Human Platelet-Initiated Formation and Uptake of the C5-9 Complex of Human Complement

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ABSTRACT We have studied the interaction of radiolabeled complement components with normal human platelets, platelets from a patient with paroxysmal nocturnal hemoglobinuria, and rabbit platelets in the absence of known complement activators or in the presence of coba venon factor (CVF). When unwashed platelets in platelet-rich plasma, or washed platelets suspended in serum or autologous plasma, were incubated for 30 min, C3 and terminal components (C5, C8, and C9) were found to bind to them. The terminal components were shown to be bound as the C5-9 complex, rather than as individual proteins, by eluting them from the platelet membrane and examining their behavior on ultracentrifugation. They cosedimented at a rate characteristic of the stable C5-9 complex (22S). As many as 370–1,380 C5-9 complexes/platelet were calculated to have been bound during the incubation period. The complex so formed did not differ by ultracentrifugational criteria from that binding to rabbit platelets after CVF activation of complement. Though C3 was not included in the complex, it did not appear to be bound by nonspecific absorption. It could not be removed by washing but rather was eluted by the freeze-thaw technique used to elute the C5-9 complex. Incubation of radiolabeled components in platelet-free plasma did not result in C5-9 complex formation, indicating an initiating role for platelets in this reaction. In contrast to platelets, erythrocytes incubated in analogous plasma did not induce detectable C5-9 formation. Neither EDTA, phenylmethylsulfonylfluoride, nor epsilon-amino-*N*-caproic acid prevented platelet-initiated formation of C5-9, suggesting that the reaction may involve mechanisms of complement activation not previously described.

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INTRODUCTION

Human platelets and platelets of other mammalian species may respond with the release reaction and/or lysis after activation of the classical complement pathway with anti-platelet antibody (1, 2), immune aggregates (3–5), or activation of the alternative pathway with immune aggregates, endotoxin, inulin, cobra venom factor (CVF),[1] or zymosan (6–17). Description of a coagulation abnormality in the blood of rabbits congenitally deficient in C6 (18) and demonstration of the role of platelets in this coagulation defect (2, 19–21) has suggested that complement-platelet interaction may proceed in the absence of known complement activators. More recently, deficient platelet aggregation in response to collagen and ristocetin have been demonstrated in human subjects deficient in C5 (21), suggesting a role for complement in normal human platelet function.

Utilizing radiolabeled complement components, we have now demonstrated that normal human platelets as well as those of rabbits can themselves initiate formation and binding of the C5-9 complex, the mediator of complement-directed membrane damage (22, 23). This reaction proceeds in the presence of EDTA and epsilon-amino-*N*-caproic acid (EACA), suggesting that it is not initiated by the classical or alternative pathways of complement activation and also differs from C5-cleaving platelet factor of Weksler and Coupal (24).

METHODS

Preparation of platelet-rich plasma, washed platelets, washed erythrocytes, platelet-free plasma, and serum. Fresh whole blood was anticoagulated with acid citrate dextrose or citrate phosphate dextrose (25). Platelet-rich plasma was immediately prepared by centrifugation of whole blood at

[1] *Abbreviations used in this paper:* BSA, bovine serum albumin; CVF, cobra venom factor; DFP, diisopropylfluorophosphate; EACA, epsilon-amino-*N*-caproic acid; PMSF, phenylmethylsulfonylfluoride; PNH, paroxysmal nocturnal hemoglobinuria.

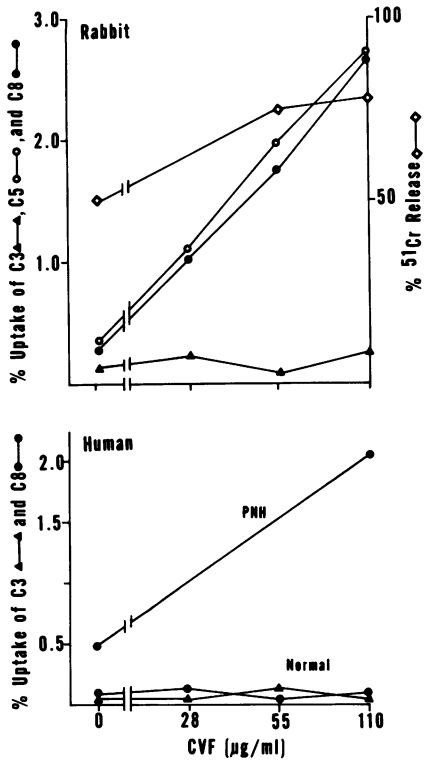

FIGURE 1 Binding of complement components to platelets with and without CVF activation of complement. Platelets were suspended in normal human serum containing ^{125}I-C3, ^{125}I-C5, and ^{131}I-C8, in various combinations or individually. CVF or buffer was then added. After a 30-min incubation, platelets or platelet membrane fragments were collected on Millipore filters and retained radioactivity was determined. In parallel experiments, platelets were labeled with ^{51}Cr and percent release was determined by measuring radioactivity remaining in the supernates after centrifugation. Upper panel: Binding of C3, C5, and C8 to rabbit platelets and comparison with ^{51}Cr release. Almost 50% of ^{51}Cr release occurred in serum without added CVF. However, increased ^{51}Cr release and visible lysis as well as markedly increased C5 and C8 uptake were seen upon addition of CVF. No significant increase in C3 uptake with CVF (as compared to serum alone) was seen in this experiment though it did occur occasionally. Lower panel: Absence of effect of CVF on uptake of C3 or C8 onto normal human platelet preparations as compared with a marked enhancement of C8 uptake onto PNH platelets. C3 uptake onto PNH platelets (not shown) did not exceed the control (0.05–0.09%). On the left ordinate is indicated the percent of radiolabeled complement components in the incubation mixture that specifically bound to the platelets. On the right ordinate, top panel, is the percent of ^{51}Cr released from the platelets.

