The Secretory Release Reaction Initiated by Complement Proteins C5b-9 Occurs Without Platelet Aggregation Through Glycoprotein IIb-IIIa

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The secretory and aggregation responses of stirred platelets exposed to complement proteins C5b-9 was investigated. C5b-9 assembly on the platelet surface resulted in the release of dense granule adenosine triphosphate (ATP) accompanied by a decrease in sample turbidity, but no detectable cell lysis. Inhibition of cellular protein kinase C completely blocked the C5b-9 initiated release of ATP, confirming the secretory nature of this response. Addition of fibrinogen (up to 1 mg/mL) had no effect on either the release of ATP or the decreased turbidity observed for C5b-9 cells. Addition of the peptides Arg-Gly-Asp-Ser (RGDS) and fibrinogen γ-chain carboxyl-terminal (γ397-411) at concentrations sufficient to completely block fibrinogen binding to GP IIb-IIIa had no effect on either C5b-9 induced dense granule secretion or the associated turbidity change. Microscopic examination and electronic particle counting of the stirred platelet suspensions confirmed that no aggregation of C5b-9 platelets occurred even when these cells were stirred in the presence of fibrinogen. The capacity of the C5b-9 proteins to initiate platelet secretion without activation of cell surface glycoprotein (GP) IIb-IIIa suggests a mechanism for intravascular dissemination of activated platelets during complement activation in vivo.

Although membrane assembly of the C5b-9 proteins has been shown to activate secretory release of platelet storage granules and to express the procoagulant properties of this cell, it remains unresolved whether these proteins concomitantly initiate platelet adherence and aggregation, responses that are mediated, in part, through expression of the activated complex of membrane glycoproteins IIb and IIIa (GP IIb-IIIa), the cell-surface receptor for fibrinogen and other adhesive plasma proteins. This question is of particular interest in light of recent observations that indicate that partially activated platelets circulate in the blood of patients with acute respiratory distress syndrome and during extracorporeal circulation, conditions associated with intravascular complement activation. In this article we present evidence that platelets exposed to C5b-9 proteins while stirred in the presence of fibrinogen are stimulated to secrete but not to aggregate, providing a possible explanation for the persistence of activated platelets in the circulation after complement activation.

MATERIALS AND METHODS

Materials. ADP, adenosine triphosphate (ATP), apyrase, bovine serum albumin (globulin and fatty acid free), phorbol 12-myristate 13-acetate (PMA), and D-sphingosine sulfate (sphingosine) were obtained from Sigma Chemical Co (St Louis); peptide Arg-Gly-Asp-Ser (RGDS) was synthesized by Dr Kenneth Jackson (St. Francis of Tulsa Research Institute, Oklahoma City); fibrinogen γ-chain carboxyl-terminal pentadecapeptide (γ397-411) was from Bachem Inc (Torrance, CA); luciferase-luciferin reagent was from Chrono-Log Corp (Havertown, PA); and bovine thrombin was a gift from Dr Charles T. Eason (Oklahoma Medical Research Foundation, Oklahoma City). All other chemicals were of reagent or analytical grade.

Solutions. Solution 1 consisted of 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl₂, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 0.1% bovine serum albumin, and 5 mmol/L PIPES, pH 6.8. Solution 2 consisted of 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl₂, 0.5 mmol/L sodium phosphate, 0.1% glucose (wt/vol), 1% bovine serum albumin, and 5 mmol/L HEPES, pH 7.4.

