Complement Proteins C5b-9 Stimulate Procoagulant Activity Through Platelet Prothrombinase

By Therese Wiedmer, Charles T. Esmon, and Peter J. Sims

The capacity of platelets treated with nonlytic concentrations of the C5b-9 proteins to catalyze prothrombin activation and thereby trigger clot formation has been investigated. When suspended in the presence of exogenous factors Xa and Va, gel-filtered platelets treated with purified C5b-9 proteins catalyzed prothrombin to thrombin conversion at rates up to tenfold above controls, and exceeded by up to fourfold the prothrombinase activity observed for thrombin-stimulated platelets. In the absence of added factor Va, C5b-9 assembly on the platelet surface significantly shortened the lag period before prothrombinase expression that was observed for untreated platelets and increased the maximum catalytic rate of thrombin formation. A comparison with other platelet stimuli revealed that the C5b-9-induced activation of platelet prothrombinase closely paralleled the effects mediated by calcium ionophore A23187. Our data suggest that the C5b-9 proteins promote the release of platelet factor V and the assembly of the prothrombinase complex, thereby potentiating the effects of thrombin on the activation of prothrombinase. Membrane assembly of the C5b-9 proteins was also observed to markedly accelerate the rate of platelet-catalyzed plasma clotting, suggesting a direct link between C5b-9-mediated prothrombinase activation and procoagulant activity accompanying immunologic damage to the platelet.

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MATERIALS AND METHODS

Materials. Prostaglandin E1 (PGE1), nystatin, and bovine serum albumin (fatty acid–free) were purchased from Sigma, St Louis, and A23187 was obtained from Calbiochem, San Diego. Spectrozyme TH was from American Diagnostica, Greenwich, Conn. All other chemicals were of reagent or analytical grade.

Solutions. Medium I: 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% (wt/vol) bovine serum albumin, 25 mmol/L PGE1, and 5 mmol/L PIPES, pH 6.8. Medium II: 145 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl2, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 1% (wt/vol) bovine serum albumin, and 5 mmol/L HEPES, pH 7.4. Medium III: Medium II containing 2.5 mmol/L CaCl2.

Platelets. Freshly prepared platelet-rich plasma from normal volunteers was obtained through the facilities of the Oklahoma Blood Institute. PGE1 was added at a final concentration of 25 mmol/L. Except where indicated, platelets were kept at room temperature and plastic was used throughout. After an initial low-speed spin to remove contaminating red cells (four minutes, 200 g), the platelets were concentrated by centrifugation (20 minutes, 500g). After careful suspension in 0.5 to 1 mL of Medium I, the platelets were gel-filtered on a column (0.9 × 30 cm) of Sepharose CL-2B (Pharmacia, Piscataway, NJ) equilibrated in Medium I. Cell counting and resistive sizing were performed using Models ZBI and Channelizer (C 1000), Coulter Electronics, Hialeah, FL.

Coagulation proteins. Bovine factor Va, factor Xa, prothrombin, and human and bovine thrombin were isolated by minor modifications of the methods cited above.

Complement proteins. Human complement proteins C5b6, C7, C8 and C9 were purified and analyzed for functional activity as previously described. Before addition to platelet suspensions, the proteins were dialyzed into Medium II.

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various amounts of C8 as indicated in the text. iig and 5 g, respectively, per 1 x 10 cells) with rapid mixing in a purified C8. incubated for 10 minutes at 37 °C in presence of4 g/mL ofC9 and platelets treated with complement proteins, thrombin, or ionophores) were the C5b67 platelets were recovered and used immediately. 100 .tl.plexes, gel-filtered platelets were suspended with C5b6 and C7 (15 solution containing 10 mmol/L EDTA, 10 mmol/L TES, 150 of the reaction mixture were removed into 450 iL of an ice-cold absorbance at 405 nm thrombin activity was calculated using imol/L Spectrozyme TH as substrate. From the rate of change in was assayed in a medium containing 10 mmol/L TES, generated was assayed in a medium containing 10 mmol/L TES, 150 mmol/L NaCl, and 1% (wt/vol) albumin, pH 7.5. Thrombin generated was initiated by addition of factor Xa (final concentration 1 nmol/L), and at the times indicated in figure legends, 50-µL aliquots of the reaction mixture were removed into 450 µL of an ice-cold solution containing 10 mmol/L EDTA, 10 mmol/L TES, 150 mmol/L NaCl, and 1% (wt/vol) albumin, pH 7.5, and 100 µmol/L Spectrozyme TH as substrate. From the rate of change in absorbance at 405 nm thrombin activity was calculated using purified thrombin as a standard. 