190 *g* for 15 min at 22–25°C. The platelet-rich plasma was then recentrifuged at 620 *g* for 60 s to reduce the contaminating red and white cells to less than 1%. The final platelet count was between 1.5 and 2.0×10^5/mm^3. Washed platelets were prepared in modified Tyrode's buffer containing 0.2% albumin and 0.03 M adenosine, as described elsewhere (26). Final platelet suspensions contained less than 0.1% red or white blood cells.

Erythrocytes were prepared free of platelets in the following manner: After removal of platelet-rich plasma, the erythrocyte-containing infranate was placed on a 6–35% albumin gradient. The gradient was constructed by successively freezing 0.7-ml quantities of 35%, 28%, 21%, 14%, and 6% albumin in 15-ml Corning polystyrene conical centrifuge tubes (no. 25310, Corning Glass Works, Science Products Div., Corning, N. Y.). Gradients were thawed before the erythrocytes were applied. 1 ml of the erythrocyte-rich infranate was layered on each gradient. The gradients were centrifuged at 2,300 *g* for 30 min and the erythrocytes were removed and washed three times in Tyrode's buffer. The erythrocytes so prepared contained less than 0.2% leukocytes and no detectable platelets.

Fresh plasma was prepared from citrated whole blood by centrifugation at 2,300 *g* for 15 min and recentrifugation of the supernate at 82,500 *g* for 45 min. Human serum was prepared by allowing fresh whole blood to clot at 37°C for 2 h in a glass tube. The clot was removed by centrifugation at 2,300 *g* for 15 min and the serum recentrifuged at 82,500 *g* for 45 min. The serum was stored at −70°C.

Complement components and reagents. Complement components (27–29) and CVF (30) were prepared as described previously. Complement components were radiolabeled with ^{125}I or ^{131}I by the chloramine T method of McConahey and Dixon (31). Specific activity was between 0.5 and 1.9×10^6 cpm/µg protein. 50% or more of the radiolabeled complement proteins were functionally active. After being labeled, each component was dialyzed for 48 h against 4×10 liters of 0.15 M sodium chloride containing 50 mM chloramphenicol (donated by Parke, Davis & Co., Detroit, Mich.) and 25 µM kanamycin sulfate (Bristol Laboratories, Inc., Div. Bristol-Myers Co., Inc., Syracuse, N. Y.). The dialyzed preparations were centrifuged for 2 h at 98,000 *g* to remove aggregates. Bovine serum albumin (BSA) (Pentex Biochemicals, Kankakee, Ill.) was added to a final concentration of 1 mg/ml and the preparations were stored at 0°C.

Specific binding of complement components to platelet membranes. Platelets were concentrated by centrifugation at 1,000 *g* and resuspended in normal human serum at a final concentration of $1–3 \times 10^6$ platelets/mm^3. ^{125}I or ^{131}I-labeled complement components were added to the above suspension to give $1–5 \times 10^6$ cpm/ml. Complement was activated in each mixture by adding increasing quantities of CVF (28–110 µg/ml). Control incubations consisted of: (*a*) Tyrode's buffer substituted for CVF, and (*b*) activation of serum by the indicated amount of CVF in the absence of platelets. After a 30-min incubation at 37°C, the platelets were examined visually under phase microscopy for lysis. Platelet fragments were collected on 0.45-µm Millipore filters (Millipore Corp., Bedford, Mass.) and washed with 40 vol of normal saline, 40 vol of distilled water, and 40 vol of normal saline. Radioactivity remaining on the filters was determined in a gamma scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). Control serum, which contained radiolabeled complement components but no platelets and had otherwise been treated like the platelet serum mixtures, was also filtered, and counts retained on the filters were subtracted from the counts remaining on the appropriate filters from the platelet-serum samples.

Sucrose density gradient ultracentrifugation. 10–40% linear sucrose density gradients were made with barbital-buffered saline, pH 7.5, ionic strength 0.15, in 5-ml (½ inch × 2 inch) cellulose nitrate tubes. The sample size was

100–200 μl and centrifugation was performed in a Beckman SW 50.1 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 16 h at 114,000 *g*. 125-μl fractions were collected and counted on a gamma scintillation spectrometer.

Sucrose density gradient ultracentrifugational analysis of complement components bound to platelet membranes or erythrocyte membranes. For these experiments freshly prepared (less than 3 h from venipuncture) platelet-rich plasma ($1.5–2.0 \times 10^5$/mm^3) was used or washed platelets or erythrocytes were suspended in normal human serum or autologous plasma. In the initial experiments concentrations of platelets of $1–5 \times 10^6$/mm^3 were used. However, lower concentrations, similar to those in platelet-rich plasma, subsequently proved more efficient in initiating formation of the C5–9 complex, and erythrocytes were therefore used at those concentrations ($1.5–2.0 \times 10^5$/mm^3). Complement components were added to give $10–20 \times 10^6$ cpm/ml. CVF, when added, was employed at a final concentration of 110 μg/ml. The mixture was incubated at 37°C for 30 min and the platelets separated by centrifugation for 3 h at 123,000 *g*. Supernatant plasmas were stored at −70°C before ultracentrifugational analysis. Platelets or erythrocytes were washed four times by suspension in phosphate-buffered saline with a Potter-Elvehjem-type tissue grinder and centrifugation at 123,000 *g* for 2 h. Radiolabeled complement components were eluted from the platelet or erythrocyte membrane fragments by suspending them in 0.5–0.7 ml of phosphate-buffered saline, freezing to −70°C, and thawing. The freeze-thaw process was repeated four times. Particulate matter was removed by centrifugation at 123,000 *g* for 2 h.

