Prothrombin Complex Proteins as Cofactors in Platelet Aggregation. I. Inhibition of Aggregation by Antiserum

By John B. Miale and Jessie W. Kent

Data are presented to support the concept that the vitamin K-dependent coagulation factors are adsorbed onto the platelet membrane and that these make up part of the "plasma atmosphere" necessary for the aggregation of platelets by various agents. Together with evidence that other coagulation factors also are part of the plasma atmosphere, it is suggested that the aggregation reaction is part of the coagulation sequence. The immunologic approach to demonstrating constituents of the platelet membrane promises to be a highly specific technique for studying further the constituents of the platelet membrane and their reactions in hemostasis.

The role of plasmatic cofactors for platelet aggregation has been investigated by many. The reports that platelet aggregation is diminished in platelet-rich plasma from subjects with congenital afibrinogenemia and in vitro studies focused attention on fibrinogen, plasmatic or intracellular, as a cofactor. Recent investigations of platelet aggregation in von Willebrand's disease indicate that failure of ristocetin to aggregate these platelets can be corrected by adding factor VIII, which then may be the plasmatic cofactor in this very special circumstance.

The data presented here indicate that the vitamin K-dependent coagulation factors II, VII, IX, and X (for convenience, prothrombin complex) are adsorbed onto the platelet membrane and are cofactors for the aggregation of normal platelets in platelet-rich plasma by ADP, epinephrine, collagen, and thrombin and for the aggregation of washed normal platelets by bovine fibrinogen.

MATERIALS AND METHODS

Aggregation was measured in the Platelet Aggregometer Model 300, Chrono-Log Corp., Broomall, Pa., equipped with a recorder and integrator (Linear Instrument Corp.), Model 703. All tests were performed at 37°C using a sample volume of 0.5 ml.

Platelet-rich plasma (PRP) was obtained from centrifugation at 1700 rpm for 7 min of fresh citrated (3.8% sodium citrate, 0.129 M, 1 part to 9 parts of venous blood) normal human blood obtained from donors carefully screened for intake of aspirin and other drugs known to affect platelet aggregation.

Washed platelets (WP) were obtained in the following way: PRP was centrifuged at 4000 rpm for 10 min, the sedimented platelets washed three times with 0.85% saline (0.1434 M) and re-suspended in saline to a concentration of 50,000-60,000 platelets/cu mm.

Epinephrine was obtained from Parke-Davis adrenalin chloride solution, containing 1 mg epinephrine/ml.
Adenosine diphosphate (ADP) was adenosine-5'-diphosphate-disodium (2 H2O), obtained from Schwarz-Mann. A 4.57-mM solution in 0.85% saline was diluted as needed, and 0.02 ml was added to 0.5 ml of PRP or WP.

Collagen was obtained from bovine Achilles tendon (Sigma Chemical Co., St. Louis, Mo.), prepared according to Hovig, and modified in the following way: 4 of reagent powder was added to 100 ml of chilled 0.85% saline and mixed in a Waring Blender for 5 min at maximum speed. The suspension was centrifuged at 4000 rpm for 5 min at 4°C and the supernate centrifuged once more at 4000 rpm for 5 min at 4°C. The milky supernate was frozen in aliquots at −105°F and used as indicated.

Thrombin used was human, Fibrinex (Ortho Pharmaceutical Co., Raritan, N.J.), reconstituted and diluted with 0.85°C saline to contain 7.5 U/ml. Working solutions were prepared by diluting the stock solution with saline.

Fibrinogen used was bovine fibrinogen (General Diagnostics, Morris Plains, N.J.) reconstituted with equal parts of H2O and saline to a concentration of 150 mg/100 ml.

Antiprothrombin complex antiserum (anti II-VII-IX-X) was obtained in the following way: The antigen was prepared by adsorption of fresh human oxalated plasma with BaSO4 powder (50 mg/ml of plasma), elution from the BaSO4 with a small volume of 0.2 M sodium citrate, reabsorption of the eluate with dry Sephadex DEAE A-50 (Pharmacia Fine Chemicals, Piscataway, N.J.) 30 mg/ml of eluate, and elution from the Sephadex with borate buffer (0.05 M boric acid-NaOH, 1 M NaCl added). The antigen averaged 1 g of protein/100 ml, and individual assays for factors II, VII, IX, and X ranged from a 40-fold concentration of factor II to a 200-fold concentration of factor IX. There was no demonstrable factor V, factor VIII, factor XI, or factor XII, nor any thrombin activity. By immunoelectrophoresis against specific antisera, the antigen showed no precipitin lines with antifibrinogen, anti-β-lipoprotein, anti-IgG, anti-IgM, anti-C3, antihaptoglobin, anticeruloplasmin, antitransferrin, or anti-α-trypsin.

