Mechanism of Complement-Mediated Activation of Human Blood Platelets In Vitro

COMPARISON OF NORMAL AND PAROXYSMAL NOCTURNAL HEMOGLOBINURIA PLATELETS

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ABSTRACT The paroxysmal nocturnal hemoglobinuria (PNH) platelet differs from the normal human platelet in its interaction with activated complement components: (a) when complement is activated by the alternative pathway, greater amounts of C3 are fixed to the PNH platelet than to the normal platelet; (b) the platelet-release reaction, as measured by serotonin release, occurs after C3 fixation to the PNH platelet. This reaction does not occur with normal platelets; (c) although serotonin release mediated by antibody alone was the same for normal and PNH platelets, antibody-initiated complement activation resulted in the fixation of greater amounts of C3 to PNH platelets and greater consequent serotonin release; and (d) nearly maximal serotonin release from PNH platelets occurs after the fixation of C3 (or perhaps C5) to the membrane without completion of the terminal sequence. In contrast, completion of the terminal complement sequence beyond C5 is required for maximal serotonin release from normal platelets. These abnormalities of interaction of complement components and PNH platelets may explain the occurrence of thromboses in this disease.

INTRODUCTION

Antiplatelet antibody from a number of sources has been shown to interact with platelets and to fix complement to the platelet surface. The result of this interaction is activation of the platelet, release of vasoactive amines, and possibly platelet lysis (1-3). Zymosan, a substance known to activate the alternative pathway of complement, will produce similar reactions but only in the presence of plasma (4, 5). These reactions are readily demonstrated with rabbit platelets (6-10), and the activation of complement has been thought to result in intravascular coagulation in these experimental animals (11). Normal human platelets, however, are markedly unresponsive in these reactions and do not readily release serotonin or platelet factor 3 when complement is activated (4, 12, 13).

Patients with paroxysmal nocturnal hemoglobinuria $(PNH)^1$ are particularly prone to the development of serious thrombotic disease, frequently in association with severe hemolytic episodes. These episodes are thought to be due to activation of complement, and hemolysis is thought to occur because a portion of the red cells of patients with PNH are abnormal in that they bind more complement and are lysed more readily than are normal erythrocytes (14). Thus the cells are lysed by smaller amounts of activated complement in vivo.

Increased complement-mediated lysis of PNH platelets has been demonstrated in vitro (15), but activation of the platelet has not been investigated in PNH. If the platelet membrane had a defect like that of the red cell, the sensitivity to complement might also manifest itself by increased activation, much like the rabbit platelet. If this were true, the increased thrombosis seen in these patients might be related to some membrane defect.

In the present investigation, we used release of $[^{14}C]$ serotonin as a measure of platelet activation

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¹Abbreviations used in this paper: CoF, cobra venom factor; PNH, paroxysmal nocturnal hemoglobinuria; VBS, Veronalbuffered saline.

(16). We showed that activation of the complement sequence by either the classic or the alternative pathways does indeed activate the release of serotonin more readily in PNH than normal platelets. This is in part due to the fixation of greater amounts of complement components. thus, the membrane abnormality of the platelets is probably similar to that of the erythrocytes and may account for the increased thromboses seen in these patients.

METHODS

Platelets. 10 patients with PNH (defined by increased sensitivity of erythrocytes to antibody-mediated complement lysis) were studied on multiple occasions. All patients were receiving up to 30 mg of prednisone on an alternate day basis, and none were significantly thrombocytopenic. The normal platelets were obtained from healthy, age-matched volunteers. Blood from all subjects was collected in plastic containers and anticoagulated with 10% EDTA. Platelets were prepared by differential centrifugation, washed four times in isotonic EDTA-Veronal-buffered saline (VBS), and resuspended in VBS containing calcium and magnesium with gelatin or in platelet-poor plasma. Serial dilutions of platelets were counted by phase microscopy.