Platelets. Venous blood from aspirin-free healthy adult volunteers was drawn into 1/3 volume of NIH formula A acid-citrate-dextrose solution (ACD-A). Platelet-rich plasma was prepared by centrifugation for 20 minutes at 200 g, and 0.1 U/mL apyrase was added. Platelets were concentrated by centrifugation (15 to 20 minutes, 500 g) onto a 36% (wt/vol) albumin cushion adjusted to...
PLATELET ACTIVATION BY COMPLEMENT C5a-9

295 mOsm, by a modification of the methods of Timmons and Hawiger,9 and then gel-filtered on Sepharose CL-2B (2.5 x 16 cm) equilibrated in solution 1. Platelets prepared by these methods were determined to be essentially free of contaminating plasma C8 (<0.5 ng/10^9 platelets), fibrinogen (<0.005% of plasma-derived protein), or von Willebrand factor (vWF; none detected by criterion of ristocetin-induced aggregation).10 For certain experiments, platelets were pretreated with sphingosine (100 μmol/L, five minutes, 37°C) to inhibit activation of protein kinase C, according to published methods.5,11

Complement proteins. Human complement proteins C5b6, C7, C8, and C9 were purified and analyzed for functional activity according to methods previously described.1

C5b67 platelets. To assemble membrane-bound C5b67 complexes, gel-filtered platelets were suspended with C5b6 (4 μg/10^9 platelets, or as otherwise indicated) and C7 (1 μg/10^9 cells) in a total volume of 50 to 100 μL. After incubation for three minutes at 37°C, the C5b67 platelets were recovered and used immediately.

Fibrinogen. Human fibrinogen was purified and assayed according to methods previously described.10 Coagulability of the fibrinogen by purified thrombin consistently exceeded 95%.

Platelet aggregometry. Platelet aggregometry and ATP measurements were performed with a Model 400 Lumi-Aggregometer (Chrono-Log Corp, Havertown, PA). All measurements were performed in stirred siliconized glass cuvettes maintained at 37°C. Stirring was routinely maintained at 1,200 rpm. In each experiment, 5 x 10^9 C5b67 platelets (or, matched controls) were suspended in 0.5 mL solution 2 containing 2 μg C9 and 1 mg luciferase-luciferin reagent (Chrono-log). After one minute of pre-equilibration, C5b-9 assembly was initiated by addition of C8 (0 to 2 μg) from 0.5 mg/mL stock solution. In certain experiments, fibrinogen (usually, 200 μg/mL) was added to the cell suspension before C8 addition. For studies with sphingosine-inhibited platelets, the cells were always maintained in 100 μmol/L sphingosine. Comparison was made to platelets activated by either thrombin (0.5 U/mL or as otherwise indicated), ADP (usually, 20 μmol/L) or PMA (100 nmol/L).12 Decreased turbidity was monitored as a downward deflection of the transmittance channel (upper tracings), and ATP release monitored by increased luminescence (upward deflection; lower tracings). The luminescence channel was calibrated in each experiment by addition of known concentrations of ATP.

Platelet counting. Platelets were manually counted without dilution using a hemocytometer under phase contrast light microscopy (Leitz, Dialux 20, magnification x400), and electronically counted using a Coulter Model ZBI interfaced to a Cell-Dyn 1000 Channel-yzer. Electronic counting was performed after dilution to approximately 10^10/μL. The lower threshold of the particle counter was adjusted to exclude C5b-9 platelet-derived microparticles from the platelet count.1 Cells were examined and counted within five minutes after removal from the aggregometer cuvette. Results obtained by these two methods did not significantly differ for either C5b-9 or control platelets. In certain experiments, platelets were fixed by the addition of nine parts of 0.5% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA) according to Verhoeven et al.11 Identical results were obtained for both fixed and unfixed samples (C5b-9 and controls) counted either microscopically or electronically.

