Complement-Induced Vesiculation and Exposure of Membrane Prothrombinase Sites in Platelets of Paroxysmal Nocturnal Hemoglobinuria

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Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired stem-cell disorder in which the glycolipid-anchored membrane proteins, including the cell-surface complement inhibitors, CD55 and CD59, are partially or completely deleted from the plasma membranes of mature blood cells. To gain insight into the pathogenesis of thrombosis that is frequently observed in this disorder, the procoagulant responses of PNH platelets exposed to the human terminal complement proteins C5b-9 were investigated. C5b-9 complexes were assembled on gel-filtered platelets by incubation with purified C5b6, C7, C9, and limiting amounts of C8. Platelet microparticle formation and exposure of plasma membrane-binding sites for coagulation factor Va were then analyzed by flow cytometry. PNH platelets exhibiting undetectable levels of surface CD59 antigen showed an approximately 10-fold increase in sensitivity to C5b-9-stimulated expression of membrane-binding sites for factor Va when compared with platelets from normal controls. Expression of catalytic surface for the prothrombinase complex (VaXa) paralleled the exposure of factor Va-binding sites; the rate of prothrombin conversion by C5b-9-treated PNH platelets exceeded that of C5b-9-treated normal controls by approximately 10-fold at the maximal input of C8 tested (500 ng/mL). These data indicate that PNH platelets deficient in plasma membrane CD59 antigen are exquisitely sensitive to C5b-9-induced expression of prothrombinase activity, and suggest that the tendency toward thrombosis in these patients may be due, at least in part, to the deletion of this complement inhibitor from the platelet plasma membrane.

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MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA; globulin- and fatty acid-free), prostaglandin I2, and the chromogenic substrate S2238 ([D-Phe-L-pippecolyl-Arg-p-nitroanilide] were from Sigma Chemical (St Louis, MO); fluorescein-5-isothiocyanate (FITC) was from Molecular Probes (Eugene, OR); and phycoerythrin-streptavidin conjugate was from Southern Biotechnology Associates (Birmingham, AL).
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AL). Purified human plasma factor V was prepared and activated with the factor V activator from Russell’s viper venom as previously described. Human thrombin, factor Xa, and prothrombin were from Haematologic Technologies (Essex Junction, VT). Murine monoclonal antibody (MoAb) W5, specific for membrane glycoprotein (Gp) Ib, was a gift from Dr Rodger P. McEver (University of Oklahoma Health Sciences Center, Oklahoma City, OK). The MoAb 162 is specific for CD59 (courtesy of Dr Marilyn J. Telen, Duke University, Durham, NC). The light chain of bovine factor Va and MoAb V237, specific for the light chain of factor Va, were gifts from Dr Charles T. Esmon (Oklahoma Medical Research Foundation). All other chemicals were of reagent or analytical grade.

**Solutions.** Buffer 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% (wt/vol) BSA, and 5 mmol/L PIPES, pH 6.8. Buffer 2 consisted of 137 mmol/L NaCl, 4 mmol/L KCl, 1 mmol/L MgCl₂, 1 mmol/L CaCl₂, 0.5 mmol/L sodium phosphate, 0.1% (wt/vol) glucose, 0.1% (wt/vol) BSA, 20 mmol/L HEPES, pH 7.4. Platelets. Venous blood from aspirin-free healthy adult volunteers or from patients with PNH was drawn into 1/7 vol of acid citrate dextrose solution (National Institutes of Health formula A), equilibrated with buffer 1.

**Activation of platelets by C5b-9.** Gel-filtered human platelets were suspended in buffer 2 at 3 to 6 × 10⁸/mL. Human complement proteins C5b6,18 C7,20 C8,21 and C922 were purified and analyzed for functional activity according to published procedures. To assemble membrane-bound C5b-9 complexes, gel-filtered platelets (3 to 6 × 10⁸/mL) were incubated for 3 minutes at 37°C with C5b (10 μg/10⁸ platelets) and C7 (2.5 μg/10⁸ cells). To assemble membrane-bound C5b-9 complexes, C5b-67 platelets were suspended to 10⁸ platelets/mL in buffer 2. Then, C9 (10 μg/10⁸ cells) and C8 at the concentration indicated in the legends to the figures were added, and the cells incubated for 10 minutes at 37°C. In all experiments, comparison was made to identical matched-pair controls (platelets incubated in the absence of the C5b-9 proteins).

**Labeling of MoAbs.** The MoAb V237 was labeled with FITC23 and MoAb W5 was conjugated with N-hydroxysuccinimide biotin as previously described.