^{51}Cr release from platelets. Platelets suspended in Tyrode's buffer at a concentration of 2×10^5/mm^3 were incubated with 10 μCi/ml of ^{51}Cr. The platelets were washed twice in Tyrode's buffer and resuspended in serum as in the procedure for quantitating specific uptake of radiolabeled complement components. However, after incubation with CVF or buffer, platelets were removed by centrifugation at 7,000 *g* for 15 min and the ^{51}Cr radioactivity remaining in the supernatant was determined. A 100% value was determined by counting a platelet suspension not subjected to centrifugation.

RESULTS

Comparison of uptake of C3 and C8 onto human and rabbit platelets with and without activation of the alternative pathway by CVF. Normal human platelets from 10 different donors were suspended in serum and C3 and C8 uptake compared in the presence or absence of added CVF. No increase in uptake of C8, and usually no increase in uptake of C3, could be discerned with CVF present, as compared to uptake in its absence (Fig. 1, lower panel). CVF did not induce visible lysis or release detectable amounts of ^{51}Cr from normal human platelets. In contrast, platelets from individuals with paroxysmal nocturnal hemoglobinuria (PNH platelets) did show a marked increase in C8 uptake when complement was activated with CVF, though C3 uptake was

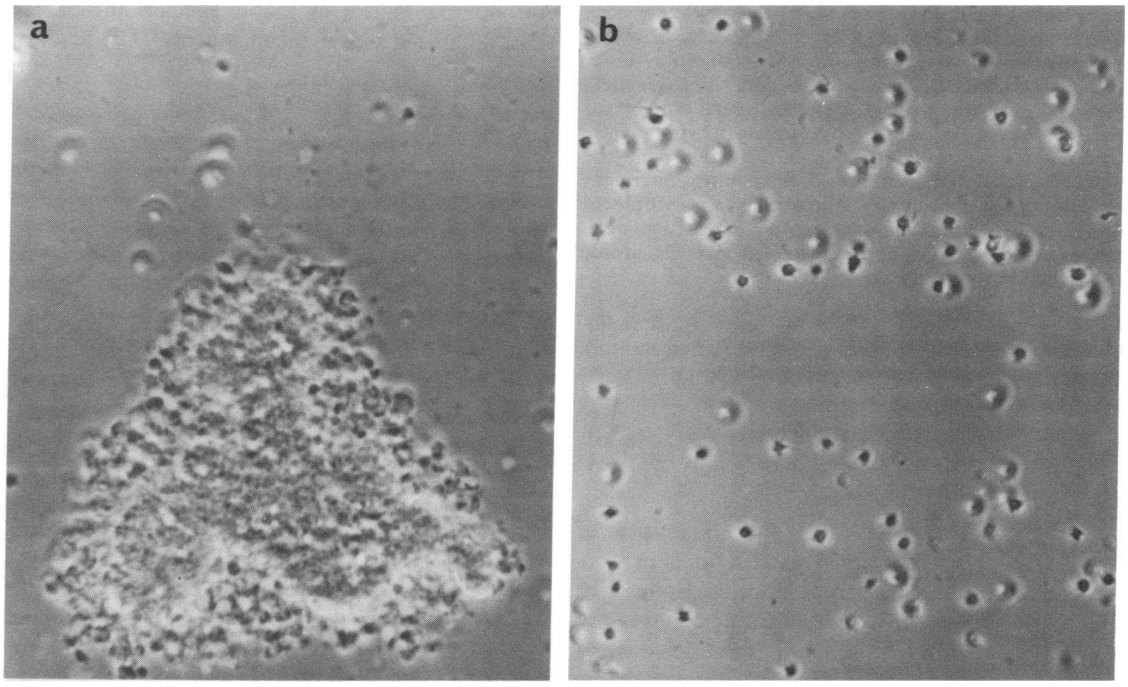

FIGURE 2 Photomicrograph of PNH and normal human platelets after exposure to CVF-activated complement. PNH or normal human platelets were suspended in serum and CVF to a concentration of 110 μg/ml was added. (*a*) Marked clumping and lysis of PNH platelets was seen. (*b*) Normal human platelets remained freely suspended without visible lysis. (× 1,000)

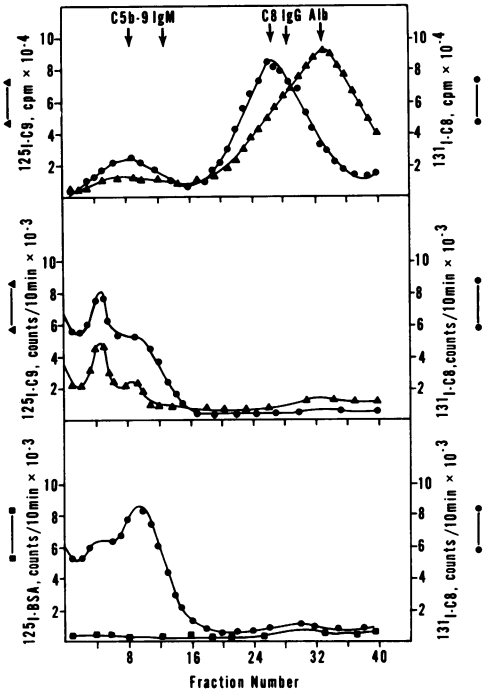

FIGURE 3 Ultracentrifugational demonstration of C5–9 complex in eluates from rabbit platelets with and without CVF activation of complement. Platelets were incubated for 30 min in normal human serum with and without CVF. Platelets were removed by centrifugation and the supernate containing CVF was subjected to ultracentrifugation in a 10–40% linear sucrose density gradient at 114,00 *g* for 16 h. Direction of sedimentation was to the left. The platelets were homogenized and washed and the membrane-bound complement components eluted by freeze-thawing. Particulate material was removed by centrifugation and the eluates then subjected to sucrose density ultracentrifugational analysis. Upper panel: The sedimentation profile of C8 and C9 in whole human serum after incubation with CVF and platelets. Middle panel: The C8 and C9 profile of eluates from rabbit platelets subjected to CVF activation. Lower panel: The C8 profile of eluates from platelets incubated in serum without CVF. In the lower panel radiolabeled albumin has been included in the incubation mixture as a control.

only minimally increased (from 0.05 to 0.09%). In addition, the PNH platelets were visibly lysed (Fig. 2). Rabbit platelets, on the other hand, reacted like human PNH platelets. Addition of increasing quantities of CVF to platelet serum mixtures resulted in a proportionate increase in C8 uptake, visible lysis, and release of ^{51}Cr (Fig. 1, upper panel). Increased C3 uptake was not the usual concomitant of C8 uptake after CVF activation of the alternative pathway (Fig. 1), though it was occasionally observed.