Clotting assays. Gel-filtered C5b67 platelets (untreated, or treated with complement proteins, thrombin, or ionophores) were suspended at 37 °C in Medium III at 8 x 106/mL in the presence of 2.7 µmol/L prothrombin, and, either 0 or 2 nmol/L factor Va. The reaction was started by addition of factor Xa (final concentration 1 nmol/L), and at the times indicated in figure legends, 50-µL aliquots of the mixture were removed into 450 µL of an ice-cold solution containing 10 mmol/L EDTA, 10 mmol/L TES, 150 mmol/L NaCl, and 1% (wt/vol) albumin, pH 7.5. Thrombin generated was assayed in a medium containing 10 mmol/L TES, 150 mmol/L NaCl, 0.1% (wt/vol) albumin, pH 7.5, and 100 µmol/L Spectrozyme TH as substrate. From the rate of change in absorbance at 405 nm thrombin activity was calculated using purified thrombin as a standard. 

Clotting assays. Gel-filtered C5b67 platelets (and matched-pair controls) were prepared as described above and each suspended to 4 x 104/mL in Medium II. In the first stage of the assay, 0.1 mL of platelets (4 x 104 cells) were mixed with 0.1 mL of platelet-poor plasma (PPP), C8-deficient PPP (C8D-PPP), or C8D-PPP reconstituted with various amounts (0 to 10 µg) of C8. After incubation for three minutes at 37 °C (to allow C8/C9 binding) clotting was initiated by addition of 0.1 mL each factor Xa (4 x 10−10 mol/L) and CaCl2 (25 mmol/L) stock solutions at 37 °C (final volume, 0.4 mL). 

Lactate dehydrogenase release. Platelet lysis was estimated from the release of cytoplasmic lactate dehydrogenase (LDH) determined under conditions identical to those used in the assay for platelet prothrombinase activity or platelet factor 4 release (below). Enzyme assay was performed according to Wroblewski and LaDue, with total LDH obtained by lysing platelets in 0.1% Triton X-100 (Calbiochem, La Jolla, Calif). Platelet factor 4 release. Platelet α-granule release was quantitated by platelet factor 4 (PF4) radioimmunoassay (Abbott Laboratories, North Chicago, Ill). 4 x 107 gel-filtered platelets (controls or C5b67-treated) were suspended at 37 °C in 0.5 mL Medium III in the presence of either 120 ng C8 + 1.6 µg C9, 0.5 µmol/L, or 2.5 µmol/L A23187. After ten minutes, 25 µL thrombocyte reagent (Abbott) was added, the samples transferred to an ice-water bath (30 minutes), and centrifuged (2500 g) for 20 minutes. The supernatants were saved and immediately assayed for PF4 and LDH. 

**RESULTS**

Effect of complement proteins C5b-9 on platelet procoagulant activity. It has recently been demonstrated that incubation of platelet-rich plasma with zymosan leads to membrane deposition of C3b and to enhanced platelet prothrombinase suggesting a role for serum complement in the modulation of platelet procoagulant activity.1 In this study, we examined the specific role of the terminal C5b-9 proteins in the procoagulant activity of platelets observed after complement activation. As shown by the data of Table 1, incubation of platelets under conditions leading to membrane assembly of the C5b-9 proteins significantly increased the rate of plasma clot formation. The enhanced procoagulant activity observed for C5b67 platelets suspended in autologous plasma (providing a source of C8 and C9 for membrane assembly of C5b-9) was completely reversed in C8-depleted plasma (Table 1 and Fig 1), confirming that accelerated plasma clotting required binding of the terminal complement proteins (at least through C8) to the platelet surface, and was not due to effects of the added C5b67 proteins per se. As shown by the data of Fig 1, the procoagulant activity observed for C5b67 platelets was restored in a dose-dependent fashion by addition of C8 to C8D-PPP, plasma clotting times approaching that observed for these cells in normal plasma when C8 was added to C8D-PPP in amounts equivalent to the normal plasma concentration of the protein (55 µg/mL). By comparison, clotting times observed for control platelets suspended under these conditions were unaffected by changes in the plasma concentration of C8 (Table 1).

