation of a C5b-like activation product was accomplished by use of chloramine-T as described by Vogt et al. (19,20). Briefly, 10 μg purified C5 was added to 10 μl veronal-buffered saline (VBX; Sigma Chemical Co., St. Louis Missouri) and incubated in the presence of 10 μl 0.32 mM chloramine-T (Sigma Chemical Co., St. Louis Missouri) in water for 10 min at room temperature. At the end of the incubation period, 10 μl 1 mM methionine (Sigma) was added to the mixture to inactivate the remaining chloramine-T. To form the modified C5b-like C6 complex (C5C6*), 20 μg purified C6 in 300 μl serum-free media was incubated (24 h, 37°C) with the chloramine-T-treated C5 (19).

Assembly of the Membrane Attack Complex (MAC). Confluent HUVEC monolayers were maintained in 96 well plates for 24 h before use (see preceding method). Cells were washed with serum-free media followed by a 15 min preincubation (37°C) with, unless otherwise noted, 0.5 μg/well of the modified C5C6* activation product and C7 (1 μg/well). The cells were then washed with serum-free medium to remove excess C5C6* activation product and C7. At time zero, complement components C8 and C9 (1 μg/well) were added to HUVEC monolayers and allowed to incubate for the indicated time. Total volume per well was 100 μl.

Membrane Attack Complex Functional Assays. A turbidometric red blood cell (RBC) lysis assay was employed to test the functional capacity of the C5C6* complex, when combined with C7–C9, to form a lytically active MAC. Citrated (3.7% sodium citrate) human blood was collected and the RBCs isolated by centrifugation for 6 min at 500 *g* followed by repeated washing in phosphate buffered saline (PBS, pH 7.4). The RBCs were diluted in PBS to achieve a final concentration of 10% RBC (vol/vol). The total assay volume was 200 μl. Ten microliters of RBCs were added to the assay buffer (PBS, pH 7.4) containing 1 μg/ml C5C6* and 1 μg/ml C7. After 5 min, the assay was initiated by the addition of complement components C8 and C9 (1 μg/ml) to the suspension. Negative controls included MAC assembly in the absence of either C8 or the C5C6* activation product. One hundred percent light transmittance was achieved by lysing RBCs for 5 min with water. Data are expressed as the time required for 50% hemolysis. Light transmittance was monitored for a maximum of 10 min.

Immunohistochemical Localization of the MAC on Endothelial Cells. HUVEC monolayers were grown to confluence in gelectin-coated, two-well cell culture chamber slides (Nunc, Inc., Naperville, Illinois) (see above). After formation of the MAC (30 min), monolayers were washed with PBS containing 1% BSA and fixed with acetone (5 min). Cells were incubated with a murine monoclonal antibody (1 : 500 dilution) to the human MAC neoantigen (Quidel, San Diego, California) for 45 min. This monoclonal antibody has been shown to be specific for the fully assembled MAC. Following repeated washings, cells were incubated with a biotinylated goat anti-mouse secondary antibody (1 : 1000 dilution) (Vector Laboratories, Inc., Burlingame, California). Detection of the primary antibody was accomplished using a Vectastain ABC kit (Vector Laboratories) with three-amino-9-ethyl-carbazole as the substrate. Controls included chambers in which the primary antibody was omitted and chambers in which an isotype-matched murine IgG1 antibody was substituted for the anti-MAC monoclonal antibody.

Effect of MAC on HUVEC Membrane Integrity. Alterations of membrane integrity due to formation of the MAC were determined by measuring the release of the intracellular enzyme lactate dehydrogenase (LDH). LDH activity was measured using a kit purchased from Sigma Chemical Co. (St. Louis Missouri, procedure DG1340-UV). The assay is based on the rate of LDH-mediated oxidation of NADH to NAD+, resulting in decreased absorbance at 340 nm. The amount of LDH in the supernatant was expressed as the percent of total lactate dehydrogenase released from lysed HUVECs. The chloramine-T-induced C5C6* activation product was utilized as the limiting factor in measurements of membrane integrity. The maximal amount of C5C6* at which no detectable increase in LDH release above negative controls was the concentration utilized for subsequent neutrophil adhesion studies.