**Preparation of platelets for flow cytometry.** A quantity of 2 × 10⁹ C5b-9–treated or control platelets were incubated in the dark in a total volume of 35 μL for 10 minutes at 37°C in the presence of bis-VC (2 μg/mL) and factor Va light chain (2 μg/mL). FITC-V237 (30 μg/mL) and phycoerythrin-streptavidin (5 μL of a 1:10 dilution) were added, and the cells were incubated for an additional 10 minutes. Then, 1-mL aliquots of isotonic saline (Baxter Scientific Products, McGaw Park, IL) were added and the samples analyzed by flow cytometry. For quantitation of CDS9 antigen levels, 25 μL of untreated platelet suspension (2.5 × 10⁶ platelets) was incubated for 30 minutes with 25 μL of anti-CDS9 MoAb 162 (1:1,000 diluted cell supernatant). The cells were washed twice and resuspended to the original volume, and 25 μL of FITC-labeled goat F(ab)₂ antihuman IgG (TAGO, Burlingame, CA) was added. Following incubation for 30 minutes in the dark, samples were analyzed by flow cytometry.

**Flow cytometry.** Samples were analyzed in a Becton Dickinson FACScan flow cytometer formatted for two-color analysis as described previously.15,23 In brief, the FL2 (red fluorescence) threshold was first set so as to exclude from analysis background noise and light-scattering particles that were not distinctly positive for platelet GPIb antigen (detected with phycoerythrin-labeled MoAb W5; see above). For each sample, 10,000 GPIb-positive events were recorded and analyzed for forward (FSC) and right-angle (SSC) light scatter and for FL1 (green) fluorescence, representing the binding of FITC-labeled antibody to factor Va light chain. For each experiment, two-dimensional analysis gates of forward and right-angle light scatter (FSC × SSC) were set using results for normal platelets (control and complement-activated), so as to maximally discriminate platelet microparticles from platelets.24 All data were acquired in linear mode and analyzed using CellQuest software (Becton Dickinson). Platelet prothrombinase activity was measured essentially as described.24 Briefly, C5b-9–activated gel-filtered platelets (normals and patients) were suspended at 2 × 10⁸/mL in 50 mmol/L Tris-Cl, pH 7.9, 175 mmol/L NaCl, 5 mg/mL BSA (TBS-BSA) in the presence of 2.7 mmol/L prothrombin, 2.5 mmol/L CaCl₂, and 2.0 mmol/L factor Va. The reaction was initiated by the addition of factor Xa (final concentration, 1.0 mmol/L). At times 0, 30, 60, and 120 seconds, 25-μL aliquots of the reaction mixture were removed and diluted into 225 μL of ice-cold TBS-BSA containing 20 mmol/L EDTA. Thrombin generated was assayed using the chromogenic substrate S2238 (final concentration, 0.1 mmol/L) and measuring the rate of change in absorbance at 405 nm using a Vmax microtiter plate reader (Molecular Devices, Menlo Park, CA). Purified human thrombin was used as standard.

**RESULTS**

**Generation of microparticles and factor Va-binding sites by activation of complement.** PNH platelets were initially examined for their susceptibility to complement-induced microvesiculation. PNH platelets selected for these studies lacked detectable CD59 antigen. C5b-9 complexes were assembled on gel-filtered platelets by incubation with the purified complement proteins C5b6, C7, C9, and limiting amounts of C8. Platelet activation responses were analyzed by fluorescence-gated flow cytometry, using FITC-labeled MoAb V237 to monitor factor Va-binding sites. Figure 1 shows dot blots of FITC-V237 fluorescence versus forward light scatter (logarithmic scales). As illustrated by these data, platelets from the PNH patients tested were significantly more sensitive to complement C5b-9 when compared with normal controls, as evidenced by a marked increase in (1) the formation of plasma membrane-derived microparticles, and (2) exposure of new binding sites for factor Va on both platelets and microparticles. In these experiments, platelet microparticles were discriminated from platelets by analysis of forward and side-angle light scatter (see Materials and Methods).25 As is also evident from these data (see untreated samples of Fig 1), PNH platelets generally showed more basal activation in the absence of added complement proteins than normal platelets. This is likely to reflect the effects of prior complement activation at the surface of these cells either in vivo or during processing of platelet-rich plasma, due to deficiencies of the plasma membrane complement regulatory proteins CD55 (decay-accelerating factor) and CD59.