Effect of C5b-9 on platelet prothrombinase. The capacity of the C5b-9 proteins to stimulate assembly of the platelet prothrombinase was investigated in a plasma-free system. C5b67 platelets (or controls) were incubated at 37 °C in the presence of factors Va and Xa, prothrombin, and excess C9, and at the time indicated C8 was added to initiate the assembly of the C5b-9 complex. As illustrated by the data of Fig 2, addition of C8 to the C5b67 platelets (suspended with excess C9) resulted in a rapid increase in the rate of thrombin production. In addition to increasing the platelet’s capacity to assemble the prothrombinase, treatment with the C5b-9 proteins (in the absence of Xa, Va, or prothrombin) also resulted in a partial release of platelet factor 4 without causing cell lysis (Table 2), suggesting that prothrombinase activity observed after membrane binding of these proteins may be related to a triggered secretion of α-granule contents. By contrast, prothrombinase activity and PF4 release of control cells was unaffected by C8 and C9 additions. In some experiments, prothrombinase activity of C5b67 cells suspended in the absence of C8 was slightly increased compared to complement-free controls, suggesting either (a) a small effect of membrane-bound C5b67 on the rate of prothrombinase or (b) the presence of trace amounts of contaminating C8 associated with the gel-filtered platelets.

In Fig 3, the effect of the preformed C5b-9 complex on prothrombinase activity was measured both in the presence and absence of added factor Va. When no exogenous Va was
Fig 1. Effect of complement proteins C5b-9 on platelet procoagulant activity. Gel-filtered C5b67-platelets were prepared as described in Materials and Methods and suspended to 4 x 10^9/mL in Medium II. After incubation in C8D-PPP reconstituted with various amounts (0 to 10 ng) of C8, plasma clotting times at 37 °C were measured as described in Materials and Methods.

present (Fig 3A), control and C5b67 platelets produced very small amounts of thrombin, with a lag period of several minutes. Assembly of C5b-9 on platelets augmented prothrombinase activity in a dose-dependent fashion (compare Fig 1) and the lag period before thrombin formation was shortened with increasing input of C8. Following this initial lag phase, maximal rates of prothrombin conversion approaching tenfold control levels were observed at the maximal input of C8 (120 ng per 4 x 10^9 C5b67 platelets) employed in these experiments. Supplementation with factor Va (Fig 3B) resulted in near-linear rates of prothrombin conversion (observed for both control and C5b-9–treated cells) detected immediately after factor Xa additions (ie, no lag phase was apparent). Under these conditions, maximal rates of prothrombin conversion approaching tenfold that of controls were again observed for the C5b-9–pretreated platelets. Under conditions depicted in Fig 3, no cell lysis was detected, even at the highest input of C8 (120 ng per 4 x 10^9 platelets). Incubation of C5b67 platelets with greater amounts of C8 (above 120 ng per 4 x 10^9 cells; not shown) increased the rate of thrombin generation above that shown in Fig 3, but also resulted in a detectable increase in cell lysis above that observed for controls.