Isolation and Fluorescent Labeling of Human Neutrophils. Human neutrophils were isolated from citrated (3.7% sodium citrate) peripheral venous blood donated from healthy volunteers. Blood was placed over Ficoll/Hypaque (Pharmacia, Uppsala, Sweden) and centrifuged at 300 × g for 30 min followed by sedimentation with 5% dextran (MW 200,000; Sigma Chemical Co.) for 40–60 min. Contaminating erythrocytes were removed by hypotonic lysis followed by addition of 2.7% NaCl to restore appropriate tonicity. Purified neutrophils were washed two additional times PBS (pH 7.4) and resuspended in cold calcium and magnesium free Hanks buffer (pH 7.4, $HBSS^-$) containing 0.1% BSA to achieve a concentration of 5×10^6 cells/ml. Cells preparations were ≥90% pure with a viability of approximately 95% as determined by trypan blue exclusion.

Neutrophils were fluorchrome-labeled as described previously using 2′,7′-bis-(2-carboxyethyl)-5(and 6)-carboxyfluorescein, acetoxymethyl (BCECF-AM; Molecular Probes, Eugene, OR) (21). Briefly, BCECF-AM was dissolved in DMSO to achieve a final concentration of 1 μg/μl. One microliter of BCECF-AM was added per 1.0 ml of cells (final concentration of 1 μM) and incubated for 30 minutes at 37°C. The cells were washed two times in $HBSS^-$/BSA and resuspended in serum free HUVEC medium to achieve a final concentration of 1×10^6 cells/ml.

Cell Adhesion Assay. Adherence of BCECF-AM-labeled neutrophils to HUVEC monolayers was quantitated as previously described (21). Confluent HUVECs were prepared as described above and incubated at 37°C 24 h before use. After assembly of the MAC, monolayers were washed two times with warm serum-free HUVEC media followed by the addition of BCECF-AM-labeled neutrophils (1.0×10^5 cells/well, 100 μl total volume). After a 15 min incubation, non-adherent neutrophils were removed by gently washing the monolayers with PBS four times with a 12-channel multi-pipetter followed by a second cycle with fresh PBS. After washing, PBS (100 μl) was added to each well and the amount of fluorescence determined using a Cytofluor 2300 fluorescent plate reader (Millipore). The excitation filter was a 20 nm bandwidth filter centered at 485 nm and the emission filter was a 25 nm bandwidth filter centered at 530 nm. A standard curve relating fluorescence to cell number was established using two-fold serial dilutions of labeled neutrophils (21). Where indicated, the murine monoclonal antibody Throm/6 (Biodesign Int., Kennebunkport, Maine) was utilized to block P-selectin-mediated adhesion. An isotype-matched murine antibody was utilized a

control. Antibodies (10 μg/ml) were added to endothelial monolayers five min before addition of BCECF-AM-labeled neutrophils. Cells were allowed to incubate at room temperature. Antibodies were utilized as the intact IgG_1 molecule.

Whole Cell EIA Measurement of P-Selectin Expression. Cell surface P-selectin expression on HUVECs (± MAC assembly) was determined by a modified whole cell EIA as described previously (22). HUVECs were plated at a concentration of a 5×10^5 cells/well in 96 well flat-bottom, gelatin-coated plates and grown to confluence at 37°C. Following MAC formation, cells were washed with PBS and fixed with 1% paraformaldehyde for 30 min. Non-fat dry milk (5% in PBS) was added to the monolayers to reduce non-specific binding. Cells were incubated with anti-P-selectin monoclonal antibody Thromb/6 (1 μg/ml) or isotype-matched murine antibody (1 μg/ml) for 45 minutes followed by incubation with the peroxidase-conjugated goat anti-mouse secondary antibody (Dako Corporation, Carpinteria, California) for 45 min. Cells were washed with PBS and the cells exposed to the substrate (*o*-phenylenediamine dihydrochloride, Sigma) for 30 min. The reaction was halted by addition of 3M sulfuric acid. Optical density was determined by an automated microplate reader (EL340, Bio-Tek Instruments, Winooski, Vermont) set to a wavelength of 495 nm.