Demonstration that terminal components bound in the presence or absence of CVF were incorporated into the C5-9 complex. Rabbit and human platelets, incubated for 30 min in human serum with or without CVF, were homogenized and washed. The membrane-bound complement components that remained were then eluted by freeze-thawing and the eluates analyzed by sucrose density gradient ultracentrifugation. Terminal components (C8 and C9) eluted from CVF-treated rabbit platelets not unexpectedly cosedimented with a sedimentation coefficient of 22S, the same rate as C5-9 formed in fluid phase by CVF activation (Fig. 3). Surprisingly, however, the terminal components eluted from rabbit and human platelets incubated in serum without CVF also cosedimented with a sedimentation-rate of 22S (Figs. 3 and 4). Examination of eluates from platelets incubated in serum with or without CVF usually revealed the incorporation of terminal components into a heavier complex as well (28S). Though the quantity of terminal components bound to rabbit platelets in the presence of CVF was usually greater than that seen in its absence, no qualitative differences in the C5-9 complex formed by either method could be detected by ultracentrifugational analysis.

To obviate a possible role of isoantibodies or aggregated immunoglobulin as complement activators in the absence of CVF, human platelets were incubated in fresh autologous plasma and the components so bound eluted and examined by ultracentrifugation. Results of four such experiments are summarized in Figs. 4 and 5. As with the human or rabbit platelets incubated in serum, the eluted terminal components (C5, 8, and 9) co-

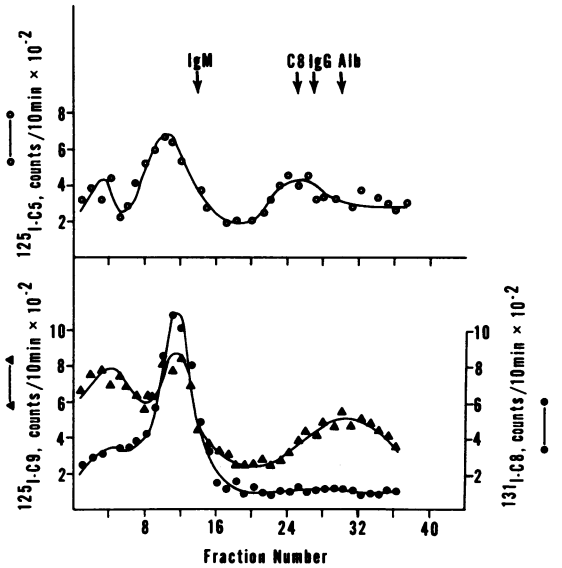

FIGURE 4 Demonstration of a C5–9 complex in eluates from human platelets incubated in fresh autologous plasma. In the upper panel radiolabeled C5 had been added to the incubation mixture and in the lower panel both C8 and C9 had been added. Small quantities of C5 and C9 were observed in their native position.

sedimented as the C5-9 complex, with a sedimentation coefficient of 22S (Fig. 4). A heavier complex, with a sedimentation coefficient and 28S, was described in most experiments. C3, on the other hand, was not included in the complex and sedimented at its native rate of 8.4S (Fig. 5). However, binding of C3 did not appear to be due to nonspecific absorption because C3 was present on platelet membranes that had been washed entirely