The antiserum was prepared in young female virgin albino rabbits by three intramuscular injections of 1 ml of antigen mixed with 1 ml of Al(OH)3 suspension (Amphogel, Wyeth Laboratories, Inc., Philadelphia, Pa.) and one injection of 1 ml intravenously at 10-day intervals. Ten days after the last injection the rabbits were bled by cardiac puncture and the serum separated and frozen in aliquots at −104°F.

The antisera were assayed for both coagulation-inhibition activity and immunologic specificity. Coagulation-inhibition activity was assayed by determining the inhibition of a modified one-stage prothrombin time test, using normal oxalated plasma diluted with BaSO4-adsorbed plasma to a prothrombin time of about 40 sec, incubated for 15 min at 37°C with antiserum serially diluted with the plasma and previously adsorbed with BaSO4 powder (0.1 M sodium oxalate added, 9 parts of antiserum per 1 part of sodium oxalate, then adsorbed with BaSO4 powder, 0.1 g/ml of serum) and a prothrombin time performed on the plasma-antiserum mixture using Simplastin. The antiserum used for these studies (R80) was tested at dilutions of 1:10, 1:20, 1:30, 1:40, and 1:50. A typical inhibition study is shown in Table 1; there is very marked prolongation of the prothrombin time.

Table 1. Inhibition of the Prothrombin Time of Normal Plasma by Anti-prothrombin Complex Antiserum

<table>
<thead>
<tr>
<th>Plasma* (ml)</th>
<th>Antiserum† Concentration</th>
<th>Thromboplastin-† Calcium (ml)</th>
<th>Prothrombin Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0</td>
<td>0.2</td>
<td>41.5</td>
</tr>
<tr>
<td>0.1</td>
<td>1.10</td>
<td>0.2</td>
<td>328.4</td>
</tr>
<tr>
<td>0.1</td>
<td>1.20</td>
<td>0.2</td>
<td>86.6</td>
</tr>
<tr>
<td>0.1</td>
<td>1.30</td>
<td>0.2</td>
<td>59.5</td>
</tr>
<tr>
<td>0.1</td>
<td>1.40</td>
<td>0.2</td>
<td>51.9</td>
</tr>
<tr>
<td>0.1</td>
<td>1.50</td>
<td>0.2</td>
<td>49.4</td>
</tr>
</tbody>
</table>

*Normal citrated human plasma diluted with normal BaSO4-adsorbed human plasma.
†Antiserum dilutions made in dilute plasma, incubated for 15 min at room temperature and the clotting time determined immediately after the 15-min incubation period.
‡Simplastin (General-Diagnostics, Morris Plains, N.J.).
Table 2. Inhibition of Assays of Prothrombin Complex Factors by Anti-prothrombin Complex Antiserum*

<table>
<thead>
<tr>
<th>Factor</th>
<th>Normal Plasma</th>
<th>Normal Plasma + Antiserum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II</td>
<td>100%</td>
<td>42%</td>
</tr>
<tr>
<td>Factor VII</td>
<td>&gt;100%</td>
<td>1.2%</td>
</tr>
<tr>
<td>Factor IX</td>
<td>&gt;100%</td>
<td>1.1%</td>
</tr>
<tr>
<td>Factor X</td>
<td>100%</td>
<td>0.5%</td>
</tr>
</tbody>
</table>

*Normal citrated plasma diluted 1:10 with buffered saline, 0.1 ml, plus antiprothrombin complex antiserum, 0.02 ml, incubated at room temperature for 15 min.

Thrombin time by 1:10 antiserum, and the effect is still evident at an antiserum concentration of 1:50. A similar system was used to determine inhibition of the partial thromboplastin time. A 1:10 dilution of antiserum prolonged the PTT by 80%, a 1:20 dilution by 50%. The antiserum was also strongly inhibitory in individual assay systems for factors II, VII, IX, and X (Table 2). Normal human citrated plasma diluted 1:10 with buffered saline was incubated for 15 min at room temperature with the antiserum and then assayed for factors II, VII, IX, and X. As shown in Table 2, there was significant reduction in the assays of each of the factors, but the specificity of the assays in the presence of polyvalent antibody is questionable.