PAF Receptor Antagonist. The PAF receptor antagonist CV 3988 has previously shown to be a specific PAF receptor antagonist (23). CV 3988 was purchased from Biomol (Plymouth Meeting, Pennsylvania) and dissolved in warm (56°C, 10 min) HUVEC medium. Neutrophils were incubated in either control media (M199) or 30 μM CV 3988 for 10 min before addition to HUVEC monolayers.

Statistical Analysis. All values are expressed as the mean ± the *SEM*. Neutrophil adhesion studies and EIA assays were conducted in triplicate and repeated four times with similar results. Data from EIA experiments were not merged due to intrinsic variation in optical density values between experiments. LDH determinations included three replicates repeated three times. Statistical significance was determined by one-way analysis of variance (ANOVA). A P-value of <0.05 is considered significant.

RESULTS

MAC Assembly: Functional and Structural Integrity. We employed a C5b-9-dependent red blood cell lysis assay and immunohistochemical localization of MAC neoantigen on HUVECs in order to ascertain the functional and structural integrity of the MAC produced using chloramine-T-treated C5. Addition of modified C5C6*, followed by C7-C9, to red blood cells resulted in concentration-dependent red blood cell lysis (Table I). Omission of either C5C6* or C8 completely abrogated red blood cell lysis. Addition of any individual complement components (C5–C9), including C5C6*, did not induce red blood cell lysis (data not shown). These data indicate that functional MAC can be assembled using chloramine-T-treated C5 to form a C5b-like molecule coupled to C6 (C5C6*).

Assembly of the MAC on HUVECs was confirmed by immunohistochemistry using a monoclonal antibody directed against the C5b-9 neoantigen. This antibody has previously been shown to react specifically with the MAC neoantigen, a structure that is dependent on the conformation of the completely assembled MAC (24). HUVECs that contained the MAC exhibited a gener-

Table 1. Assembly of Functional MAC Using Modified C5C6[a] as Determined by Red Blood Cell Lysis

C5C6* Concentration	C8 Concentration	Time to 50% Cell Lysis (Sec)
1.0 μg/ml	1.0 μg/ml	106
1.0	0.0	>600*
0.5	1.0	128
0.0	1.0	>600*

[a]Assay was monitored for a maximum of 600 s (see text for details).

ally diffuse staining pattern, but in some cases also exhibited punctate staining (Figure 1A). No staining was observed in HUVECs that had been incubated with distal complement components (i.e. C5C6*, C7 and C9) exclusive of C8 (Figure 1B). No staining was observed in the absence of the primary antibody or when an equal concentration of isotype-matched nonspecific antibody was substituted for the primary antibody (reults not shown).

Effect of Sublytic Concentrations of MAC on HUVEC Membrane Integrity. The direct effect of MAC assembly on HUVEC membrane integrity was assessed by release of lactate dehydrogenase (LDH). In these experiments, the C5C6* complex was utilized in limiting dilutions while maintaining fixed concentrations (see above) of C7, C8 and C9. Concentrations of C5C6* at or below 1.0 μg/ml did not result in significant increases in concentrations of released LDH when compared to HUVECs that had not been incubated with C5C6* (Figure 2). A C5C6* concentration of 0.5 μg/ml was chosen for all subsequent experiments since LHD was released when the concentration was increased to 1.0 μg/ml.

MAC-Induced Neutrophil-Endothelial Cell Adhesion. Assembly of the MAC on confluent HUVECs resulted in a concentration-dependent and time-dependent increase in subsequent neutrophil adhesion (Figure 3). Maximum neutrophil adhesion was observed with a C5C6* concentration of 0.5 μg/ml. This concentration of C5C6* induced a greater than 2.0 fold increase in adhesion over monolayers devoid of C5C6* (19.78 ± 1.9% versus 8.01 ± 0.52%; $P < 0.05$; one-way ANOVA).