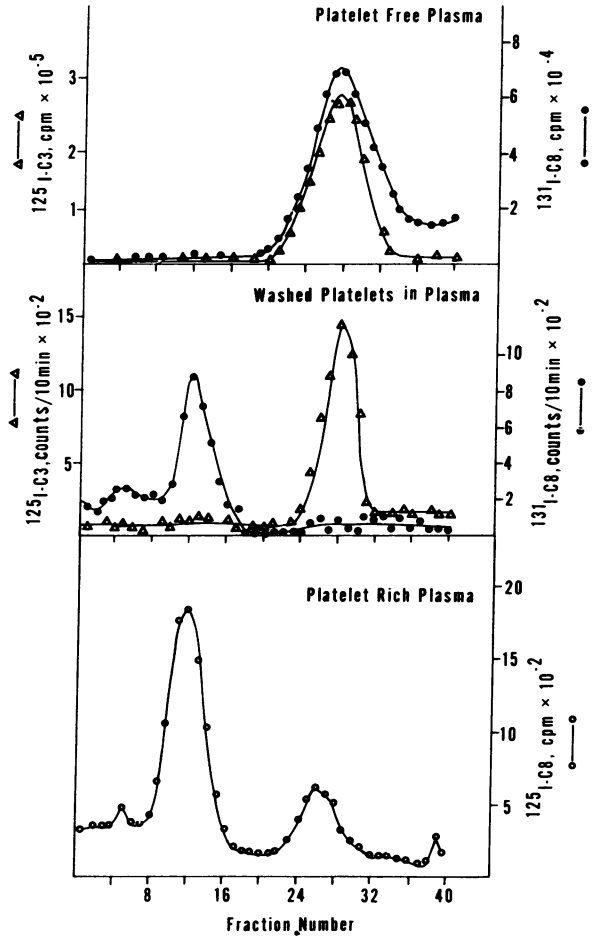

FIGURE 5 Comparative sedimentation velocities of C3 and C8 incubated in plasma with C3 and/or C8 eluted from prewashed or unwashed platelets. Radiolabeled ^{125}I-C3 and ^{131}I-C8 were incubated with previously washed human platelets suspended in autologous plasma and platelet-free plasma, and ^{125}I-C8 was incubated with human platelet-rich plasma. Platelet-rich plasma was used to provide a source of unwashed platelets. The platelet-free plasma (upper panel) and the freeze-thaw eluates from the platelets (middle and lower panels) were examined by sucrose density ultracentrifugation as described for Fig. 3. In the eluates from both preparations of platelets, most or all of the C8 sedimented at a rate characteristic of the C5–9 complex. In the platelet eluate in which no native C8 could be detected, all of the C3 sedimented at its native rate (middle panel). In the platelet-free plasma, C8 sedimented at its native rate, with none sedimenting at the rate of the C5–9 complex.

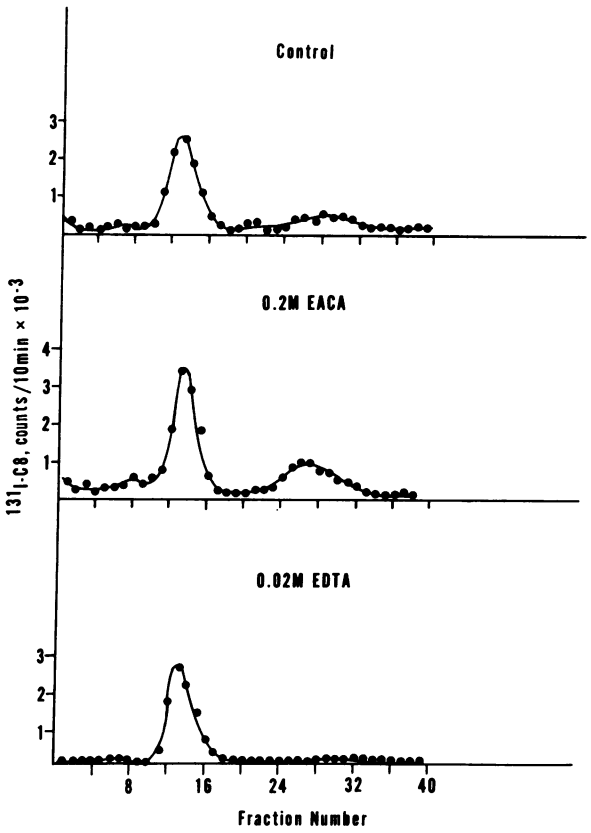

FIGURE 6 Failure of EACA or EDTA to inhibit formation of the 22S C5–9 complex. Normal human platelets were preincubated for 30 min at 37°C in either Tyrode's buffer alone, or Tyrode's buffer containing 0.2 M EACA or 0.02 M EDTA. They were then suspended in normal plasma containing these inhibitors and C5–9 elution was conducted as in Fig. 3. It can be seen that there was no inhibition of formation of the 22S C5–9 complex. The 28S complex was not seen in this experiment. PMSF, 0.001 M also failed to inhibit formation of the C5–9 complex.

free of native C8 and could only be removed by the same techniques used to elute the C5-9 complex. The formation of platelet-bound C5-9 complex did not appear to be the result of platelet injury induced by the washing process. The same reaction was demonstrated with the unwashed platelets present in platelet-rich plasma (Fig. 5, lower panel).

Evidence for platelet-initiated C5-9 formation. To determine if C5-9 binding in the absence of CVF was the result of a platelet-initiated process, or rather resulted from fluid phase activation of complement by another mechanism, platelet-free plasmas were incubated in parallel with platelet-plasma mixtures and were subjected to sucrose density ultracentrifugation. Terminal components sedimented in their native position (Fig. 5), with no evidence for spontaneous fluid phase formation of the C5-9 complex in the absence of platelets (32).

Supernatant plasmas from the platelet-plasma mixtures were also subjected to sucrose density gradient ultracentrifugation and little or no C5-9 could be detected, suggesting that most, if not all, C5-9 formed in the presence of platelets was bound to the platelet surface.

Failure of EDTA, EACA, phenylmethylsulfonylfluoride (PMSF), *or diisopropylfluorophosphate* (DFP) *to inhibit platelet-initiated formation of the C5-9 complex.* Preincubation of platelets with 0.02 M EDTA and 0.2 M EACA and subsequent inclusion of these inhibitors in the platelet-plasma incubation mixtures failed to prevent platelet-initiated formation of the C5-9 complex (Fig. 6). Similarly, no inhibition was seen if platelets were preincubated with 0.02 M DFP and 0.02 M PMSF was included in the platelet-plasma incubation mixtures.

Absence of C5-9 complex formation on erythrocytes incubated in homologous plasma. When erythrocytes were incubated with radiolabeled C8 in autologous plasma, no C8 with the 22S sedimentataion characteristics of the C5-9 complex could be subsequently eluted from them. In contrast, a parallel experiment run with platelets and plasma from the same blood sample showed characteristic formation of platelet-bound C5-9 complex.

C5-9 complexes bound per platelet. The number of C5-9 complexes bound per platelet during the 30-min incubation was estimated at 370–1,380 in three separate experiments. This figure was determined by calculating the number of C8 molecules bound per platelet which, when subsequently eluted, sedimented at a rate of 22S and were therefore included in the complex. It has previously been shown that there is one C8 molecule per C5-9 complex (22). These calculations were made for those experiments in which the platelet concentrations similar to those normally seen in plate-rich plasma were used.[3] These concentrations of platelets were far more efficient in generating the C5-9 complex than the 10-fold higher concentrations used in earlier experiments (compare Fig. 5, bottom panel, with other experiments). In making these calculations it was assumed that the ^{125}I-C8 used was 70% active. A lower percentage of active C8 would give a higher number of complexes bound per platelet.

