HUMAN COMPLEMENT IN
THROMBIN-MEDIATED PLATELET FUNCTION
Uptake of the C5b-9 Complex*

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We have previously reported (1) that thrombin-induced platelet aggregation and release is enhanced in the presence of complement. Only components C3, C5, C6, C7, C8, and C9 are required. No known activating mechanism of the classical or alternative pathways is involved in this reaction. This system enters the known complement sequence at the C3 stage. Because the sequence of addition of thrombin and C3-C9 was important, we postulated that the thrombin initially reacted with the thrombin receptor on the platelet surface, and then activated the complement sequence directly on the platelet surface. In the present communication we will make three major points. First, we will present data which demonstrate that if complement is activated by an enzyme such as trypsin for which the platelet has no receptor, no enhancement of platelet function is obtained subsequent to the addition of complement. Second, when complement is activated on the platelet surface by thrombin, C5b-9 complexes are produced which can be eluted from the platelet membrane and can be demonstrated physicochemically as 33S multimolecular complexes. These multimolecular complexes can be visualized ultrastructurally. Third, we will present data demonstrating that the complement-induced enhancement of platelet function may be mediated via the arachidonic acid transformation pathway because it is inhibited by known inhibitors of cyclo-oxygenase such as aspirin and indomethacin.

Materials and Methods

Preparation of Washed Platelets and Subcellular Fractions. A suspension of human platelets was obtained using the Ardlie buffer system modified as previously described (1). Platelet membranes were prepared previously as described (2).

Preparation of Aluminum Hydroxide-absorbed Serum. Fresh human serum was absorbed with aluminum hydroxide as previously described (3) under conditions that were shown to remove prothrombin but not to inactivate complement. Serum was considered to be prothrombin-free if no clot was formed after incubation with fibrinogen (1.5 mg/ml) for 48 h. Assays were performed to measure the activity of whole complement and components of both the classical and alternate mechanism as previously described (3).

Preparation of Complement Components. C3 (4), C4 (5), C5 (6), C6, C7 (7), C8 (8), and C9 (9) were prepared by methods described earlier. In addition, in some experiments purified complement components prepared by Cordis Laboratories Inc., Miami, Fla. were utilized.

Radiolabeling of the Fifth Component of Complement (C5). C5 was labeled with I251 by the method of McConahey and Dixon (10). The sp act was = 100,000 cpm/μg.

Platelet Aggregation. Assays for platelet aggregation were performed in a Payton dual channel

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aggregometer using a Riken Denshi recorder (Payton Associates Inc., Buffalo, N. Y.). Platelets were suspended at 200,000/μl in Ardlie II buffer in which bicarbonate was replaced with Tris (1). All reagents added to the washed platelets were dialyzed before use with cacodylate buffer, pH 7.4, a buffer shown to be optimal for aggregation of washed platelets (11). Highly purified human thrombin (2.05 U/μg) was prepared and kindly supplied by Dr. John Fenton, New York State Department of Health, Albany, N. Y. 0.1 U (50 ng) in 10 μl was added to 0.3 ml washed platelets. Trypsin (0.245 U/μg) was purchased from Worthington Biochemical Corp., Freehold, N. J. Various concentrations in 10-μl vol were added to 0.3 ml washed platelets. The mixture was stirred for 5 min, then twice the concentration of soybean trypsin inhibitor was added (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.).

Release of [14C]Serotonin. Platelets suspended in plasma were labeled with [14C]serotonin by the method of Valdorf-Hansen and Zucker (12). The radiolabeled platelets were then washed in the usual way (1, 13). For the experiment, 0.3 ml of the washed platelet suspension was utilized to which 10-μl aliquots of various reagents were added. Aggregation was recorded for 5 min, then the tube was centrifuged and the supernate removed. 14C was counted in both the supernate and the cell button in a Packard Liquid Scintillation Counter (Packard Instrument Co., Inc., Downers Grove, Ill.) and the percentage of release was calculated.