The temporal pattern of neutrophil adhesion (Figure 4) was determined using a C5C6* concentration of 0.5 μg/ml. HUVECs were incubated to the C5C6* complex and C7 for 10 min followed by addition of C8 and C9 for the times indicated. After washing, BCECF-AM-labeled neutrophils were added to monolayers for 15 min. The basal level (without MAC formation) of neutrophil adhesion was found to be 8.75 ± 0.86%. The maximal level of adhesion, 22.10 ± 1.93%, was observed 30 min following formation of the fully assembled MAC. After 60 min, the level of adherence was not significantly different from the basal

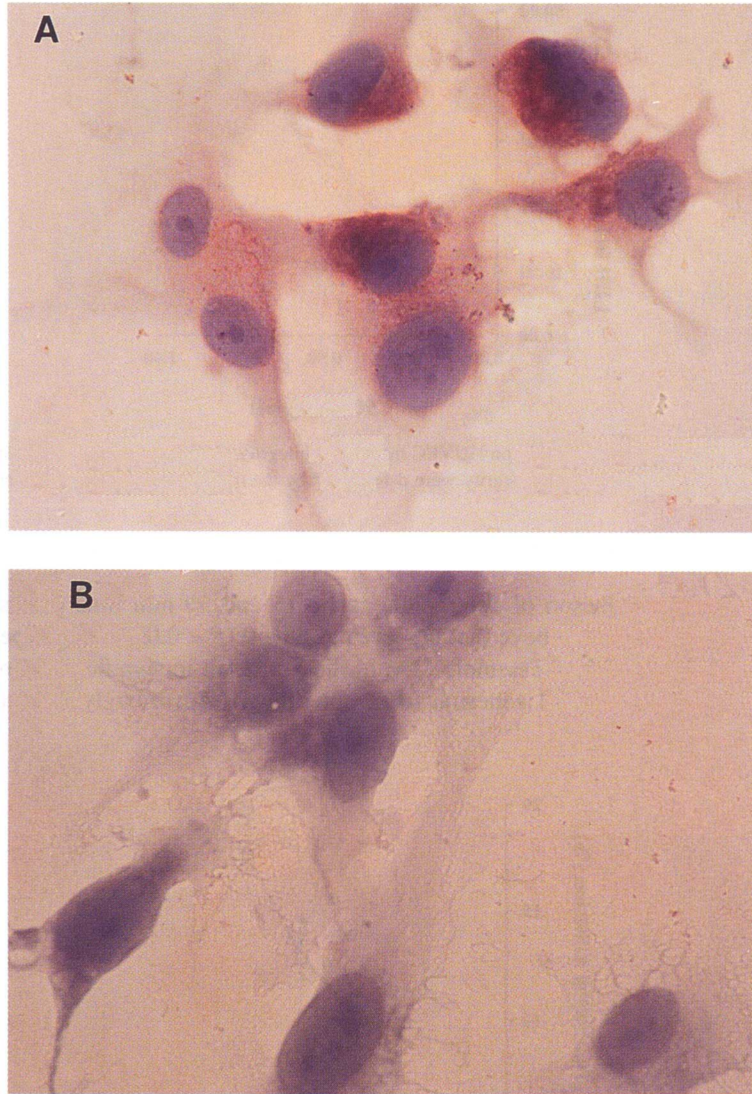

Fig. 1. Localization of the intact membrane attack complex on HUVEC monolayers. A) HUVECs were incubated with 0.5 μg/well C5C6* and 1 μg/ml C7 for 10 min followed by addition of C8 and C9 (1 μg/well) for an additional 30 min. B) Omission of C8 resulted in failure to assemble the MAC. Original magnificants ×200. Complexes were visualized by addition of an antibody to the MAC neoantigen followed by a biotin-labeled secondary antibody (see METHODS).