DISCUSSION

The experiments reported here demonstrate that normal human and rabbit platelets can initiate formation of the C5-9 complex in the absence of known activators of the complement system. Platelets appeared essential for the formation of C5-9 in this fresh plasma system as there was no evidence for C5-9 complex formation in plasma from which they had been removed. It is not certain whether other plasma factors are also required. The presence of C3 on the platelet membrane suggests a possible role for this protein in the activation process. However, the sucrose density gradient ultracentrifugation techniques employed are not sensitive enough to determine if the bound radiolabeled C3 was in its native form or had been converted to C3b.

The failure of 0.02 M EDTA to block platelet-initiated C5-9 formation suggests that participation of either the classical or alternative pathways of complement activation, in the usual sense, is unlikely. It is possible that one or more components of these pathways may play a role, however. It is also possible that one or more of the constituents of the clotting or fibrinolytic systems play a role in platelet-initiated C5-9 formation, even though the serine esterase inhibitor, PMSF, and the plasmin inhibitor, EACA, failed to block the reaction.

Weksler and Coupal (24) have demonstrated that a factor present in an acid-soluble extract of human platelets can release chemotactically active C5a from C5. At the present time it is not certain whether the property of human platelets that initiates formation of the C5-9 complex is related to the C5-splitting activity described by these investigators. In their experiments the evolution of chemotactic activity was blocked by as little as 0.02 M EACA. Yet 0.2 M EACA failed to block the ability of human platelets to induce formation of the C5-9 complex, suggesting that a different mechanism is involved.

C3 activation, without the participation of C5-9, may induce the release reaction in rabbit or human platelets after complement activation by such agents as zymosan or immune aggregates (1, 14-17). Nevertheless, it is clear from these studies that binding of C3 to PNH or rabbit platelets is not necessary for C5-9 binding (with resultant lysis) after alternative pathway activation of complement with CVF. These findings agree with those of Götze and Müller-Eberhard (23), who have shown

[3] A typical calculation was performed as follows: The specific radioactivity of the ^{125}I-C8 used was 1.6×10^6 cpm/μg. 15% of the platelet-bound ^{125}I-C8 could be eluted and 11.28×10^3 cpm sedimented in the 22S peak on ultracentrifugation. Therefore, $(11.28 \times 10^3)/0.15 = 7.5 \times 10^4$ cpm were incorporated into the C5–9 complexes bound to the 2.4×10^9 platelets in the 15-ml incubation mixture. Therefore, cpm/platelet = $(7.5 \times 10^4$ cpm$/2.4 \times 10^9$ platelets$) = 3.125 \times 10^{-5}$ cpm/platelet and μg of ^{125}I-C8/platelet = $(3.125 \times 10^{-5}$ cpm/platelet$/1.6 \times 10^6$ cpm/μg$) = 1.953 \times 10^{-11}$ μg/platelet. Total (cold plus ^{125}I-labeled) C8 per platelet was calculated as follows: 100 μg of ^{125}I-C8 (70% of which was hemolytically active) was initially added to 15 ml of platelet-rich plasma containing 80 μg/ml $\times$ 15 ml = 1,200 μg of cold C8. Thus the ratio of hemolytically active ^{125}I-C8 to total hemolytically active C8 was $(100$ $\mu g \times 0.7/1{,}200$ $\mu g + (100 \times 0.7)$ $\mu g) = 0.055$. And total bound C8 = $(1.953 \times 10^{-11}$ μg/platelet$/0.055) = 3.54 \times 10^{-10}$ μg/platelet. Molecule of C8/g = [6.02×10^{23} molecule/g-mol (Avogadro's number)$/1.53 \times 10^5$ g/g-mol (mol wt of C8)] $= 3.9 \times 10^{18}$ molecules/g or 3.9×10^{12} molecules/μg. Thus, molecules of C8 per platelet = 3.54×10^{-10} μg C8/platelet $\times$ 3.9×10^{12} molecules/μg = 1.38×10^3 molecules/platelet.

that the $\overline{C423}$ enzyme can effect binding of C5, 6, and 7 to the surfaces of cells distinct from those to which the $\overline{C423}$ enzyme is bound.

The existence of a coagulation defect in the blood of rabbits congenitally deficient in C6 has been previously related to the action of the terminal components on platelet coagulant activity. Activation of the alternative complement pathway with such agents as inulin and endotoxin leads to lysis of rabbit platelets with marked enhancement of their coagulant activity, an effect not seen in the absence of one or more of the terminal components (2, 20, 21). The observations reported here suggest a mechanism by which complement-induced enhancement of platelet coagulant activity might occur in the absence of such extrinsic activators. The platelet, by activating complement at its own surface, could enhance its own coagulant activity, thus accelerating the rate of whole-blood clotting. In C6-deficient blood the clotting time would, therefore, be relatively prolonged.

Though normal human platelets, like rabbit platelets, can initiate formation of the C5-9 complex, they differ from rabbit platelets in being considerably less sensitive to the effects of C5-9. It is therefore not surprising that in human C6 (33) and C7 (34) deficiency, no significant prolongation of the whole-blood clotting time is seen. On the other hand, PNH platelets are more sensitive than normal platelets to the effect of complement (35) and behave much like those of the normal rabbit. The mechanism we have described might therefore play a role in the thrombotic tendency known to complicate PNH (36, 37).

We have calculated that as many as 370–1,380 C5-9 complexes bind per platelet during the 30-min incubations in these experiments. It therefore seems unlikely that a small subpopulation of platelets could be responsible for this reaction. As little as one C5-9 complex has been found sufficient to induce lysis in sheep erythrocytes (38), thus emphasizing the relative resistance of human platelets to the lytic effects of C5-9.