Preparation of Complement Source Lacking Anti-Thrombin III or Albumin. Antibodies to anti-thrombin III and anti-albumin (Behring Diagnostics, American Hoechst Corp., Somerville, N. J.) were conjugated to Sepharose 4B (Pharmacia Fine Chemicals, Div. of Pharmacia, Inc., Piscataway, N. J.) by the method of Cuatrecasas (14) and 0.2 ml of the conjugated sepharose was added to 1 ml of aluminum hydroxide-absorbed serum. The tube containing the mixture was clipped onto a rotator and was allowed to rotate for 60 min at 4°C. The tubes were then centrifuged and the supernatant serum was recovered. 10 μl of the serum absorbed either with anti-anti-thrombin III or with anti-albumin was added to 0.3 ml platelet suspension after the addition of 0.1 U thrombin.

Preparation of Whole Platelet-Thrombin-C5b-9 Complexes. 5 U of thrombin was added to 6 ml platelet suspension (200,000 platelets/μl). To this mixture was added 0.2 ml each of C3, C6, C7, C8, and C9; and 0.3 ml [12SI]C5 containing 1.4 × 10⁷ cpm. A second tube contained 6 ml platelets, 5 U of thrombin and 0.4 ml of aluminium hydroxide-absorbed serum as the source of complement. A similar amount of [12SI]C5 was added to the mixture. Two sets of controls were used. One contained the same reagents as the test sample but included heparin at 10 U/ml. The second control included platelets and complement components including [12SI]C5, but contained no thrombin.

Samples were incubated at 37°C for 45 min with constant shaking. The tubes were then centrifuged for 15 min at 23,300 g. The supernate was removed and the platelet button was washed five times with phosphate-buffered saline, pH 7.4 (PBS). Each centrifugation was for 15 min at 23,300 g. After the last centrifugation, 0.3 ml PBS containing 0.1% Triton X-100 was added to the cell button. The samples were then frozen and thawed five times, and finally centrifuged at 43,500 g for 2 h, and the supernate recovered.

Preparation of Platelet Membrane-Thrombin-C5b-9 Complexes. Platelet membranes from 7 U of blood were utilized. 1 ml modified Ardlie II buffer (1) was added to the membrane button with thorough mixing, and 0.25 ml was then aliquoted into each of four tubes. 3.75 ml of the same buffer was added to each tube. To two tubes was added 10 U thrombin and 0.7 ml aluminium hydroxide-absorbed serum further absorbed with anti-anti-thrombin III (Results) containing 0.5 ml [12SI]C5 (1.8 × 10⁷ cpm). To the other two tubes was added 10 U thrombin and 0.4 ml each of C3, C6, C7, C8, and C9; and 0.5 ml [12SI]C5. Heparin at 10 U/ml was added to one tube of each pair. In some experiments, instead of the heparin-containing control, control tubes contained platelet membranes and aluminium hydroxide-absorbed serum or purified complement components and [12SI]C5, but no thrombin.

All four tubes were incubated at 37°C for 30 min with rotation. The samples were then centrifuged for 3 h at 37,500 g. The cell button was washed three times in PBS, each centrifugation being 2 h at 121,000 g. Elution of the putative C5b-9 complexes was performed according to the method of Biesecker et al. (15). After the final wash, 1.0 ml of 0.05 M Tris-acetate buffer, pH 8.9, containing 10% deoxycholate (DOC) (Sigma Chemical Co., St. Louis,

Abbreviations used in this paper: DOC, deoxycholate; PBS, phosphate-buffered saline.
Mo.) was added to the membranes. After a thorough mixing process, the mixture was allowed to stand at room temperature for 2 h and was then centrifuged at 121,000 g for 3.5 h. The supernate was removed and 0.7 ml was applied to a Bio-Gel A15 m (Bio-Rad Laboratories, Richmond, Calif.) column (23.5 × 1 cm) equilibrated in 0.05 M Tris-acetate buffer, pH 8.6, containing 2% DOC. 0.5 ml fractions were collected and 0.3 ml of the peak tube from the first peak of radioactivity to elute from the column was subjected to density-gradient ultracentrifugation.