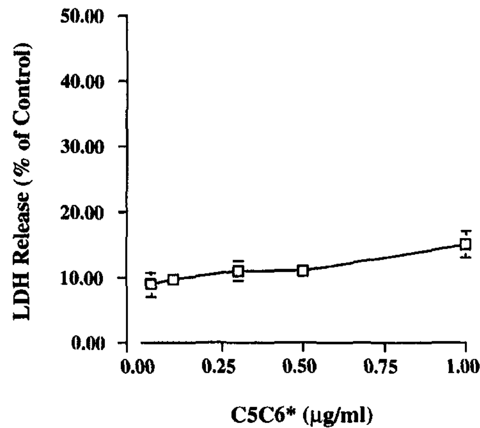

Fig. 2. Effect of MAC assembly on HUVEC membrane integrity. C5C6* was used in limiting dilutions. Alterations in membrane integrity were determined by the release of the intracellular enzyme LDH.

level (time 0). Adheson of neutrophils at the 15 and 30 min time points was significantly increased above that observed at time 0 ($P < 0.05$). Longer periods of time following MAC assembly (2–6 h) did not result in significant increases in subsequent neutrophil adhesion (data not shown). Accordingly, subsequent

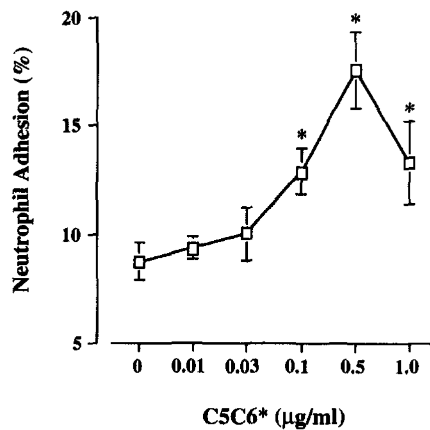

Fig. 3. Concentration-response curve for MAC-mediated neutrophil adhesion. Incremental concentrations of C5C6* and C7 (1 μg/well) were incubated for 10 min followed by the addition of 1 μg/well C8 and C9. After washing, BCECF-AM-labeled neutrophils were added to monolayers for 15 min and the neutrophil binding determined. *$P < 0.05$ versus control (0 C5C6*).

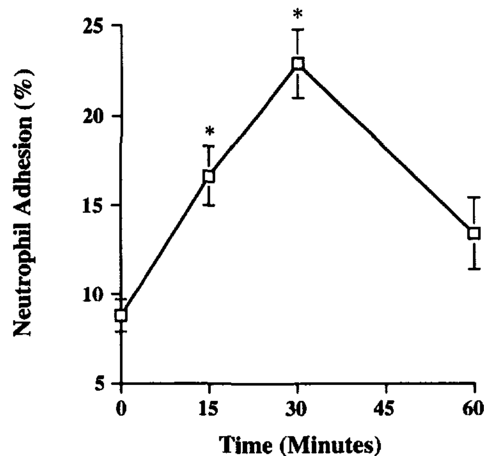

Fig. 4. Temporal characteristics of neutrophil adhesion following formation of the MAC. Endothelial monolayers were incubated with 0.5 μg/well C5C6* and C7 (1 μg/well) for 10 min followed by the addition of C8–C9 (1 μg/well) for the times indicated. After washing, BCECF-AM-labeled neutrophils were added to monolayers for 15 min and the neutrophil binding determined as described in METHODS. $^*P < 0.05$ versus 0 time point.

experiments which addressed the role of P-selectin and expression of P-selectin utilized a C5C6* concentration of 0.5 μg/ml and time of 30 min for formation of the MAC.

Requirement of P-Selectin for Neutrophil-Endothelial Cell Adhesion. The rapid rise in neutrophil adhesion (30 min) suggested that the endothelial adhesion molecule P-selectin might be involved in mediating the increase in adhesion (5,6). Studies utilizing the anti-P-selectin monoclonal antibody Throm/6 were conducted to determine the role of P-selectin in mediating neutrophil adhesion following deposition of the MAC. The Throm/6 antibody has been previously shown to inhibit P-selectin mediated adhesion. As shown in Figure 5, addition of the Throm/6 antibody, at a concentration of 1.0 μg/ml, resulted in a significant decrease in neutrophil adhesion when compared to cells pretreated with non-specific isotype-matched antibody ($P < 0.05$). In the presence of the Throm/6 antibody, the level of adhesion was not significantly different when compared to that of control. Antibody concentrations greater than 1.0 μg/ml did not increase the degree of inhibition (results not shown).