Buffers and complement reagents. VBS, buffered 0.015 M and 0.1 M EDTA acid and disodium salt (EDTA-VBS) were prepared (9). Serum prepared from whole blood at 37° C was stored at -90° C and used as a complete complement source. Purified human C1 and C2 were prepared as previously described (17). Highly purified human C3 and rabbit anti-human C3 were prepared (10). Partially purified cobra venom factor (CoF) was obtained from Sephadex G-100 (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) (18). CoF-activated serum was prepared by incubation of purified CoF with normal human serum as described by Kabakçi et al. (19). Serum from a patient with congenital C6 deficiency was used in the study of the terminal complement sequence (kindly donated by Dr. Ralph Snyderman). This serum was free of hemolytically active C6, as demonstrated by failure to lyse PNH erythrocytes in the presence of excess coldreacting antibody (6). The titer of C6 by hemolytic assay was less than 25 U (normal 6,000 U). Lytic activity could be restored by addition of hemolytically active human C6 (Cordis Laboratories, Miami, Fla.).

Antibodies. The presence of antiplatelet antibody in patients with thrombocytopenia was confirmed by the method of Dixon et al., a quantitative antiglobulin absorption technique (20). The presence of C-fixing antibodies was confirmed using the platelet surface C3 assay as described below. Using this technique, a C-fixing antibody in the serum of several patients with thrombocytopenia and lupus erythematosus was demonstrated. The antibody was an IgG immunoglobulin. Serum from several patients with drug-induced purpura was also employed in this study. With the drug-induced antibody, complement fixation was shown to be increased in the presence of the drug. Antibodies were used as dilutions of either whole serum heated to 56°C for 30 min or were purified by absorption to platelets and acid elution according to the technique of Shulman et al. (21). Eluates were free of hemolytically active C components. Purified cold-reactive antibody from serum containing anti-I was prepared by the method of Rosse et al. (18).

Measurement of [14C]serotonin release. Measurement of newly absorbed serotonin released from platelets was used as a measure of release of endogenous serotonin, as the two release patterns are identical (16). Platelets suspended in 10 cm³ of plasma were incubated with 50 μ Ci of D,L-3-14C hydroxytryptamine (New England Nuclear, Boston, Mass.) according to the method of Hiershman and Shulman (1). Labeled platelets were then washed in 10 vol of 0.015 M EDTA-VBS and resuspended in VBS. Labeled platelets were used immediately. The usual reaction mixture contained 0.2 ml of labeled platelets, an equal volume of dilution of reactant (antibody, CoFactivated serum, pH 6.4, or C3b), and in the case of antibody and CoF, an equal volume of C source (normal serum or C6-deficient serum). Platelets and reactants were incubated at 37°C for 30 min. For kinetic studies, the reaction was terminated by the addition of 0.1 M EDTA at specified times. In the case of cold-reacting antibodies, the initial incubation was performed at 30°C with subsequent warming to 37°C. After incubation, platelets were immediately separated from the supernatant fluid at room temperature at 2,000g for 10 min. 0.2 ml of the supernatant fluid was solubilized in didodecyl dimethylammonium hydroxide (Eastman Organic Chemicals Div., Rochester, N. Y.) in a counting vial and 10 ml of 2,5-diphenyloxazole and p-bis(O-methylstyryl)benzene in toluene (New England Nuclear) was added. Radioactivity was measured in a Beckman scintillation counter (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). In each instance, serotonin release was calculated as a percentage of the maximal release (determined by the radioactivity in the supernate after freeze-thawing labeled platelets three times). Spontaneous release of serotonin by platelets alone or when incubated with the C source alone was subtracted in each instance. Absolute counts of 14C obtained varied over a wide range of 100-25,000 cpm. Spontaneous release from PNH and normal platelets was identical. Uptake of exogenous [14C]serotonin was identical in normal and PNH platelets.

