Immunologic Studies of Platelet Protein

By RALPH L. NACHMAN

ANY PLATELET FUNCTIONS are probably related directly or indirectly to adsorbed plasma proteins on the platelet membrane.1,4 The mechanism of this adsorptive phenomenon is not clear; in this regard, the platelet has been aptly described as a sponge.5 Many clotting factors such as prothrombin, factors IX, X and VII,1 factor V,2 factor VIII,3 have been found to be avidly attached to the platelet surface. In addition, fibrinogen4 has been shown to be intimately associated with the platelet and is considered by some8 to be the primary substrate for the action of thrombin in the production of platelet aggregation. Recent evidence also indicates that fibrinogen may actually form a part of the intrinsic platelet structure.7,4 The purpose of this report is to immunologically characterize some of the proteins intimately associated with platelets. In addition, studies on the action of thrombin and trypsin on platelet protein will be reported.

MATERIAL AND METHODS

All glassware was treated with silicone (Silicad, Clay-Adams, New York). Venous blood was obtained from human donors through a 15 gauge needle attached to plastic tubing. An 0.1 volume of a 2 per cent solution of EDTA was used as anticoagulant.

Platelet rich plasma (P. R. P.) was prepared by differential centrifugation at 300 g for 15 minutes at room temperature. The platelets were separated from the P. R. P. using the oil bottle technic of Green.9 P. R. P. was introduced into a 100 ml. siliconized oil bottle with a .5 ml. stem 4 cm. long with a diameter of 3.8 mm. (Corning Glass Co., Corning, N. Y.). The oil bottle was spun at 3000 r.p.m. for 30 minutes at room temperature. The platelet button with negligible numbers of white and red cells was harvested using siliconized Pasteur pipettes and washed twice at 4 C. with Alsever’s solution and finally with veronal buffer.10

Antibodies were produced in rabbits by repeated weekly intramuscular injections of washed human platelets for a period of 4 months. A total number of approximately 100 x 109 platelets were used. Antifibrinogen antibody was obtained commercially (Hyland Laboratories, Los Angeles, California).

Platelets were trypsinized by the method of Schmid, Jackson and Conley.6 Platelet “sonicates” were prepared by separating platelets as described, washing 10 times in veronal buffer to ensure optimum removal of adsorbed plasma protein, and sonicating for 10 seconds in buffer at 20 KC under ice water using a Branson sonifier at full amplitude (Heat Systems, Great Neck, L. I.). The sonicated protein was centrifuged at 6000 g for 15 minutes and the clear supernate concentrated by ultrafiltration.

Cohn fraction 1 human fibrinogen was obtained commercially (Pentex). Purified plasminogen free fibrinogen was obtained commercially (Mann Research Laboratories, New York). Pooled normal human beta globulin was supplied by Dr. L. Kornfeld, and fibrinogen-free purified plasminogen by Dr. Nils Bang.

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Fig. 1.—(A) Immunoelectrophoretic analysis of platelet sonicate. Trough at the bottom was filled with antihuman platelet serum. The fibrinogen line is indicated by the arrow. (B) Immunoelectrophoretic analysis of normal plasma. Trough at the bottom was filled with antihuman platelet serum. The major antigen detected is fibrinogen, indicated by the arrow.

Immunoelectrophoresis was carried out by a modification of the Scheidegger technic. Immunodiffusion studies were performed by a modified Ouchterlony technic. Starch gel electrophoresis was performed according to the method of Smithies. Protein concentration was determined by ultraviolet absorption at 280 m\(\mu\).

RESULTS

Immunoelectrophoresis of the Platelet Sonicate

At least 5 discrete precipitin lines were evident in the platelet sonicate using the antiplatelet antibody (fig. 1A). The innermost precipitin line appeared to move only minimally in the electrophoretic field and was identical to Cohn fraction I fibrinogen. Immunoelectrophoretic analysis of normal plasma (fig. 1B) using the same platelet antibody revealed 2 discrete antigens, one identical to the platelet fibrinogen, the other probably plasminogen.