RESULTS

The aggregation of platelets exposed to the terminal complement proteins was first assessed by monitoring changes in light transmittance associated with membrane assembly of C5b-9 complexes (Fig 1). C5b67 complexes were first deposited on the cell surface by incubation of gel-filtered platelets with C5b6 + C7, and these cells then suspended in a stirred cuvette at 37°C for C8 and C9 additions (see Materials and Methods). As illustrated in Fig 1A, addition of increasing C8 to C5b67 platelets (suspended in the presence of fibrinogen and excess C9) resulted in a slow C8 dose-dependent increase in light transmittance that paralleled secretion for dense granules. No lysis (measured by release of cytoplasmic LDH) was observed under these conditions. Also, no change in light transmittance (or ATP release) was observed when C8 (1 μg) was added to control cells not exposed to the C5b67 proteins. The secretory nature of the ATP release was confirmed by blocking α and dense granule secretion with the protein kinase C inhibitor sphingosine.5,11 As illustrated in Fig 1B, sphingosine completely abolished ATP release but did not affect the changes in light scattering observed with C5b-9 assembly. These data suggest that the release of ATP occurs through dense granule secretion and not through diffusional efflux of cytoplasmic ATP. These results are also consistent with our previous data relating to secretion from platelet α granules induced by the C5b-9 proteins.5

As illustrated in Fig 2A, the change in light transmittance observed after C5b-9 assembly was unaffected by the concentration of fibrinogen in the suspending medium (cf, data for ADP-stimulated platelets). Furthermore, when subsequently challenged with ADP, C5b-9-treated platelets showed a large decrease in turbidity, equivalent to the aggregation response observed for ADP-stimulated platelets not exposed to the complement proteins (Fig 2B). These results raised the possibilities that C5b-9-induced secretion but only partial platelet aggregation (eg, due to incomplete expression of activated GP IIb-IIIa), or that the change in sample turbidity observed for these cells in the absence of a second stimulus was unrelated to either expression of fibrinogen receptors or platelet aggregation per se (see below).

As noted above, the small decrease in turbidity observed on C5b-9 assembly suggested either (a) the formation of small platelet aggregates, reducing the number of individual scattering particles in the sample, or (b), a change in the scattering properties of the individual cells, arising from shape change or degranulation, independent of aggregation. In order to determine whether platelet micro-aggregates were formed after C5b-9 assembly, aliquots of the cell suspension were removed from the measuring cuvette at various times after C8 addition and the cells counted and microscopically examined without dilution. As shown in Fig 3, the number of individual platelets was unaffected by C5b-9 assembly and there was no increase in the number of micro-aggregates detected by phase contrast light microscopy. Identical results were obtained regardless of whether C5b-9 assembly was performed in the presence or absence of added fibrinogen (cf, Fig 2A). By contrast, activation of GP IIb-IIIa by addition of ADP in the presence of added fibrinogen resulted in gross clumping of the cells with removal of platelets from suspension, which was readily detected by decreased turbidity, a decrease in platelet count, and the appearance of numerous platelet micro-aggregates (Figs 2 and 3A). In the experiment shown in Fig 3A, submaximal ADP (4 μmol/L) stimulation was used to
GP IIb-IIIa, experiments were performed in the presence of the fibrinogen-derived peptides RGDS and γ-chain (γ397-411). These peptides have been shown to interact with GP IIb-IIIa in a competitive fashion with the fibrinogen α and γ chains, and to inhibit binding of both fibrinogen and vWF to this receptor complex. As illustrated in Fig 4, addition of these peptides at concentrations sufficient to completely block either platelet aggregation or binding of fibrinogen to its receptor abolished the transmittance change observed on activation of platelet GP IIb-IIIa by ADP, but had no effect on either the C5b-9-induced secretory response or the concomitant changes in light transmittance (cf, Figs 4A and 4B). In the presence of both RGDS and sphingosine, dense granule secretion was completely blocked, but the C5b-9 induced change in turbidity was virtually unaffected (Fig 4C). In order to confirm that the light transmittance change observed for C5b-9 platelets was consistent with activation of dense granule secretion independent of cell aggregation or fibrinogen binding to its receptor, platelets were stimulated to secrete with PMA in the presence of concentrations of RGDS sufficient to block fibrinogen binding and platelet

achieve a transmittance change equivalent to that observed for C5b-9 platelets (cf, Figs 3B and 3C).