Measurement of surface-bound C3 and IgG. The quantity of C3 on the surface of platelets was determined by the anti-C3 absorption technique of Borsos and Leonard (22) as modified by Rosse et al. (18). Sheep cells coated with human C3 were prepared by incubation in normal human serum, washed, and reacted with 0.02 M 2mercaptoethanol. Anti-C3 was made in rabbits according to the method described (23). Highly purified C3 was made by the method of Nilsson and Müller-Eberhard (24), as modified by Logue et al. (23). An appropriate amount of anti-C3 was incubated with known quantities of pure C3 and the amount remaining was determined by its ability to lyse E^{sh}C43^{hu}. A calibration curve was drawn relating the decrease in lysis of the sheep cells to the amount of C3 which had been incubated with the anti-C3. In a test system, 0.2 ml of washed platelets containing 100,000-200,000 platelets/mm³ were incubated with dilutions of the reactant and various complement sources as for the serotonin assay. After washing in 10 vol of 0.015 M EDTA, platelets were resuspended in VBS, counted, and reacted with the standard concentration of anti-C3. Further washing caused no further reduction in detectable surface C3. The amount of C3 on the membrane surface was determined by reference to the calibration curve and expressed as femtograms of C3 per platelet. In experiments where C3 binding was compared to release, C3 was determined on the surface of labeled platelets after removal of the supernatant fluid. Kinetic studies were performed as described for [14C]serotonin release.



FIGURE 1 Fixation of C3 to normal and PNH platelet membrane by CoF-activated serum. Incubation of CoFactivated serum with PNH platelets results in a fourfold increase in surface-bound C3 (open symbols). No increase in membrane C3, over that observed with normal serum, occurs on the normal platelet (closed symbols).

Quantitation of platelet surface IgG after incubation with antibody was performed as previously described by Dixon et al. (20). This method is similar to the C3 assay except that purified human IgG and rabbit anti-human IgG were employed.

RESULTS

Approximately the same yield (88%) of normal and PNH platelets from platelet-rich plasma was achieved by differential centrifugation. In no instance was leukocyte or erythrocyte contamination greater than 0.1%. Washed normal and PNH platelets have approximately the same amount of C3 detectable on the membrane surface (less than 1.0 fg/platelet or 3,250 molecules/platelet). When incubated with exogenous [¹⁴C]serotonin, uptake of the label occurred at approximately the same rate with normal and PNH platelets as when incubated with normal serum. Both types of platelets, when untreated, gradually released the serotonin at the same rate into the supernate, hence all experiments were performed immediately after labeling.

Activation of the alternative pathway of complement

Cobra venom-mediated activation. Incubation of normal or PNH platelets for 30 min in normal serum results in an increment in membrane C3 to less than 30 fg, or 97,000 molecules, per platelet. Less than 30% of exogenous [¹⁴C]serotonin is released. No further increment in normal platelet surface C3 or serotonin release occurs during incubation of normal platelets with serum dilutions in the presence of CoF-activated serum. In contrast, incubation of CoFactivated serum with PNH platelets results in a fourfold increase in surface-bound C3 at any given serum dilution (Fig. 1).

CoF-activated serum is ineffective in causing serotonin release from normal platelets but is effective in doing so from PNH platelets. The relationship between C3 fixation and serotonin release is shown in Fig. 2. The fourfold increment in membrane C3 fixation to PNH platelets resulted in proportional increase in serotonin release from these platelets. CoF alone or cobra venom-activated serum alone (depleted of C3, C5, C6, C7, C8, and C9) produced no increment in membrane C3 or serotonin release from normal or PNH platelets.

Acidified serum-mediated activation. As shown in Fig. 3, substitution of dilutions of serum at pH 6.4, in the reaction mixture results in a fourfold increase in C3 measurable on the PNH platelet surface but no appreciable increase in C3 on normal platelets. Similarly, a proportionally greater amount of serotonin release from PNH platelets is observed (Fig. 4). In no instance did acidified serum or isotonic



FIGURE 2 Serotonin release from normal and PNH platelets in response to membrane C3 fixation by CoF-activated serum. Serotonin released from normal platelets is not increased over that observed with normal serum (closed symbols). A fourfold increase is observed from PNH platelets (open symbols).



FIGURE 3 Fixation of C3 to normal and PNH platelet membrane by acidified serum, pH 6.4.

buffer, pH 6.4, effect a measurable increment in serotonin release from normal platelets.