Immunodiffusion Comparison of Platelet Proteins

Platelet sonicate, normal serum, Cohn fraction 1 fibrinogen, and pooled normal human beta globulin were compared to each other in an immunodiffusion plate using the antiplatelet antibody (fig. 2). Four platelet antigens were observed in the sonicate. The slowest moving dense line of the platelet sonicate was identical to fibrinogen. Close to the antibody well, the fastest moving platelet antigen of the sonicate formed a smeared precipitin line which gave a reaction of partial identity with an antigen present in normal serum. This latter antigen was markedly diminished in serum obtained from native platelet-poor plasma. A second antigen present in Cohn fraction 1 fibrinogen, gave a line of identity with pooled human beta globulin, which in turn was identical to an antigen present in the platelet sonicate. This beta globulin contaminant of fibrinogen was absent when purified plasminogen-free fibrino-
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Fig. 2.—Immunodiffusion analysis of the antigens in platelet sonicate, normal serum, Cohn fraction I fibrinogen, and pooled human beta globulin. The center well contained antihuman platelet serum.

gen was used in the antigen well. The antiplatelet antibody reacted with purified plasminogen, which gave a line of identity with the beta globulin contaminant of fibrinogen (fig. 3).

Thrombin Effect on the Platelet Sonicate

Starch gel electrophoresis of the concentrated platelet sonicate revealed a single line in the beta globulin region (fig. 4). The platelet fibrinogen did not move into the gel but remained at the origin. The electrophoretic pattern of the platelet sonicate changed significantly after the addition of 10 units of bovine thrombin. No additional calcium was added. The beta globulin line diminished in intensity and a faster-moving smeared line was seen. No gross clot was seen in the thrombin-treated platelet sonicate; however, with the addition of greater amounts of thrombin up to 100 units, a small clot was observed.

The thrombin effect was also visualized in the double diffusion studies. With the addition of 10 units of thrombin to a concentrated sonicate, there was diminution but not disappearance of the fibrinogen in the platelet sonicate; however, another platelet antigen was no longer visible. To rule out the possibility that small amounts of platelet protein were being trapped in a microscopic clot, the studies were repeated using a highly concentrated platelet sonicate obtained from the blood of a patient with thrombocytosis.
Fig. 3.—Immunodiffusion analysis of the antigens in platelet sonicate, Cohn fraction 1 fibrinogen, pooled human beta globulin and purified plasminogen. The center well contained antihuman platelet serum.

Two units of thrombin were added to this sonicate. It was then compared immunologically to an untreated aliquot of platelet sonicate of equal protein concentration (fig. 5). Six antigen lines were evident in the untreated platelet sonicate. The fibrinogen line appeared to be split. It is possible that the increased number of antigens plus the splitting of the fibrinogen line in this sonicate was due to the high concentration of platelet protein. In the thrombin-treated sonicate, 5 antigen lines were seen, with little or no change in the fibrinogen. The small inner line, however, disappeared after the addition of thrombin.

The Effect of Trypsin on the Platelet Sonicate

Sonicates were prepared from washed trypsinized platelets. Aliquots of these platelets were not clumped by thrombin. The concentrated sonicate was then divided into 2 aliquots, one of which was studied by immunoelectrophoresis using the antiplatelet antibody. The other aliquot was treated with 100 units of thrombin. A small clot formed; the clot-free material from this thrombin-treated sonicate was compared to the untreated sample by immunodiffusion using an antifibrinogen antibody. The fibrinogen line was present in the immunoelectrophoretic pattern of the sonicate obtained from trypsinized platelets (fig. 6A). Following the addition of a large amount of thrombin, this fibrinogen was no longer detectable (fig. 6B).
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Fig. 4.—Starch gel electrophoretic pattern of (A) normal serum, (B) platelet sonicate, (C) platelet sonicate plus thrombin.

Fig. 5.—Immunodiffusion analysis of the antigens in highly concentrated platelet sonicate, sonicate plus thrombin, Cohn fraction 1 fibrinogen, and thrombin. The arrow indicates the antigen in the platelet sonicate which disappeared following thrombin action. The center well contained antihuman platelet serum.
Fig. 6.—(A) Immunoelectrophoretic analysis of a sonicate from trypsinized platelets. The fibrinogen line is indicated by the arrow. The trough contained antihuman platelet serum. (B) Immunodiffusion analysis of the fibrinogen antigen in Cohn fraction 1 fibrinogen, sonicate from trypsinized platelets, and sonicate from trypsinized platelets plus thrombin. The center well contained antihuman fibrinogen. An antigen identical to fibrinogen was present in the sonicate from trypsinized platelets, and disappeared following the addition of a large amount of thrombin.