Sucrose Density-Gradient Ultracentrifugation. Two methods of sucrose density-gradient ultracentrifugation were employed. 10-40% linear, sucrose density gradients were prepared with barbital-buffered saline, pH 7.4, ionic strength 0.15, in 5-ml cellulose nitrate tubes; or 10-50% linear gradients were prepared with 0.05 M Tris-acetate buffer, pH 8.6, containing 1% DOC (15). The sample size was 0.2-0.3 ml and centrifugation was performed in a Beckman 50.1 rotor (Beckman Instruments Inc., Science Essentials Co., Mountainside, N. J.) for 18 h at 96,000 g. Seven-drop fractions were obtained with a Buchler (Buchler Instruments Div., Searle Diagnostics, Fort Lee, N. J.) piercing unit and counted in a Searle gamma counter (model 1185, Searle Radiographies Inc., Des Plaines, Ill.).

Preparation of C5b-9 Complexes for Electron Microscopy. Samples of the peak tubes from the appropriate area of the density gradients were pooled and dialyzed overnight against Tris-acetate buffer, pH 8.6, containing 1% DOC. Appropriate dilutions were prepared and the samples were stained with 2% sodium silicotungstate, then directly applied to a collodion-and-carbon-coated grid and viewed in a Philips 301 Electron Microscope (Philips Electronic Instruments, Inc., Mahwah, N. J.).

Treatment of Platelets with Aspirin and Indomethacin. Two inhibitors of cyclo-oxygenase were used in this study, namely, aspirin (acetylsalicylic acid) and indomethacin. Both drugs were obtained from Sigma Chemical Co. They were dissolved in ethanol, then diluted to the desired concentration with Ardlie II buffer. Two methods of treatment were employed. First, platelets were pretreated with the drug. For this treatment aspirin (100 μM) or indomethacin (28 μM) was added to the platelet-rich plasma and the mixtures were incubated at 37°C for 30 min. Platelets were then washed in the usual manner. The second method was to have the drugs present in the reaction mixture, and for this method, the same final concentration of the drugs was employed. 0.3 ml washed platelet suspension was pipetted into the aggregometer cuvette. An appropriate amount of the drug was added in a 10-μl vol then allowed to incubate for 15 min at 37°C before either thrombin alone or thrombin followed by a source of complement was added.

In Vivo Aspirinization of Platelets. Normal volunteer donors ingested 2 × 300 mg aspirin tablets ≈10 h before donating blood to be used for the platelet preparation.

Results

[^14C]Serotonin Release Mediated by Trypsin and Thrombin. As shown in Fig. 1, 500 ng (0.12 U) of trypsin induced 33% release of serotonin. This finding agrees with that obtained by Ganguly (16). The addition of C3, C5, C6, C7, C8, and C9 caused very little additional release of serotonin. On the other hand, 50 ng (0.1 U) of thrombin induced 20% release of serotonin, but the addition of the same purified complement components, increased serotonin release threefold (62%).

The Effect on Complement-dependent Thrombin-induced Serotonin Release of Removal of Anti-Thrombin III from the Complement Source. It has been previously reported (17) that anti-thrombin III inhibits the C5b, 6, 7 complex. Therefore, it was of interest to determine the effect of the removal of this inhibitor on the complement-dependent serotonin release reaction. Aluminum hydroxide-absorbed serum was further absorbed with antibody to anti-thrombin III conjugated to Sepharose. A second aliquot of the serum was absorbed with anti-albumin conjugated to Sepharose under identical conditions to serve as the control. The results of three separate experiments are shown in Fig. 2. Removal of anti-thrombin III caused an ≈15–20% increase in complement-dependent thrombin-mediated release of serotonin over that obtained with the control anti-
aluminum-absorbed serum.