These data suggested that the secretory response and light transmittance change observed on C5b-9 assembly occurred without platelet aggregation, and raised the possibility that the GP IIb-IIIa receptor complex is not activated under these circumstances. In order to explore whether the observed platelet response to the C5b-9 proteins was affected by low-level binding of secreted fibrinogen to partially activated
aggregation (Fig 4D). As illustrated in Fig 4, when cell aggregation is blocked by RGDS, PMA-stimulated platelets also exhibit an activation-associated change in light transmittance that completely mimics the platelet response to C5b-9.

The input of C5b-9 proteins used for these experiments (chosen to avoid detectable cell lysis; see Materials and Methods) generally resulted in release of 30% to 60% of the dense granule storage pool (see Table 1). This raised the possibility that the amount of ADP secreted by C5b-9 platelets was insufficient to provide feedback to ADP receptors required for GP IIb-IIIa activation and platelet aggregation. In order to address this possibility, we investigated the quantitative relationship between dense granule secretion and platelet aggregation, for cells submaximally stimulated by either C5b-9 or thrombin (Table 1). Partial secretion of the dense granule storage pool induced by low level concentrations of thrombin always resulted in detectable platelet aggregation (monitored by both light microscopy and decreased platelet counts). By contrast, no aggregation was observed when comparable levels of secretion were induced by exposure to the C5b-9 proteins.

DISCUSSION

The data of the present study indicate that membrane assembly of the C5b-9 proteins results in secretory release of the dense granule storage pool without platelet aggregation, and that this response occurs independently of any potential interaction of fibrinogen with its cell surface receptor. Combined with the results we have obtained by direct measurement of fibrinogen binding to C5b-9 platelets in an unstirred system, these data indicate that functionally competent GP IIb-IIIa complexes are not expressed in platelets stimulated to secrete by the C5b-9 proteins. This is, to our knowledge, the first demonstration of a naturally occurring platelet agonist that can induce secretion from the storage granules without activation of the platelet’s aggregation response. Since C5b-9 activated platelets also express de novo membrane receptors for coagulation factors Va and Xa and can actively catalyze thrombin generation through the cell surface prothrombinase, these results raise the possibility that when deposited on platelets in vivo, the C5b-9 proteins can initiate episodes of disseminated intravascular coagulation by generating procoagulantly active but nonadherent platelets with the capacity to persist within the circulation.

In addition to potential implications relating to the role of these proteins in the pathogenesis of thrombotic vascular disease, the capacity of the C5b-9 proteins to induce secretion of the platelet granule storage pools without activation of the aggregation response raises several questions relating to the mechanism of fibrinogen receptor expression through the normal receptor-coupled stimulatory pathways. Activation of platelet GP IIb-IIIa has been linked to elevated intracellular Ca²⁺ and to the activation of protein kinase C (or other cellular protein kinases), mediated through receptor-coupled stimulation of phospholipase C. On the other hand, both ADP and epinephrine can stimulate fibrinogen receptor expression without stimulating phospholipase C catalyzed hydrolysis of phosphatidylinositol polyphosphates (PI), and without activating protein kinase C or causing granule secretion. It has also been reported that fibrinogen binding to thrombin-stimulated platelets can be blocked by inhibitors of the ADP receptor, implying a requirement for feedback of secreted ADP, even in the circumstance that
PI hydrolysis and protein kinase C activation has been initiated. Finally, Kunicki et al have noted that secretory release from α granules without fibrinogen binding can be achieved by activating platelets with calcium ionophore in the presence of prostaglandin E, implying participation of cyclic AMP-dependent kinases in regulating expression of functional GP IIb-IIIa.