C3b-mediated activation. The active component of C3, C3b, was prepared from purified human complement components. Sheep red cells were coated with C4 by the method of Borsos and Rapp (25). Functionally purified C1 and C2 were sequentially added. The cells were washed at 0°C, and C3 purified to electrophoretic and immunochemical purity was added. The mixture was incubated in the presence of normal and PNH platelets labeled with serotonin.

Incubation of PNH platelets with dilutions of this fluid-phase C3b resulted in release of up to 50% of exogenous [14C]serotonin, whereas no release was evident after incubation with normal platelets (Fig. 5). Control incubations were performed using purified C3 not activated with early complement components, and with $E^{sh}AC4^{hu}$, C1 and C2, and no C3. No release of serotonin occurred in these reactions.

Activation of complement by the classical pathway

Relationship of antibody fixation to platelet membrane C3 binding and release. An identical amount of complement-fixing antibody found in the serum of several thrombocytopenic patients with lupus erythematosus was detected on normal or PNH platelets by quantitation of membrane-bound IgG. However, as shown in Fig. 6, for a given amount of antibody detectable on the surface, four times as much



FIGURE 4 Serotonin release from normal and PNH platelets in response to membrane C3 fixation by acidified serum.

C3 was fixed to PNH platelets as compared to normal platelets.

Similar results were obtained using serum from patients with drug-induced purpura (three instances of quinidine and one of codeine-induced thrombocytopenia [Table I]). In each instance, the amount of IgG fixed to the platelet surface was the same for normal and PNH, and varied with the proportion of drug and serum in the reaction mixture. In



FIGURE 5 Serotonin release from normal and PNH platelets in response to fluid-phase C3b. This product is ineffective in activating normal platelets but will produce a release reaction in PNH platelets.



FIGURE 6 Fixation of C3 to normal and PNH platelet membrane by quantitative amount of antiplatelet antibody. C3 is fixed to normal and PNH platelets but a fourfold increase in surface C3/fg of antibody is observed in PNH.

each instance, the increment in membrane C3 varied in proportion to the drug or antibody concentration, but was always greater for PNH platelets. Similarly, when antibodies obtained from a patient sensi-

Antibody	Normal		PNH	
	C3	Release	 C3	Release
	fg/platelet	%	fg/platelet	%
Lupus 1	12.4	31.1	35.4	92.7
2	15.8	46.6	46.3	91.7
3	6.4	—	25.9	—
Codeine	11.9	26.6	14.9	71.7
Quinidine 1	10.6	52.5	47.7	89.3
2	_	73.8		95.5
3	10.9	68.4	29.1	90.0
HLA	9.5	_	27.0	_
Anti-I 1	10.1	42.9	33.0	63.1
2	7.5	_	60.0	
3	12.5	54.1	49.6	98.4
Mono	10.6	47.5	12.0	92.0

TABLE I Antiplatelet Antibody, C3, and Release

C3 fixation and serotonin release from normal and PNH platelets in response to several antibodies. Increased membranebound C3 and increased serotonin release was invariably observed from PNH platelets.

tized by multiple platelet transfusion, as well as a patient with mononucleosis and thrombocytopenia, and several patients with an IgM cold agglutinin were employed, C3 fixation was greater on the PNH platelet.

When the amount of membrane-bound C3 was compared to the degree of serotonin release, the increased serotonin release from PNH platelets paralleled the greater C3 fixation on the PNH platelets (Fig. 7). The C3 fixed was no more effective in causing serotonin release from PNH than from normal platelets; the increased serotonin release by PNH platelets was related to greater amounts of membrane C3 fixed at any antibody dilution.

Kinetics of antibody-mediated release. To assess the role of complement in the antibodymediated release reaction, the response of normal and PNH platelets was studied over time (Fig. 8). Both normal and PNH platelets show an identical pattern of serotonin release during the first 2 min of the release reaction; approximately 25% of the labeled serotonin is released. This occurs before the fixation of detectable amounts of C3 to the platelet surface, and appears to be independent of complement fixation. Further, this initial release reaction can be demonstrated to occur in heat-inactivated serum. When an antibody which does not fix complement is employed, this initial phase of serotonin release is observed and is identical to that observed with complement-fixing antibodies. Incubation of



FIGURE 7 Serotonin release from normal and PNH platelets in response to C3 activation by antiplatelet antibody. Maximal serotonin release from normal platelets is 40% at 150 fg C3/platelet. Substantially more C3 fixation and release occurs with PNH platelets. This is a representative study.