**Physicochemical Demonstration of the Presence of C5b-9 Complexes on the Platelet Surface; the Formation and Uptake Mediated by Thrombin.** In the initial experiments a suspension of whole platelets was utilized. The platelet suspension was incubated with thrombin and either purified C3, C5, C6, C7, C8, and C9 or aluminum hydroxide-absorbed serum as the source of complement. Radioactively labeled C5 was added to each. After a thorough washing procedure, the putative complexes were eluted from the platelets with Triton X-100 plus freezing and thawing, and the eluate was subjected to density-gradient ultracentrifugation. As shown in Figs. 3 A and 3 B, no radiolabeled C5 was found in the 33S region which would have indicated C5b-9 complex formation (15, 18). Instead, 90% of the C5 in the case of aluminum hydroxide-absorbed serum and 50% when the purified components were utilized was found in a position midway between the two markers, sheep hemoglobin (4-5S) and IgG(7S). This observation suggested that the C5 was cleaved by some platelet-associated proteolytic enzyme because native C5 has an $s$ of 8.7S.

To overcome this probable proteolytic degradation of C5, a similar experiment was performed in which a preparation of isolated platelet membranes was used instead of whole platelets. Figs. 4 and 5 demonstrate the results of such experiments.

Fig. 4 demonstrates the results of molecular-sieve chromatography on Bio-Gel A15m of the platelet eluate. The peak tube from the first peak (arrow) to elute from the column was subjected to sucrose density-gradient ultracentrifugation. The results of this experiment are shown in Fig. 5. Complex formation was indicated when $[^{125}I]C5$ eluted in the heavy part of the gradient. Two peaks of radioactivity were identified. The heavier peak had an $s$ of 33S and the lighter peak, of 29S. A similar result was obtained whether the source of complement was aluminum hydroxide-
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FIG. 2. The effect of removal of anti-thrombin III from the complement source on thrombin-induced release of platelet serotonin. Th, thrombin; AIS, fresh human serum absorbed with aluminum hydroxide; R-Alb, further absorbed with anti-albumin; R-anti-Th III, AIS further absorbed with antibody to anti-thrombin III (A) and (B) indicate sequence of addition.

absorbed serum or purified components C3, C5, C6, C7, C8, and C9.

Morphological Demonstration of C5b-9 Complexes Eluted from the Platelet Membrane. Appropriate fractions from the sucrose density gradient were dialyzed to remove sucrose, then stained and prepared for electron microscopy. The material with a sedimentation rate of 33S was a suspension of lesions (Fig. 6). It has recently been reported that complement-dependent lesions previously seen on various cell types were dimers of the C5b-9 complex with a sedimentation rate of 33.5S (15). The material which eluted from the density gradient 33S peak was morphologically identical to the C5b-9 dimer previously reported (15). Some similar structures were also seen in the material that eluted at 29S.

No complexes were demonstrable in controls lacking thrombin or in the control samples containing heparin.

Inhibition of the Complement-mediated, Thrombin-dependent Platelet Function by Drugs. The effect of aspirin and indomethacin on the complement-dependent release of serotonin was determined in two ways. First, platelets in plasma were incubated with the drugs and then the platelets were washed free of the drugs then treated with thrombin with and without purified components C3–C9. As shown in Fig. 7, aspirin at 100 μM inhibited the complement-induced enhancement of serotonin release. However, indomethacin (28 μM) had little or no effect. The second method of treatment was to treat the washed platelets with the drugs, then to add either thrombin alone or thrombin together with complement when the drugs were still present. Fig. 8
Fig. 3.Sucrose density ultracentrifugation of native [\textsuperscript{125}I]C5 and [\textsuperscript{125}I]C5 after incubation with whole platelets in the presence of thrombin and a source of complement. A 10-40% linear sucrose gradient was employed. (A), Aluminum hydroxide-absorbed serum used as a source of complement. (B), The complement source was highly purified C3, C5, C7, C8, and C9 which were added to the platelets together with [\textsuperscript{125}I]C5 and thrombin. (O), [\textsuperscript{125}I]C5; ( ), native [\textsuperscript{125}I]C5. Fractions were counted until statistical significance was achieved.

demonstrates the results of two such experiments. Both aspirin and indomethacin inhibited the complement effect. Neither drug caused inhibition of the thrombin-mediated release in the absence of complement (Figs. 7 and 8).