Expression of P-Selectin on HUVECs. A modified whole cell EIA was utilized in order to quantitate MAC-induced increase in expression of P-selectin. This assay has been employed previously to determine adhesion molecule expression on endothelial cells (18,22). Incubation of endothelial cells with increasing concentrations of C5C6*, followed by addition of C8 and C9, resulted

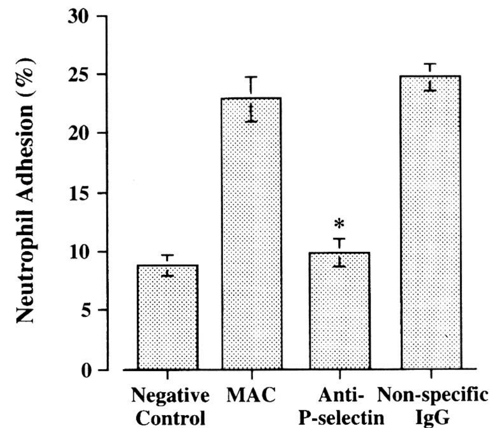

Fig. 5. Inhibition of neutrophil adhesion by addition of the anti-P-selectin monoclonal antibody Throm/C6. Antibody (1 μg/well) was added to monolayers during the last five min of incubation with the MAC components. Antibody (10 μg/ml) also was added to BCECF-AM-labeled neutrophils. *$P < 0.05$ versus MAC-treated HUVECs.

in a concentration-dependent increase in P-selectin expression (Figure 6). Concentrations of less than 0.3 μg/ml C5C6* did not result in increased expression of P-selectin above that observed in untreated HUVECs. Concentrations ranging from 0.3 μg/ml to 1.0 μg/ml significantly increased expression ($P < 0.05$) (Figure 6).

Effect of PAF Receptor Blockade. The contribution of PAF to neutrophil-HUVEC adhesion following MAC assembly on HUVECs was evaluated using

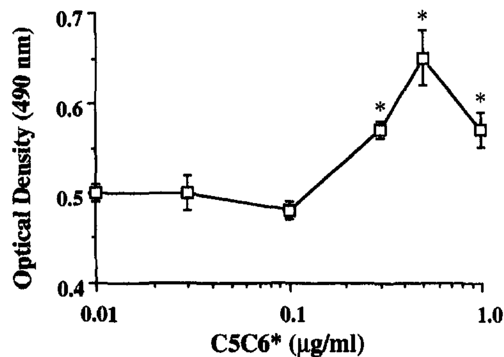

Fig. 6. Increased expression of P-selectin after formation of MAC as determined by whole-cell EIA. The expression of P-selectin by HUVECs is related to the concentration of C5C6* present monolayers. *$P < 0.05$ versus non-C5C6*-treated HUVECs.

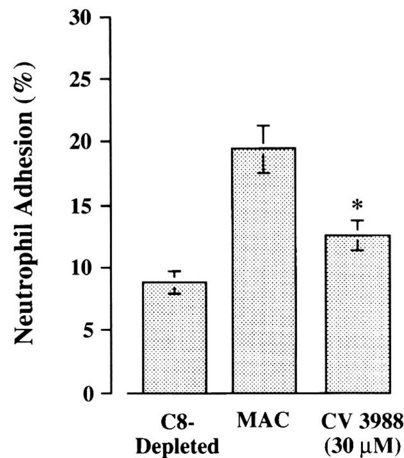

Fig. 7. Requirement of PAF for maximal neutrophil adhesion following formation of the MAC. Neutrophils were pretreated with 30 μM CV3988 for 10 min before addition to cell monolayers. Negative controls received vehicle (saline). Following a 15 min incubation with labeled neutrophils the fluorescence was determined. *$P < 0.05$ versus MAC-treated HUVECs.

the specific PAF receptor antagonist CV 3988 (Figure 7). Neutrophils were incubated with 30 μM CV 3988 for 10 min at room temperature before addition of the neutrophils to HUVECs. MAC was assembled on endothelial cells for 30 min. Pretreatment of neutrophils with CV 3988 resulted in a significant decrease ($P < 0.05$) in neutrophil adhesion (14.12 ± 0.78% versus 19.78 ± 1.9%; $P < 0.05$; ANOVA). Neutrophils treated with vehicle (saline) did not result in decreased adhesion.