The formation of the C5-9 complex on unwashed platelets in freshly prepared platelet-rich plasma suggests that this phenomenon may be an ongoing in vivo process. A small degree of activation of the clotting system may occur as the blood is drawn and the clotting enzymes so formed participate in platelet-initiated C5-9 formation. Such an occurrence does not mitigate against the same process occurring in vivo in normal individuals, as well as in patients in whom intravascular coagulation is clincally evident. Continuous intravascular thrombin formation in normal individuals has been postulated for three quarters of a century (39, 40), and recent evidence for its occurrence has been provided by the demonstration of small amounts of fibrinopeptide A in the plasmas of normal individuals (41).

At this time the physiological or pathological role(s) of this phenomenon can only be postulated. The failure to demonstrate initiation of C5-9 formation by human erythrocytes indicates that this reaction is not common to all cell membranes. It is possible that modulation of platelet membrane by complement is necessary for proper platelet function. Such a role is consistent with the markedly reduced to absent response to ristocetin recently observed in the platelet-rich plasma of two individuals congenitally deficient in C5 (21). The receptor necessary for aggregation by these agents may not be exposed unless the platelet is acted upon by the C5-9 complex.

A pathologic role for the C5-9 complex in atherogenesis has been suggested by a recent study performed with C6-deficient and normal rabbits fed a cholesterol-rich diet (42). Though both groups of rabbits developed atheromas, the normal group had significantly more advanced disease. The mechanisms by which complement may participate in the formation of atheromas is not certain. However, it is possible that the platelet participates by forming aggregates at the sites of insipient plaques. Platelets in C6-deficient animals might be less prone to form such aggregates because of the absence of complement-dependent platelet membrane receptors.

Total hemolytic complement, as well as immunologically measured C3, have been shown to be decreased in several clinical conditions characterized by disseminated intravascular coagulation (43). Recent studies utilizing rabbits have shown a role for the platelet in the depression of total hemolytic complement and C3 that occurs with intravascular clotting (44). The demonstration here that the platelet can bind C3 and initiate formation of the C5-9 complex is consistent with these in vivo observations. It is possible that these processes may be accelerated secondary to platelet damage induced by intravascular clotting.

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REFERENCES

1. Henson, P. M. 1970. Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. II. Interaction of platelets with neutrophils. *J. Immunol.* **105**: 490–501.
2. Zimmerman, T. S. 1973. The platelet in complement-blood coagulation interaction. *Adv. Biosc.* **12**: 291–310.