**DISCUSSION**

The present experiments demonstrate at least 5 discrete platelet antigens, some of which were not detected in plasma. One of these antigens was found in normal serum, but was greatly diminished in serum derived from platelet-poor plasma. It is conceivable that it was released from platelets during the coagulation process. One of the most prominent platelet antigens is a protein clottable by thrombin and immunologically identical to fibrinogen. Although platelet fibrinogen was first described in 1948 by Ware, Fahey and Seegers and more fully studied in 1952 by Johnson et al., there is still some controversy as to whether fibrinogen forms an integral part of the interior structure of the platelet or whether it is only found on the plasma membrane. Utilizing immunologic technics, Salmon demonstrated fibrinogen in bovine...
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platelets after 7 saline washings and concluded that it was a part of the intracellular structure of platelets. Schmid et al. who were unable to detect platelet fibrinogen after exposure of the platelets to trypsin, favored the view that the fibrinogen is on the surface of the platelets. Grette was able to extract thrombin clottable protein from trypsinnized platelets, and concluded that fibrinogen is an intracellular constituent of platelets. Using a fluorescent antibody technic, Gokcen and Yunis reported the presence of fibrinogen in washed platelets treated with trypsin or fibrinolysin. Our studies have confirmed the observations reported by these investigators. We have demonstrated that a thrombin-clottable protein immunologically identical to fibrinogen was present in sonicates obtained from trypsinnized platelets. It is important to note that these trypsinnized platelets did not form aggregates in the presence of thrombin, indicating that membrane fibrinogen was probably digested. We conclude that fibrinogen forms an intricate part of the internal platelet structure as well as being adsorbed to the plasma membrane. The sensitive qualitative immunologic technics used in this study to detect fibrinogen may explain the different results obtained by other investigators using trypsinnized platelets.

The observation that the antibody to washed platelets reacts with purified fibrinogen-free plasminogen, strongly suggests that plasminogen is one of the platelet antigens. The immunodiffusion studies demonstrated that the beta globulin contaminant of Cohn fraction 1 fibrinogen which is also present in the platelet sonicate, is plasminogen.

After a series of extensive studies, Schmid, Jackson and Conley concluded that fibrinogen on the surface of platelets is the substrate of thrombin in the production of platelet aggregation, viscous metamorphosis, and clot retraction. Grette, studying the release of intracellular constituents following the action of thrombin on platelets, concluded that the thrombin effect follows the hydrolytic cleavage of a nonfibrinogen substrate categorized as "yet unknown protein."

Our studies demonstrate that in addition to its action on fibrinogen, thrombin clearly altered the immunologic and electrophoretic characteristics of another platelet protein. The beta globulin portion of platelet sonicate was grossly changed in starch gel following electrophoresis. It is of interest that Salmon in a similar study reported the disappearance of 2 fractions of a platelet sonicate following the addition of thrombin. The effect of thrombin on the platelet sonicate was also demonstrated in the immunodiffusion studies. Fibrinogen in the thrombin-treated sonicate diminished appreciably. In addition, a second platelet antigen disappeared. The amount of thrombin necessary to alter the immunologic character of the second nonfibrinogen platelet antigen was smaller than that required to act on the platelet fibrinogen (fig. 5). In the presence of only 2 units of thrombin, this unidentified platelet antigen disappeared, while no visual change in fibrinogen lines were noted.

These studies do not allow any conclusions regarding the physiologic significance of the nonfibrinogen effect of thrombin on platelets. It is clear from these and other investigations that thrombin acts on platelet fibrinogen. It is
conceivable, however, that interactions between thrombin and other platelet protein besides fibrinogen may play a role in platelet aggregation, viscous metamorphosis and clot retraction.

**Summary**

Antigens were demonstrated in platelets which were not detectable in plasma. One of these antigens was detected in serum and was presumed to be released during the process of coagulation. Plasminogen and fibrinogen were detected in platelets, and the fibrinogen appears to be part of the intracellular structure. Thrombin was found to act not only on fibrinogen but also on at least one other unidentified platelet protein.

**Summario in Interlingua**

Antigenos esseva demonstrate in plachettas que non esseva detegibile in plasma. Un de ille antigenos esseva detegite in sero, presumitemente in consequentia de su relaxation durante le processo del coagulation. Plasminogeno e fibrinogeno esseva detegite in plachettas, e le fibrinogeno pare esser parte del structura intracellulare. Esseva trovate que thrombina age non solmente super fibrinogeno sed etiam super al minus un alte non-identificate proteina plachettal.

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