In two separate experiments, ingestion of aspirin by the platelet donor inhibited
Discussion

The present study was prompted by several previous observations. In an earlier communication (1) we demonstrated the effect of complement on thrombin-mediated platelet aggregation and release. As a result of these observations, we postulated that thrombin first reacts with the thrombin receptor at the platelet surface and then activates complement. To support this supposition we looked for another enzyme which induces platelet aggregation and release and which activates complement but for which the platelet has no receptor. Such an enzyme is trypsin. As demonstrated in the present study, trypsin-mediated platelet function is little affected subsequent to the addition of complement. These observations strengthen the premise that binding of the complement-activating enzyme to the platelet membrane is a necessary step for the enhancement effect. A second observation made in our earlier studies (1) indicated that complement components C3, C5, C6, C7, C8, and C9 were required for complement-induced enhancement of thrombin-mediated platelet function. It appeared probable that C5b-9 complexes were forming on the platelet membrane. Attempts were made, therefore, to elute the putative complexes from the platelet membrane by a modification of the method previously described for the elution of...
C5b-9 complexes from platelets (19). Elution of radiolabeled C5 from whole platelets in six separate experiments revealed that 50–80% of the radioactive material appeared in a position on the density gradient, midway between hemoglobin (4.5S) and IgG(7S). Thus, it appeared that native C5, which has a sedimentation rate of 8.7S, was cleaved by some platelet-associated proteolytic enzymes.

Previous studies by Weksler and Coupal (20) have demonstrated that a human platelet granule protease cleaves C5 in plasma to generate a chemotactic fragment. It is not known whether the proteolytic cleavage obtained in this study was the same. The nature of the low molecular weight material has not been determined. The sedimentation rate does not correspond to the molecular weight of C5b (180,000) or C5a (11,000). It is presumably material resulting from further cleavage of the C5b molecule. This low molecular weight material might correspond to one or more of the fragments cleaved from the α-chain of C5 by trypsin (21). One fragment has a mol wt of ≈33,000 and a second of ≈22,000. Both fragments originate from the same end of the C5 α-chain. Removal of the larger of the two results in generation of C5 inter α-βIII; removal of the smaller results in generation of C5 inter α-βII.

In an attempt to obviate the platelet enzymatic activity, experiments were performed with isolated, washed platelet membranes. After incubation with thrombin and complement, an eluate of the platelet membrane was prepared utilizing DOC by a recently described method (15). The eluate was subjected to molecular-sieve chromatography and then an appropriate sample was applied to a sucrose density gradient. After ultracentrifugation, two peaks of radioactivity were obtained. The material eluting at 33S was shown to be morphologically identical to the C5b-9 dimer previously described (15, 22). A second peak of radioactivity (29S) to elute from the gradient may have represented incomplete C5b-9 dimers or complexes which had dissociated and reassociated during the ultracentrifugation. These observations

Figure 5. Sucrose density ultracentrifugation of sample from Bio Gel A15m column. 10–50% linear sucrose gradient containing 1% DOC was employed. (●), [125I]Cr5; (○), native [125I]Cr5. Fractions were counted until statistical significance was achieved.
Fig. 6. Electron microscopical analysis of fractions from density gradient depicted in Fig. 5. The fractions were dialyzed to remove sucrose, stained with 2% sodium silicotungstate, then applied directly to a grid coated with collodion and carbon. (A), × 84,000. (B), × 224,000. The black square in (A) indicates the area shown in (B). The micrographs illustrate a suspension of C5b-9 dimers, some of which are settled flat on the grid; others are seen in a side view. The arrows indicate typical dimers of the C5b-9 complex which are settled flat on the grid.
agree with our previous report (3) that ultrastructural lesions could be seen on the surface of platelets which had interacted with thrombin and complement.