DISCUSSION

While many proinflammatory activities of complement-derived products (e.g., the anaphylatoxins) have been described, little is known about the role of the MAC in modulating inflammatory processes. Hattori, et al. (6) utilized antibody-sensitized endothelial cells and whole serum to provide evidence that the MAC can promote expression of P-selectin and release of von Willebrand factor from human endothelial cells. In the present report, purified distal complement components (C5–C9) were used to assemble the MAC on HUVECs. The present approach, in which rapid neutrophil-HUVEC adhesive interactions were assessed, allowed the modulatory role of the MAC to be studied in a serum-free system unconfounded by non-MAC complement components (e.g., iC3b, C3a,

C5a). The data provided in this study support the hypothesis that assembly of the MAC on an endothelial surface promotes rapid neutrophil adhesion through a P-selectin and PAF-dependent mechanism.

A number of complement-derived products have been shown to play important roles in mediating neutrophil adhesion. The cleavage product iC3b may influence neutrophil adhesion in both an indirect and direct fashion. Indirectly, iC3b has been shown to increase the synthesis of interleukin-1 (IL-1), an event thought to ultimately result in the upregulation of ICAM-1 (16). Furthermore, Marks et al. (15) have shown that iC3b can directly mediate neutrophil adhesion by acting as a ligand for the CD11b/CD18 complex on the neutrophil. While the ability of C5a to promote neutrophil chemotaxis and activation has been studied in detail (17), its ability to act directly upon endothelial cells to promote adhesion has only recently been investigated. Foreman, et al. (18) recently reported that C5a can directly promote P-selectin expression in HUVECs and a subsequent increase neutrophil adhesion. As mentioned previously, Hattori, et al. (25) utilized antibody sensitized endothelial cells and whole serum to demonstrate the ability of the MAC to upregulate P-selectin. A limitation to this approach is that formation of complement-derived products (e.g., iC3b and C5a) could modulate or conceal MAC-mediated adhesive interactions. In light of recent evidence for the actions of the other complement-derived components in mediating adhesion and adhesion molecule expression, we utilized purified components to assemble the MAC in order to examine the ability of the MAC to modulate P-selectin expression and neutrophil adhesion.

In physiologic settings, formation of C5b is the result of a specific cleavage of the C5 molecule into C5a and C5b. Once cleaved, a C6 binding site is created on the C5b fragment, allowing for formation of the C5bC6 complex. If the newly formed C5b molecule is not rapidly complexed with C6, the binding site for C6 is lost, rendering the C5b molecule inactive (26). In order to overcome this difficulty, we created a stable, C5b-like molecule through treatment of purified C5 with the oxidizing agent chloramine-T (19). Treatment with chloramine-T does not promote cleavage of C5, but oxidizes methionine residues, leading to the formation of a C6 binding site (19). The advantages of this approach are several. Of primary importance is the increased stability of the C5b-like molecule. Whereas, the half-life of the native C5b molecule is brief (min), the modified C5C6 molecule has been shown to only lose 15% of its activity after one h at 37°C (20). Furthermore, the use of chloramine-T-treated C5 eliminates the need for model systems that require activation of the entire complement cascade, thereby reducing the extraneous effects mediated by other complement-derived products (e.g., C5a, iC3b).