FIGURE 8 A representative kinetic study. Kinetic study of C3 fixation (A) and serotonin release (B) in normal and PNH platelets in response to complement-fixing antiplatelet antibody. Identical serotonin release (25%) occurs from normal and PNH platelets before C3 fixation. Increased serotonin release from PNH occurs subsequent to increased C3 fixation to membrane.

both normal and PNH platelets with serum from a patient with immune thrombocytopenic purpura (as defined by the assay of Dixon et al. [20]) results in 25-30% release of serotonin during the first few minutes and a subsequent increase to 40% over the next 20 min. The amount of serotonin released is identical at each time point in the two groups of platelets.

In a second phase of the reaction, when a complement-fixing antibody is used, increasing amounts of C3 are detectable on the membrane and a further increment in serotonin release is observed. After 30 min, C3 has been bound to PNH platelets and more serotonin has been released. With each complement-fixing antibody studied, serotonin was released from PNH platelets only after significant increment in membrane-bound C3; i.e., after 2 min of reaction.

To study the role of the terminal complement components beyond C5 in the release reaction, purified anti-I antibody was reacted with platelets and C6deficient serum was employed as a complement source. The C6-deficient serum did not cause serotonin release from normal platelets unless purified C6^{hu} was added to the reaction (Table II). In contrast, when PNH platelets were studied, appreciable release occurred in C6-deficient serum and a 10–15% further increment in release occurred when purified $C6^{hu}$ was added back to the reaction. The terminal components of complement beyond C5 are not required for a release reaction detectable at 30 min from PNH platelets activated by antibody. A further slight increase in serotonin release may be observed at 30 min, however, when all terminal components are present.

DISCUSSION

In the rabbit, a role for complement in platelet activation has been well established (17). Rabbit platelets undergo a two-stage reaction with soluble antigen-antibody complexes. The first, requiring complement components through C3, results in platelet aggregation. The second, requiring completion of the sequence through C6, results in the release reaction (12). Similarly, agents known to interact with the components of the alternative pathway of complement activation cause rabbit platelets to undergo the release reaction subsequent to the activation of C3 (26).

In the present work, we have investigated the role of complement in the activation of human platelets from both normal donors and patients with PNH. The methods employed for the quantitative determination of membrane-bound components of the immune system have been previously shown to be

 TABLE II

 Anti-I Effect of C6-Deficient Serum

	Platelets		
C' source	Normal	PNH	
	% Serotonin release*		
Normal	76.8 ± 3.5	$90.6 \pm 7.6 \ P < 0.01$	
Decreased C6	24.2 ± 5.3	$71.4 \pm 6.9 \ P < 0.001$	
Decreased C6 + 6	68.0 ± 8.6	$85.0 \pm 3.7 \ P < 0.1$	
None	23.0 ± 2.1	$20.4 \pm 5.1 \ P < 0.6$	

Serotonin release from normal and PNH platelets by purified anti-I in the presence of C6-deficient serum. Repletion of C6 is required for maximal release from normal platelets. The ranges shown are the results of five experiments. * Mean \pm SD.

sensitive and reproduceable (18, 20). Determination of radiolabeled serotonin release has been shown to be an accurate means of assessing the plateletrelease reaction (16). These methods were used in the study of the individual steps in this reaction.