Comparison of C5b-9 with other platelet stimuli. The influence of the C5b-9 proteins on the platelet prothrombinase observed under conditions described for Fig 3 was compared to the effect mediated by thrombin and the Ca^2+ ionophore A23187, two potent platelet activators (Fig 4). Platelets were preincubated (ten minutes, 37 °C) with 1 U/mL thrombin, or 1 μmol/L A23187 to stimulate platelet activation and release reactions, and then tested for their capacity to assemble the prothrombinase. In the presence of exogenous factor Va (B), incubation with 1 U/mL bovine or human (not shown) thrombin elevated prothrombinase activity slightly over that of control platelets, whereas treatment of cells with nonlytic amounts of the C5b-9 proteins resulted in a fivefold increase in the rate of thrombin formation, which was comparable to the response observed after exposure of the platelets to 1 μmol/L A23187 under these conditions. It should be noted that increased rates of prothrombin conversion were observed when the concentrations of C8, thrombin, or A23187 were increased above levels employed in experiments described for Fig 4. Because higher concentrations of these agents also resulted in increased cell lysis (above levels detected for untreated controls), only data for cells treated with 1 U/mL thrombin and 1 μmol/L

Table 2. Platelet Factor 4 Release

<table>
<thead>
<tr>
<th>Platelets</th>
<th>PFI4 Release (ng/10^5 cells)</th>
<th>Lysis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>15 ± 6*</td>
<td>0.40</td>
</tr>
<tr>
<td>Control + C8/C9</td>
<td>17 ± 5</td>
<td>0.51</td>
</tr>
<tr>
<td>C5b67</td>
<td>23 ± 4</td>
<td>0.32</td>
</tr>
<tr>
<td>C5b-9</td>
<td>167 ± 35</td>
<td>0.42</td>
</tr>
<tr>
<td>Thrombin</td>
<td>1400 ± 72</td>
<td>0.60</td>
</tr>
<tr>
<td>A23187</td>
<td>1300 ± 103</td>
<td>1.70</td>
</tr>
<tr>
<td>Thrombin + C5b-9</td>
<td>1225 ± 176</td>
<td>1.10</td>
</tr>
</tbody>
</table>

* Mean ± SD.
were suspended in 0.4 ml of Medium III and incubated for ten minutes at 37 °C in the presence of 1.6 μg of C9 and 0 ng (○), 30 ng (△), 60 ng (▲) or 120 ng (■) of C8. After addition of prothrombin (2.7 μmol/L) and factor Xa (1 nmol/L), prothrombinase was measured in the absence (A) or presence (B) of 2 nmol/L factor Va as described in Materials and Methods. Control platelets (■) are also shown. Release of LDH amounted to 1.2% (○), 1.1% (△) and 3.0% (■), respectively.

A23187 are shown. By comparison, thrombin generation by platelets incubated with sublytic concentrations of another channel-forming agent, the Na+/K+ ionophore nystatin, was indistinguishable from controls. In the absence of added factor Va (Fig 4A), pretreatment of platelets with 1 U/mL thrombin abolished the lag period before onset of prothrombinase activity. By contrast, treatment of the cells with either A23187 or C5b-9 shortened—but did not completely abolish—this lag period. Although the length of the lag phase before prothrombin conversion which was observed in the absence of added factor Va was found to vary from experiment to experiment, in multiple separate experiments performed under conditions described for Fig 4B a distinct lag phase was always observed for those platelets (C5b-9, ionophore-treated, or controls) not previously incubated with thrombin.

As illustrated by the data in Fig 5A, treatment of the C5b-9 cells with thrombin abolished the lag period before prothrombin conversion that was observed when these cells were assayed in the absence of exogenous Va. The effect of the individual stimuli with the same platelet suspension are also shown for comparison. Since thrombin also abolished the lag period for control platelets, it is apparent that thrombin pretreatment alone is sufficient to linearize the rate of prothrombin conversion. In Fig 5B, results are shown for the prothrombinase activity of the same platelets when measured in the presence of factor Va. As shown by the data of this figure, a relatively small increase in the rate of thrombin formation was observed when platelets were pre-treated with thrombin in addition to the C5b-9 proteins. In three experiments performed under the conditions described for Fig 5B, thrombin was generated at an initial rate of 30.7 ± 1.7 units of thrombin/10^6 platelets × min (mean ± SD) for C5b-9-treated platelets as compared to 49.4 ± 4.1 for platelets treated with both agonists.