Occasional batches of antiserum showed a faint precipitin line when tested against human fibrinogen by immunoelectrophoresis and gel diffusion, but this minor antifibrinogen component could be easily removed by adsorption onto an excess of human fibrinogen. Immunoelectrophoretic

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![Fig. 1. Composite tracings, aggregation and inhibition of aggregation in PRP. (A) Controls: (1) thrombin alone, (2) collagen alone, (3) ADP, (4) epinephrine, (5) R80 antiprothrombin complex antiserum. (B) Inhibition of aggregation, preincubation with R80 antiserum followed by (1) thrombin, (2) epinephrine, (3) ADP, (4) collagen.](image-url)
analysis of the antiserum (R80) against the antigen used for injection showed four distinct precipitin lines, but no claim can be made at this time that these correspond to the four factors of the prothrombin complex.

Normal rabbit serum, for control, was obtained by cardiac puncture from a normal donor albino rabbit. It was adsorbed with BaSO₄ prior to use, as with the antiserum.

Monospecific antisera used were the following: antihuman fibrinogen (Hyland, Costa Mesa, Calif), antihuman IgG (Hyland), antihuman α-1 lipoprotein (Hoechst Pharmaceutical Co., Kansas City, Mo.) and antihuman complement C3 (Hyland).

RESULTS

Aggregation and Inhibition of Aggregation in PRP

As shown in Fig. 1A, normal patterns of aggregation were produced by ADP (final concentration 3.7 × 10⁻⁷ M), epinephrine (final concentration 0.2 mg/ml), collagen (1:2 dilution of stock solution), and thrombin (final concentration 0.3 U/ml. In contrast, the addition of antiserum alone (R80) to PRP produced no aggregation.

When PRP was incubated with 0.04 ml of R80 for 3 min followed by the aggregating agent it was found that (Fig. 2B) aggregation by ADP, epinephrine, collagen, and thrombin was inhibited, and the secondary response to ADP and epinephrine was eliminated. No inhibition could be demonstrated when other sera were used in place of the antiprothrombin complex antiserum: normal rabbit serum, antihuman fibrinogen, antihuman IgG, antihuman complement C₃, or antihuman α-1 lipoprotein.

Aggregation and Inhibition of Aggregation of Washed Platelets

As shown in Fig. 2A, washed platelets were not aggregated by ADP (final concentration 1.84 × 10⁻⁵ M), epinephrine (final concentration 0.2 mg/ml), collagen (1:2 dilution of stock), thrombin (final concentration 0.3 U/ml), or 0.04 ml of R80. They aggregated normally when 0.25 ml of bovine fibrinogen (150 mg/100 ml) were added to 0.25 ml of washed platelet suspension.

When a suspension of washed platelets was incubated with antiserum R80 (Fig. 2B) followed by bovine fibrinogen (curve 8) there was complete inhibition of aggregation. Inhibition also occurred when the addition was bovine fibrinogen and ADP, bovine fibrinogen and epinephrine, bovine fibrinogen and collagen, or bovine fibrinogen and thrombin. No inhibition could be demonstrated when other sera were used in place of the antiprothrombin complex antiserum: normal rabbit serum, antihuman fibrinogen, antihuman IgG, antihuman complement C₃, or antihuman α-1 lipoprotein.

Studies on the Specificity of the Antiserum

A series of absorption studies was done to establish that the effect noted was specific:

1. The antiserum was adsorbed with washed normal human platelets (0.5 ml of antiserum + 0.1 or 0.04 ml of a suspension of washed platelets, final platelet concentration 140,000/cu mm and 50,000/cu mm, respectively) for 30 min at room temperature and 4 hr at 4°C. The adsorbed antiserum was harvested after centrifugation at 3000 g and aggregation inhibition determined as described.
Fig. 2. Composite tracings, aggregation, and inhibition of aggregation of washed platelets. (A) Controls: (1) bovine fibrinogen added, (2) ADP, (3) thrombin, (4) epinephrine, (5) collagen, (6) R80 anti-prothrombin complex antiserum. (B) Inhibition of aggregation: (1) ADP plus fibrinogen, (2) epinephrine plus fibrinogen, (3) fibrinogen alone, (4) collagen plus fibrinogen, (5) thrombin plus fibrinogen, (6–10) preincubation with antiserum followed by: (6) collagen and fibrinogen, (7) ADP plus fibrinogen, (8) fibrinogen alone, (9) epinephrine plus fibrinogen, (10) thrombin plus fibrinogen.