In the case of C5b-9, secretion of dense granule ADP did not lead to functional expression of fibrinogen receptors, even though these cells aggregated in response to a subsequent challenge by exogenous ADP (Table 1 and Fig 2B). These results suggest that either (a) the transient electrochemical and metabolic changes that are initiated on insertion of the C5b-9 pore render the cell temporarily refractory to feedback activation by secreted ADP (see references 5 and 19), or (b) the local concentration of secreted ADP is insufficient to induce receptor expression in the absence of another synergistic stimulus. In this context it should be emphasized that although C5b-9 raises intracellular Ca⁺⁺ and causes activation of protein kinase C (and other cellular kinases), receptor initiated hydrolysis of PI by phospholipase C is not observed under these conditions. One can speculate that by dissociating these two cellular responses (secretion and fibrinogen receptor expression), the C5b-9 proteins may provide a unique tool to probe the metabolic and electrochemical events required for activation of the GP IIb-IIIa receptor complex.

Finally, comparison of the data in Figures 1 through 4 underscores the potential for misinterpretation of platelet activation reactions using turbidimetric determinations of platelet aggregation. These data demonstrate that the optical changes arising from platelet activation and degranulation can mimic those produced by partial cell aggregation, even in the circumstance that no detectable platelet aggregation has occurred. These data confirm recent observations of Pat-scheke et al, which suggested that light scattering changes arising during α-granule secretion can cause a change in transmission similar to that produced by platelet aggregation. In their experiments, platelets were stimulated to undergo shape change and degranulation in the presence of EDTA, to inhibit fibrinogen binding to cell surface GP

### Table 1. Comparison of Platelet Secretion to Platelet Aggregation

<table>
<thead>
<tr>
<th></th>
<th>ATP Released (µmol/L)</th>
<th>Platelet Count (x 10⁷/mL)*</th>
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</thead>
<tbody>
<tr>
<td>Untreated</td>
<td>0.00</td>
<td>9.18 ± 0.05</td>
</tr>
<tr>
<td>C5b-9 (µg)†</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.6 µg C8</td>
<td>1.24</td>
<td>9.35 ± 0.08</td>
</tr>
<tr>
<td>0.8 µg C8</td>
<td>0.90</td>
<td>9.56 ± 0.12</td>
</tr>
<tr>
<td>0.5 µg C8</td>
<td>0.70</td>
<td>9.28 ± 0.20</td>
</tr>
<tr>
<td>C5b-9‡</td>
<td>0.34</td>
<td>9.79 ± 0.02</td>
</tr>
<tr>
<td>Thrombin (U)§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.025</td>
<td>2.27</td>
<td>Clumped</td>
</tr>
<tr>
<td>0.013</td>
<td>1.47</td>
<td>4.03 ± 0.02</td>
</tr>
<tr>
<td>0.010</td>
<td>0.56</td>
<td>6.78 ± 0.13</td>
</tr>
<tr>
<td>0.009</td>
<td>0.63</td>
<td>6.18 ± 0.17</td>
</tr>
<tr>
<td>0.008</td>
<td>0.33</td>
<td>8.69 ± 0.05</td>
</tr>
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*Platelets were treated with 0.5% glutaraldehyde and then counted as described in Materials and Methods (mean ± SD, N = 3).
†Twenty micrograms of C5b-6, 1 µg of C7, and 4 µg of C9 per 10⁸ platelets plus amounts of C8 indicated.
‡Seven micrograms of C5b-6, 1 µg of C7, 4 µg of C9, and 2 µg of C8 per 10⁸ platelets.
§Secretion from platelets maximally stimulated by 0.5 unit of thrombin averaged 2.34 ± 0.35 µmol/L ATP.
||P < .001 (v untreated). Platelet aggregates were confirmed by phase-contrast microscope in fixed samples.
IIb-IIIa. Although these investigators conclude that the light scattering changes they observed reflect secretion from internal granules, the results we obtain for C5b-9 platelets inhibited by both sphingosine and RGDS (Fig 4C) suggest that the complement-induced change in light scattering occurs independently of either aggregation or degranulation, and may reflect plasma membrane vesiculation and cell shape changes that are known to occur secondary to the C5b-9 induced rise in cytosolic Ca\(^{2+}\).

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REFERENCES

The secretary release reaction initiated by complement proteins C5b-9 occurs without platelet aggregation through glycoprotein IIb-IIIa

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