3. Gocke, D. J. 1965. *In vitro* damage of rabbit platelets by an unrelated antigen-antibody reaction. II. Studies of the plasma requirement. *J. Immunol.* **94**: 247–252.
4. Des Prez, R. M., and R. E. Bryant. 1969. Two mechanisms of immunologically-induced injury to rabbit platelets. *J. Immunol.* **102**: 241–252.
5. Siqueira, M., and R. A. Nelson, Jr. 1961. Platelet agglutination by immune complexes and its possible role in hypersensitivity. *J. Immunol.* **86**: 516–525.
6. Siraganian, R. P., A. L. Sandberg, A. Alexander, and A. G. Osler. 1973. Platelet injury due to activation of the alternate complement pathway. *J. Immunol.* **110**: 490–497.
7. Horowitz, H. I., R. M. Des Prez, and E. W. Hook. 1962. Effects of bacterial endotoxin on rabbit platelets. II. Enhancement of platelet factor 3 activity in vitro and in vivo. *J. Exp. Med.* **116**: 619–633.
8. Des Prez, R. M. 1967. The effects of bacterial endotoxin on rabbit platelets. V. Heat labile plasma factor requirements of endotoxin-induced platelet injury. *J. Immunol.* **99**: 966–973.
9. Spielvogel, A. R. 1967. An ultrastructural study of the mechanisms of platelet-endotoxin interaction. *J. Exp. Med.* **126**: 235–249.
10. Dodds, W. J., and R. J. Pickering. 1972. Purified cobra venom factor: Effect on blood platelets. *Proc. Soc. Exp. Biol. Med.* **140**: 429–434.
11. Zimmerman, T. S., and H. J. Müller-Eberhard. 1972. Interaction of complement, platelets, and the blood coagulation system. *J. Clin. Invest.* **51**: 107*a*. (Abstr.)
12. Siraganian, R. P. 1972. Platelet requirement in the interaction of the complement and clotting systems. *Nat. New Biol.* **239**: 208–210.
13. Zucker, M. B., and R. A. Grant. 1974. Aggregation and release reaction induced in human blood platelets by zymosan. *J. Immunol.* **112**: 1219–1230.
14. Marney, S. R., Jr., D. G. Colley, and R. M. Des Prez. 1975. Alternate complement pathway induction of aggregation and release of 5-hydroxytryptamine and adenosine diphosphate by rabbit platelets. *J. Immunol.* **114**: 696–703.
15. Zucker, M. B., R. A. Grant, C. A. Alper, I. Goodkofsky, and I. H. Lepow. 1974. Requirement for complement components and fibrinogen in the zymosan-induced release reaction of human blood platelets. *J. Immunol.* **113**: 1744–1751.
16. Pfueller, S. L., and E. F. Lüscher. 1974. Studies of the mechanisms of the human platelet release reaction induced by immunologic stimuli. I. Complement-dependent and complement-independent reactions. *J. Immunol.* **112**: 1201–1210.
17. Pfueller, S. L., and E. F. Lüscher. 1974. Studies of the mechanisms of the human platelet release reaction induced by immunologic stimuli. II. The effects of zymosan. *J. Immunol.* **112**: 1211–1218.
18. Zimmerman, T. S., C. M. Arroyave, and H. J. Müller-Eberhard. 1971. A blood coagulation abnormality in rabbits deficient in the sixth component of complement (C6) and its correction by purified C6. *J. Exp. Med.* **134**: 1591–1600.
19. Zimmerman, T. S., and H. J. Müller-Eberhard. 1971. Initiation of coagulation by complement activation: Generation of platelet associated clot promoting activity. *Blood.* **38**: 791. (Abstr.)
20. Brown, D. L., and P. J. Lachmann. 1973. The behavior of complement and platelets in lethal endotoxin shock in rabbits. *Int. Arch. Allergy Appl. Immunol.* **45**: 193–205.
21. Graff, K. S., J. P. Leddy, and R. T. Breckenridge. 1975. Platelet function in man, a requirement for C5. Proceedings of the Complement Workshop. *J. Immunol.* In press.
22. Kolb, W. P., J. A. Haxby, C. M. Arroyave, and H. J. Müller-Eberhard. 1972. Molecular analysis of the membrane attack mechanism of complement. *J. Exp. Med.* **135**: 549–566.
23. Götze, O., and H. J. Müller-Eberhard. 1970. Lysis of erythrocytes by complement in the absence of antibody. *J. Exp. Med.* **132**: 898–915.
24. Weksler, B. B., and C. E. Coupal. 1973. Platelet-dependent generation of chemotactic activity in serum. *J. Exp. Med.* **137**: 1419–1430.
25. Masouredis, S. P. 1972. Clinical use of blood and blood products. Clinical use of whole blood and clinical use of erythrocyte preparations. *In* Hematology. W. J. Williams, E. Beutler, A. J. Erslev, and R. W. Rundles. McGraw-Hill Book Company, New York. 1308–1321.
26. Zimmerman, T. S., and H. L. Spiegelberg. 1975. Pneumococcus-induced serotonin release from human platelet. Identification of the participating plasma/serum factor as immunoglobulin. *J. Clin. Invest.* **56**: 828–834.
27. Nilsson, U. R., and H. J. Müller-Eberhard. 1965. Isolation of β_{1H}-globulin from human serum and its characterization as the fifth component of complement. *J. Exp. Med.* **122**: 277–298.
28. Manni, J. A., and H. J. Müller-Eberhard. 1969. The eighth component of human complement (C8). Isolation, characterization and hemolytic efficiency. *J. Exp. Med.* **130**: 1145–1160.
29. Hadding, U., and H. J. Müller-Eberhard. 1969. The ninth component of human complement. Isolation, description and mode of action. *Immunology.* **16**: 719–735.
30. Müller-Eberhard, H. J., and K.-E. Fjellström. 1971. Isolation of the anticomplementary protein from cobra venom and its mode of action on C3. *J. Immunol.* **107**: 1666–1672.
31. McConahey, P. J., and F. J. Dixon. 1966. A method of trace iodination of proteins for immunologic studies. *Int. Arch. Allergy Appl. Immunol.* **29**: 185–189.
32. Kolb, W. P., and H. J. Müller-Eberhard. 1973. The membrane attack of complement. Verification of a stable C5-9 complex in free solution. *J. Exp. Med.* **138**: 438–451.
33. Heusinkveld, R. S., J. P. Leddy, M. R. Klemperer, and R. T. Breckenridge. 1974. Hereditary deficiency of the sixth component of complement in man. II. Studies of hemostasis. *J. Clin. Invest.* **53**: 554–558.
34. Boyer, J. T., E. P. Gall, M. E. Norman, U. R. Nilsson, and T. S. Zimmerman. 1975. Hereditary deficiency of the seventh component of complement. *J. Clin. Invest.* **56**: 905–914.
35. Aster, R. H., and S. E. Enright. 1969. A platelet and granulocyte membrane defect in paroxysmal nocturnal hemoglobinuria. Usefulness for the detection of platelet antibodies. *J. Clin. Invest.* **48**: 1199–1210.
36. Crosby, W. H. 1953. Paroxysmal nocturnal hemoglobinuria. Relation of the clinical manifestations to underlying pathogenic mechanisms. *Blood.* **8**: 769–812.
37. Payne, P. R., J. M. Holt, and P. B. Neame. 1968. Paroxysmal nocturnal haemoglobinuria parturition complicated by presumed hepatic vein thrombosis. *J. Obstet. Gynaecol. Br. Commonw.* **75**: 1066–1068.

38. Kolb, W. P., and H. J. Müller-Eberhard. 1974. Mode of action of human C9: adsorption of multiple C9 molecules to cell bound C8. *J. Immunol.* **113**: 479–488.
39. Nolf, P. 1908. Contribution a l'étude de la coagulation du sang (5e mémoir). La fibrinolyse. *Arch. Int. Physiol. Biochim.* **6**: 306–539.
40. Astrup, T. 1958. The haemostatic balance. *Thromb. Diath. Haemorrh.* **2**: 347–357.
41. Nossel, H. L., I. Yudelman, R. E. Canfield, V. P. Butler, Jr., K. Spanondis, G. D. Wilner, and G. D. Qureshi. 1974. Measurement of fibrinopeptide A in human blood. *J. Clin. Invest.* **54**: 43–53.
42. Geertinger, P., and H. Sørensen. 1975. On the reduced atherogenic effect of cholesterol feeding in rabbits with congenital complement (C6) deficiency. *Artery.* **1**: 177–184.
43. Tomar, R. H., and D. Kolchins. 1972. Complement and coagulation. Serum β1c-β1a in disseminated intravascular coagulation. *Thromb. Diath. Haemorrh.* **27**: 389–395.
44. Kalowski, S., E. L. Howes, Jr., W. Margaretten, and D. G. McKay. 1975. Effects of intravascular clotting on the activation of the clotting system. The role of the platelet. *Am. J. Pathol.* **78**: 525–536.