Fig. 3. Typical tracings (composite) of the effect of adsorbed antisera on platelet aggregation in PRP by epinephrine. (1) Antiserum adsorbed with human platelets, (2) antiserum adsorbed with antigen (BaSO₄-eluate), (3) antiserum adsorbed with BaSO₄-adsorbed human plasma, (4) untreated antiserum.
above. As shown in Fig. 3(1), platelet-adsorbed antiserum loses its aggregation-inhibiting activity. This is interpreted as supporting the concept that the platelets contain specific receptor sites for the antiprothrombin complex antibodies. It should be noted that the antiserum alone has no platelet-aggregating activity [Fig. 1A(5)].

2. The antiserum was adsorbed with BaSO₄-adsorbed normal human plasma, prothrombin time > 600 sec (0.5 ml of antiserum + 0.1 ml BaSO₄-adsorbed plasma) for 30 min at room temperature and 4 hr at 4°C. The adsorbed antiserum was harvested after centrifugation at 3000 g and aggregation-inhibition determined as described above. As shown in Fig. 3(3) antiserum adsorbed with BaSO₄-adsorbed plasma retains its original capacity to inhibit platelet aggregation. This is interpreted as evidence that only antibodies present in the antigen (BaSO₄ eluate) are active in platelet inhibition. Furthermore, since the BaSO₄-adsorbed plasma is rich in fibrinogen, there is additional confirmation that the inhibition is not due to the presence of antifibrinogen.

3. The antiserum was adsorbed with a freshly prepared antigen preparation, the eluate of BaSO₄-adsorbed normal plasma (0.5 ml of antiserum + 0.1 ml of antigen), for 30 min at room temperature and 4 hr at 4°C. The adsorbed antiserum was harvested by centrifugation at 3000 g and aggregation inhibition determined as described above. As shown in Fig. 3(2), this antiserum is unable to inhibit platelet aggregation. This is interpreted as supporting the concept that the inhibition of platelet aggregation is specifically due to antibodies against prothrombin-complex proteins.

DISCUSSION

Platelet aggregation, so intimately involved in hemostasis and thrombosis, has been the subject of intensive study. Although these studies have partially clarified the classification of qualitative platelet disorders and the platelet defect in von Willebrand’s disease, the physicochemical reactions which occur when aggregation takes place are still nebulous. The concept of Apitz⁴ that platelets aggregate because a “sticky” fibrin forms on the surface has been considered too simplistic but may be in fact the last of a series of reactions. Later observations that normal platelets are aggregated by such diverse substances as ADP, epinephrine, collagen, thrombin, and ristocetin, among many others, have intrigued investigators and stimulated much research into the definitive mechanisms responsible for the aggregation reaction. Most investigators feel that ADP is the final common pathway for the aggregation produced by various agents, but there is no agreement on the molecular mechanisms that precede the final reaction.¹⁵-¹⁷

One area of special interest has been the role of plasmatic cofactors in the aggregation reaction.¹⁸ The concept that a “plasmatic atmosphere” of proteins is a requirement for normal platelet function was first proposed by Roskam in 1923.¹⁹ There followed several reports²⁰-²² dealing with the identification of the proteins involved in platelet aggregation and release reactions. In addition to calcium,²³ two coagulation proteins are known to act as cofactors, fibrinogen and factor VIII.

The definition of fibrinogen as a cofactor was derived from in vitro studies⁵-⁸
and from the observations that in clinical cases of congenital afibrinogenemia ADP-induced platelet aggregation is diminished. Platelets suspended in their own afibrinogenic plasma usually show diminished aggregation with low concentrations of ADP but aggregate normally if the concentration of ADP is high. It is noteworthy that only minute amounts of fibrinogen are required, and it has been pointed out that not infrequently so-called afibrinogenemic plasma does in fact contain small amounts of fibrinogen.

Ristocetin is an antibiotic no longer used for therapy because it causes platelet agglutination and thrombocytopenia. While it aggregates normal platelets in vivo and in vitro, it will not aggregate platelets from subjects with von Willebrand's disease unless factor VIII, or that portion of the factor VIII molecule which has von Willebrand activity, is added. The mode of action of ristocetin is now known, but the role of the von Willebrand factor as a second cofactor in platelet aggregation seems well established. A possible third cofactor is factor XII.