The newly formed population of microparticles was quantitated by setting a forward and side scatter gate so as to
exclude the majority of the platelets for analysis, as previously described. As illustrated in Fig 2, a complement dose-dependent increase in the formation of microparticles was observed for platelets from patients with PNH. For comparison, few microparticles were generated in suspensions of normal platelets under these conditions of limited deposition of C5b-9 complexes. It should be noted that, in these experiments, we observed sample-to-sample differences in the extent to which platelets and platelet-derived microparticles were resolved into distinct populations when analyzed by light scatter, and for certain samples (eg, patient PNH SS of Fig 1), the forward scatter of newly formed microparticles overlapped that of small platelets, suggesting heterogeneity in the size of the particles (activated platelets and plasma membrane vesicles) on which the factor Va-binding sites were expressed (see Discussion).

As shown in Fig 3, microparticle formation was accompanied by a complement dose-dependent expression of factor Va-binding sites, monitored by the binding of FITC-labeled V237 in the presence of a saturating amount of exogenously added factor Va light chain. Total binding of FITC-V237 was approximately 10-fold higher for C5b-9–treated PNH platelets than for normal controls at an input of C8 of 500 ng/mL. Under the conditions of these experiments, increased factor Va-binding sites were observed on both PNH platelets and platelet-derived microparticles.

Prothrombinase activity. To test whether these newly formed factor Va-binding sites detected by flow cytometry coincided with increased expression of functional procoagulant surface, the capacity of these C5b-9–treated platelets to assemble the platelet prothrombinase enzyme complex was investigated. As illustrated in Fig 4, the exposure of factor Va-binding sites was paralleled by the expression of catalytic surface for the prothrombinase complex; the rate of prothrombin to thrombin conversion by PNH platelets exposed to the C5b-9 proteins increased in a complement dose-dependent fashion, and again exceeded that of C5b-9–treated normal controls by approximately 10-fold at the maximal input of C8 tested. By contrast to results obtained with C5b-9, we were unable to detect increased sensitivity of the PNH platelets to activation by either collagen (0 to 10 μg/mL) or the calcium ionophore, A23187 (0 to 1 μmol/L; data not shown), suggesting that the increased sensitivity of PNH platelets to activation by C5b-9 is specifically related.
to the loss of the complement-inhibitory function that is normally provided by cell-surface CD59.

**DISCUSSION**

Thrombotic complications are a major cause of morbidity and mortality in patients with PNH. Previous studies have demonstrated that platelets treated with complement proteins C5b-9 release procoagulant microparticles that promote assembly of the prothrombinase enzyme complex. Data of the present study demonstrate that PNH platelets, which lack the CD59 antigen, are significantly more sensitive to activation by complement proteins C5b-9 than platelets from normal controls, as shown by a 10-fold increase in complement-induced exposure of membrane-binding sites for factor Va, which was accompanied by a corresponding increase in membrane-catalyzed prothrombinase activity. The results obtained with PNH platelets are similar to data that we previously obtained using normal platelets in which platelet activation responses were potentiated in vitro by a functionally blocking antibody against CD59.

By contrast to results obtained with normal platelets, the factor Va-binding sites exposed on complement-treated PNH platelets increased prominently on both platelets and platelet-derived microparticles. Furthermore, in certain experiments, the population of microparticles (defined by light scatter) were poorly resolved from remnant PNH platelets (see Fig 1). This apparent difference between normal and PNH platelets is likely to reflect (1) increased fragmentation of PNH platelets due to loss of complement regulatory function, resulting in membrane fragments that overlap the microparticle gate; or (2) decreased capacity of the remnant PNH platelets to resequester acidic phospholipids.
that are exposed upon C9 insertion and induced vesiculation of the plasma membrane, due to inactivation of the aminophospholipid translocase in the remnant cells and membrane fragments. In this context, it is of note that, by contrast to recent results reported for calcium ionophore, we did not observe either impaired vesiculation or diminished expression of prothrombinase activity in PNH platelets exposed to C5b-9 complexes.

This ability of the PNH platelet to shed nascent C5b-9 complexes by vesiculation may account for the normal survival in the circulation of the platelets in this disorder. In the case of erythrocytes, which also lack CD59, C5b-9 complexes accumulate on the membrane and the cells are lysed; this may explain why the survival of PNH erythrocytes is shortened to less than 10% of that of normal red blood cells, while platelet survival is unaffected.

The results of the present study indicate that deletion of the regulatory protein, CD59, from platelets is accompanied by a marked increase in the susceptibility of these cells to the effects of the C5b-9 proteins, leading to thrombin generation, which may contribute to the thrombotic risk associated with this disorder.

ACKNOWLEDGMENT

The contributions of MoAbs W5 (Dr R.P. McEver) and V237 (Dr C.T. Esmon) are gratefully acknowledged. The collaboration of Dr M.J. Telen in the use of MoAb 162 is also gratefully acknowledged.

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