Thus, we have identified by sucrose density ultracentrifugation the presence of C5b-9 complexes in an eluate from platelets which had interacted with thrombin and complement. The complexes were identified both physicochemically and morphologically. Previously, thrombin-mediated uptake of C3 and C5 was demonstrated utilizing radiolabeled components and morphologically using ferritin as a marker (1). The latter (1, 3) together with the present study demonstrate that thrombin mediates uptake of C3 and C5b-9 dimers by the platelet membrane and the presence of these complement components leads to a pronounced enhancement of platelet function.

We have earlier shown (1) that the platelet membrane is an integral component of this system, neither cellular uptake of C3 and C5 nor ultrastructural membrane-bound lesions could be demonstrated when the platelets were substituted for by either erythrocytes or leukocytes. Because activating components of either the classic or alternative pathways of complement are not required, this third mechanism of activation of complement differs from the two major pathways previously described. A working concept of the C5 convertase system is indicated in Fig. 9. Whereas C4b,2a,3b is C5 convertase of the classic pathway and C3b,Bb,P is the C5 convertase of the alternative pathway, C5 convertase of this thrombin-dependent pathway is composed of thrombin, C3b, and the platelet membrane. The need for C3 in the thrombin-induced C5 convertase is surprising because it is known that thrombin will
No Drug  Aspirin (100 uM)  Indomethacin (28 uM)

Percent release of [14C] serotonin

(A) Th Th  (B) Th Th

No Drug  Aspirin (100 uM)  Indomethacin (28 uM)

Percent release of [14C] serotonin

(A) Th  (B) Th

Fig. 8. The effect of aspirin and indomethacin on thrombin-induced release of platelet serotonin in the presence and absence of complement when the drugs are present in the reaction mixture (Results).

Fig. 9. Diagrammatical representation of C5 convertase produced by various mechanisms of activation; C4b,2a,3b by the classical mechanism; C3b,Bb,F by the alternative mechanism; C3b, thrombin, platelet membrane by the mechanism described in this paper. Illustration modified from (23) with permission.
directly cleave C5 (24). However, the cleavage of C5 by thrombin is probably different from that produced by C4b,2a,3b because thrombin cleavage of C5 does not result in the production of anaphylatoxin activity (24). The presence of C3b adjacent to the C4b,2a complex modifies the specificity of the complex from a C3 convertase to a C5 convertase. It is, therefore, tempting to speculate that the C3b molecule adjacent to the thrombin molecule on the platelet membrane modifies the specificity of thrombin from a C3 convertase to a C5 convertase.

Because thrombin can activate platelets by different mechanisms, it was of interest to determine which mechanism was involved in the complement enhancement. Aspirin and indomethacin are both inhibitors of the enzyme cyclo-oxygenase of the arachidonic acid transformation pathway. When platelets were pretreated with these drugs, only aspirin led to the inhibition of complement-enhanced aggregation and release of serotonin. However, when the drugs were present in the reaction mixture, both inhibited the effect. Ingestion of aspirin by the donor of the platelets also inhibited the complement effect. Thus, it appears that thrombin-mediated complement-dependent aggregation and release of serotonin acts via the arachidonic acid transformation pathway. The mechanism by which cell-bound C5b-9 may influence cyclo-oxygenase activity remains to be determined.

Summary

Thrombin-mediated platelet aggregation and release is enhanced by the presence of C3, C5, C6, C7, C8, and C9 of human complement. The interaction of thrombin with its receptor on the platelet membrane initiates activation of complement on the platelet surface. Trypsin-mediated platelet function is not enhanced by the addition of complement, probably because trypsin has no receptor on the platelet surface so activation of complement is triggered in the fluid phase and not on the platelet surface. Activation of complement by thrombin led to production of dimers of the C5b-9 complex on the platelet surface. These complexes were eluted from the platelet membrane and were identified physicochemically and morphologically.

The mechanism of complement-induced enhancement of platelet function is not clear, however, it probably is mediated via the arachidonic acid transformation pathway because this activity was blocked by known inhibitors of cyclo-oxygenase, namely, aspirin and indomethacin.

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