Attempts to identify aggregation cofactors by the use of plasmas having identified deficiencies of coagulation factors have not been rewarding. It seems probable that even in the most severe deficiencies there is not a complete absence of the implicated coagulation factor. In vitro experiments relying on the subtraction or addition of specific factors by chemical means are subject to the same criticism as far as substrates are concerned plus the difficulty of the purity of the additives. We began with the working hypothesis that the "plasmatic atmosphere" necessary for platelet aggregation is not primarily the immediate environment around the platelet but rather it is the adsorption of plasmatic constituents onto the platelet membrane. The concept of the platelet as a "sponge" is not new. In addition to adsorbed substances such as serotonin, immunoglobulins, plasminogen, and myxovirus, platelets adsorb coagulation factors V, VIII, IX, XI and XII and possibly XII. Other coagulation factors have as yet not been implicated.

The test of the hypothesis by immunologic techniques offers many advantages over chemical or indirect approaches. Immunologic methods are more specific and many times more precise than are the assays of coagulation proteins. The use of platelet aggregation provides a sensitive and graphic measurement of the reactions occurring at the cell membrane.

Our data indicate that one or several of the vitamin K-dependent coagulation factors in the prothrombin complex are required for the aggregation reaction of normal platelets. An antiserum prepared against the proteins of the complex inhibits aggregation of platelets in PRP by ADP, epinephrine, thrombin, and collagen. Furthermore, the inhibition is not related to an interaction between the antiserum and the normal plasma in which the platelets are suspended, for we show that the antiserum inhibits also the aggregation of washed platelets by bovine fibrinogen. We assume that the action of the antibodies in the serum is directed towards proteins of the prothrombin complex at the platelet membrane.

Data are presented which support the opinion that the aggregating-inhibiting effect on the antiserum used is specifically attributable to antibodies to prothrombin complex proteins: (1) there is no aggregation inhibition by normal
rabbit serum, antihuman fibrinogen antiserum, antihuman IgG antiserum, antihuman complement C₃ antiserum, or antihuman alpha-1 lipoprotein antiserum; (2) adsorption of the antiserum with normal human BaSO₄-adsorbed plasma does not alter its aggregation-inhibition activity; (3) adsorption of the antiserum with antigen (BaSO₄ eluate) abolishes its aggregation-inhibition activity; and (4) adsorption of the antiserum with normal human platelets also abolishes its aggregation-inhibition activity.

There is a theoretical possibility that the aggregation-inhibiting activity of the antiserum is due to an antibody to an unidentified protein in the antigen, but we feel this to be unlikely. First, we show by immunoelectrophoresis that at least some of the possible contaminating proteins are not demonstrable in the antigen. Second, the antiserum alone does not aggregate platelets, and this suggests, at least, that no specific antiplatelet antibodies are present. Third, while it is possible that as yet unidentified proteins may be adsorbed by BaSO₄ and eluted with citrate, the powerful clot-inhibiting activity of the antiserum, not altered by adsorption with BaSO₄-adsorbed plasma, suggests that the demonstrably powerful antibodies to the prothrombin complex are the ones active in aggregation-inhibition. Finally, it must be admitted that none of the above offer absolute proof of the specificity of the reaction. This can only be proved by using antisera prepared to acceptably pure coagulation factors. Work is now in progress toward this goal.

Studies are in progress to determine whether the specificity is directed towards one, several, or all four of the factors in the prothrombin complex. The powerful inhibition of the prothrombin time test by the antiserum implicates the II-VII-X group, while the less striking inhibition of the partial thromboplastin time implicates factors IX-X. The antiserum used contains no demonstrable antifibrinogen, anti-factor XI, anti-factor XII, anti-factor VIII, or antifactor V; while some of these may also be cofactors involved in platelet aggregation, they have been excluded in this study.

The demonstration that factors in the prothrombin complex are present on or in the platelet membrane, plus other coagulation factors previously implicated, expands the role of platelets in hemostasis. It can be postulated that many, if not all, of the coagulation proteins and the phospholipid involved in the sequence leading to the formation of fibrin are present on the platelet membrane. It is not improbable that the reactions at the platelet membrane replicate in a microcosmic setting the entire coagulation sequence. Aggregation may be both the manifestation of the coagulation sequence and the reaction by which a self-generating sequence is maintained. The evidence at present suggests that there is an interaction between the aggregating agent and coagulation proteins adsorbed onto the platelet. The interaction alters the platelet membrane, leading to release of ADP and other intracellular constituents. It can also be assumed that activation of the coagulation sequence leads to the conversion of prothrombin to thrombin which in turn converts fibrinogen at the membrane to fibrin. Aggregation is an expression of these reactions.

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