There appear to be several mechanisms by which activation of complement by antibody activates serotonin release from platelets. From the kinetic studies with antibody, two separate phases of serotonin release from normal and PNH platelets have been identified. The first phase occurs within the first 2 min of incubation and appears to be due to interaction with antibody. Release of serotonin in this phase is equal in normal and PNH platelets and occurs independently of the presence of complement, as judged by detectable C3 on the membrane. This reaction occurs without increment in membrane-bound C3, and the proportion of serotonin released is identical in reactions employing both complement-fixing and noncomplement-fixing antibodies. Pfueller and Lüscher have suggested this involves direct interaction of antibody (probably Fc fragment) and platelet surface (13). With PNH platelets, complement-dependent efflux of serotonin appears to result from two mechanisms of complement-platelet interaction: one due to C3 or C5 fixation, the other requiring fixation of terminal complement sequences beyond C5. As with the PNH erythrocyte, greater amounts of C3 are fixed to the membrane of the PNH platelet than to the normal platelet. The activation of C3 and C5, and its fixation to the membrane of the PNH platelet, result in release of serotonin even in the absence of terminal complement components, as shown with C6-deficient serum. This reaction does not appear to occur with normal platelets. The second mechanism which results in a further efflux of serotonin from PNH platelets does not occur in the absence of C6 and presumably requires the terminal components

of complement. This release is probably the result of membrane damage evoked by the terminal C' components. This occurs with normal platelets as well as PNH platelets. At present this cannot be confirmed as we do not have available a measure of lysis due to completion of the complement sequence.

In the experiment using purified components of complement in the generation of C3b, no C3b was detected on the platelet membrane but release of serotonin occurred. This may be due to insensitivity of the assay for the detection of C3b. Less C3 may be fixed in the system than in systems in which whole serum is present because, in the latter, bound C3 may bind activated factor B of the alternative pathway (27). This complex is capable of generating superfluous C3b at the membrane surface which would be more readily detectable in the assay.

Alternatively, the release reaction might be mediated by other proteins in the solution. Since the C3 used in these experiments was "pure" by electrophoretic and immunologic criteria, and since the reaction did not occur when this protein was omitted, the material responsible for the release reaction would have to be C3b or C3a. Previous experiments in other systems would indicate that C3b was responsible for this release reaction.

In contrast to antibody-mediated complement activation, fluid-phase complement activation through the alternative pathway results in serotonin release from PNH but not from normal platelets. Activation of this pathway by acidification of serum or treatment with CoF-activated serum results in the fixation of up to 10 fg of C3 to PNH platelets and a 10-fold increase in serotonin release, but no appreciable C3 fixation or serotonin release from normal platelets. A portion of the observed serotonin efflux from PNH platelets may well be due to platelet lysis as evidenced by the requirement for C6 to effect maximal serotonin release. However, C3 or C5 activation in the absence of terminal complement components is sufficient for serotonin release from PNH platelets. The maximal release of serotonin from platelets with C3b but without the terminal components was 50%. In contrast, when acidified or CoF-activated serum containing all C components was supplied, the proportion of serotonin released was invariably greater than 75%. Thus it is probable that C3b alone in fluid phase is capable of activating PNH platelets, resulting in serotonin release but that the further increment in serotonin release (25%) is either a result of platelet lysis after completion of complement sequences on the platelet membrane or a result of platelet interaction with other serum proteins. In contrast, the fluid-phase C3b does not bring about release of serotonin from normal platelets.

Aster and Enright showed that the PNH platelet, like the PNH red cell, is more sensitive to lysis by complement that its normal counterpart (15). The present data demonstrate that this is in part due to an increase in the amount of C3 which is fixed to the platelet membrane but the data also suggest that the terminal components of complement are more effective on PNH than on normal platelets in effecting membrane damage. Similar differences have been found between normal and the markedly abnormal variety of PNH red cells (18). It is not clear from these present studies or those of Aster and Enright (15) whether the platelets in PNH, like the red cells, consist of different populations with respect to complement sensitivity.

Activation of the platelet as manifest by serotonin release may be the mechanism by which increased thrombosis occurs in these patients, as originally suggested by Shulman et al. (21). Most major thrombotic phenomena occur during periods of complement activation as manifest by hemolytic crises. Thus, the two major clinical manifestations of PNH, hemolysis and thrombosis, may have similar causes, an abnormal sensitivity to the effects of the "normal" activation of complement.

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