DISCUSSION

The data of the present study demonstrate that membrane assembly of the C5b-9 proteins can markedly accelerate platelet-catalyzed thrombin generation, suggesting one mechanism by which the thrombotic reactivity of the platelet can be altered following complement activation. In this context it is of interest to note that (a) zymosan treatment of platelet-rich plasma has been shown to induce nonlytic platelet release reactions and to enhance the rate of thrombin formation via the platelet prothrombinase, suggesting that activated component(s) of the complement system alter the platelet procoagulant activity; (b) platelet aggregation and release reactions in response to either zymosan or low dose thrombin have been reported to be impaired in plasma deficient in C3, C5, C6, or C7, suggesting that the terminal complement proteins may specifically play a role in platelet
acid through the cyclooxygenase pathway. Taken together there is evidence that the C5b-9 complex spontaneously assembles on the surface of this cell during normal blood clotting. Furthermore, assembly of the purified C5b-9 proteins on gel-filtered platelets has been reported to elevate the intracellular pool of free arachidonic acid, with increased conversion of the fatty acid through the cyclooxygenase pathway. 

The capacity of the C5b-9 proteins to accelerate prothrombin conversion in the presence of saturating quantities of factor Va (Figs 2, 3B, 4B, and 5B) closely parallels the effects mediated by the calcium ionophore A23187. As discussed by Zwaal et al., elevated prothrombinase activity in cells treated with A23187 can be correlated with an increase in the amount of negatively charged phospholipids available for Va binding to the cell surface, due to transbilayer migration of phosphatidylserine from the cytoplasmic leaflet of the plasma membrane. Elevated prothrombinase activity is also observed after exposure of the cytoplasmic membrane surface due to cell lysis. Under the conditions of our experiments, enhanced prothrombinase activity was observed in response to sublytic quantities of the C5b-9 proteins (release of cytoplasmic LDH unchanged from control levels even at maximal C8 inputs; Fig 4), suggesting that the C5b-9 proteins mimic the effect of calcium ionophores, resulting in a change in the transmembrane distribution of phosphatidylserine. In this context it is of interest to note that the C5b-9 proteins have been shown to increase the Ca²⁺-permeability of the plasma membrane and to induce plasma membrane vesiculation. In addition to their effect on plasma membrane permeability, the possibility that the proteins directly promote transbilayer migration of phospholipids can also be suggested, based on the capacity of these proteins to disorder the normal lamellar configuration of lipid acyl chains.

The capacity of the C5b-9 proteins to stimulate platelet prothrombinase even in the absence of exogenous Va suggests that C5b-9 also promotes release of platelet factor V, ultimately providing catalytic amounts of the activated cofactor (Va) required for Xa binding to the platelet surface. Triggered release of factor V from the platelet's α-granules has previously been demonstrated following platelet stimulation by arachidonic acid and thrombin, as well as in response to calcium ionophores. The pronounced lag preceding thrombin generation by C5b-9-treated platelets suspended under these conditions (ie, in the absence of factor Va—Figs 3A, 4A, and 5A) suggests that factor V released from C5b-9-treated platelets is only slowly converted to Va, presumably requiring feedback of either Xa or thrombin on factor V. Because the C5b-9 proteins can apparently increase the number of membrane sites for assembly of the prothrombinase as well as promote release of platelet factor V, the net effect of their binding to the platelet surface is to markedly potentiate the effects of thrombin on the activation of the platelet prothrombinase (Fig 5). One might speculate that the increased procoagulant activity of the platelet following membrane deposition of the C5b-9 proteins may account at least in part for episodic vascular thrombosis associated with complement activation in vivo.

In summary, we have demonstrated that the C5b-9 proteins can increase the capacity of the platelet membrane to catalyze thrombin generation by prothrombinase and that this procoagulant activity associated with the terminal complement proteins can occur without lytic rupture of the plasma membrane. Furthermore, our data suggest that the effects of the C5b-9 proteins on platelet prothrombinase may be related to their capacity to act as a calcium ionophore, promoting both the secretion of platelet factor V and the assembly of the functional XaVa complex.

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