Endothelial cell adhesive interactions are modulated through the regulation of several specific cell surface adhesion molecules (1). Several lines of evidence presented here suggest that P-selectin plays a major role in promot-

ing increased neutrophil adhesion following non-lytic (as determined by LDH release) MAC deposition. The rapid induction of adherence (within 30 min) is consistent with that reported for P-selectin-mediated adherence, which has been shown to occur between 5 and 30 min following activation of endothelial cells with either thrombin or histamine (5). Other adhesion molecules including ICAM-1 and E-selectin, require 4–6 h for full expression (27,28) suggesting that these molecules play a limited role in the early events of neutrophil-endothelial cell adhesion. Furthermore, the ability of the anti-P-selectin antibody Throm/6 to completely inhibit the MAC-mediated neutrophil adhesion provides strong evidence that P-selectin is a major mechanism by which the MAC promotes adhesion. The increased expression of P-selectin on endothelial cells was further substantiated by a whole cell ELISA assay demonstrating increased expression of P-selectin 30 min following deposition of the MAC. These data provide compelling evidence as to the role of P-selectin in mediating neutrophil adhesion following formation of the MAC.

The reduced adhesion of neutrophils pretreated with the PAF receptor antagonist CV 3988 provides evidence that PAF also plays a role in neutrophil adhesion following MAC deposition. A number of observations support a role for PAF in mediating neutrophil adhesion: 1) PAF is synthesized by endothelial cells following stimulation by a number of agents including thrombin, histamine and H_2O_2 (5); 2) desensitization of the PAF receptor on the neutrophil leads to decreased adhesion (5); 3) there is a concentration-response relationship between the amount of PAF and the degree of neutrophil binding (5). Lorant, et al. (29) have suggested that PAF and P-selectin act in concert to adhere the neutrophil to the endothelium before the establishment of a longer-lasting adhesion. In this scenario, P-selectin acts to "tether" the neutrophil in place while PAF serves to activate the neutrophil. Thus, the cooperative interactions of P-selectin and PAF may not only adhere and activate neutrophils, but allow for enhanced leukcoyte-endothelial cell interactions.

The mechanism by which deposition of the MAC promotes PAF synthesis remains to be determined. A number of investigators have observed that calcium-mediated activation of phospholipase A2 initiates PAF synthesis, suggesting that pathological conditions associated with increased intracellular Ca^{2+} may lead to increased PAF production (30,31). Deposition of the MAC has been associated with increased levels of intracellular Ca^{2+} and synthesis of lipids (32). Cybulsky, et al. (33) have provided evidence for a MAC-induced, Ca^{2+}-dependent activation of phospholipase by glomerular epithelial cells. In light of this evidence, one can speculate that assembly of the MAC on endothelial cells promotes a rapid influx of Ca^{2+} that in turn serves to increase PAF synthesis by activating phospholipase A2.

The specific binding of C5a to an endothelial receptor has been shown to promote neutrophil adhesion through a P-selectin dependent mechanism (18), rais-

ing the possibility that the increased adhesion may be mediated by this pathway. However, it should be emphasized that the oxidation of C5 by chloramine-T does not result in cleavage of C5, thereby preventing formation of C5a (19). Thus, it is unlikely that the adhesion of neutrophils is influenced by the actions of C5a. The possibility does exist that the MAC is acting directly as an adhesion molecule. Biesecker (34) has noted that L8 myoblasts exhibited the ability to adhere and spread to a substratum coated with SC5b-9. However, this adhesion was dependent on the binding of S-protein (vitronectin) to the C5b-9 complex. In the present study, S-protein was not present, making it unlikely that the MAC is acting directly as an adhesion molecule. In addition, a monoclonal antibody to the MAC neoantigen did not decrease the degree of neutrophil adhesion in the present study (results not shown).

The observation that deposition of the MAC on endothelial cells promotes increased rapid neutrophil adhesion suggests that the MAC may be involved in the recruitment of neutrophils to the site of an acute inflammatory reaction. The ability of the MAC to upregulate P-selectin and induce PAF may advance an explanation as to the ability of neutrophils to rapidly adhere to the endothelium following complement activation. Thus, deposition of the MAC may not only directly induce cell injury, but also act indirectly by promoting the expression of adhesion molecules and thereby increasing the degree of neutrophil accumulation at sites of acute inflammation.

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