Differentiation of Certain Platelet Factors Related to Blood Coagulation

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Study of the platelet from the viewpoint of the blood coagulation mechanisms seems likely to be helpful for increasing our knowledge of thrombosis, and bleeding tendencies in thrombasthenias and thrombopathias. A logical approach to their study is the differentiation of their properties and where possible to ascribe those properties to individual components. Platelet factors 1, 3 and 4 are considered from that viewpoint. The first accelerates the formation of thrombin in a manner analogous to serum Ac-globulin. Factor 3 is required for the development of thrombo activity, and factor 4 has antiheparin properties.

The importance of platelets in several phases of the blood coagulation mechanisms is now being generally recognized. In experiments by Patton, Ware and Seegers blood was collected with special care and centrifuged in silicone coated glass to remove platelets. The plasma obtained in this manner was allowed to stand in silicone tubes for many hours and very little fibrin formed, and practically none of the prothrombin changed to thrombin. The clotting mechanisms thus remained essentially static when deprived of platelet materials. Mann, Hurn and Magath found a restoration of the clotting time of stored plasma if platelet suspensions were added in addition to brain thromboplastin. Quick concluded that the amount of thromboplastin supplied by platelets is very small, and that platelets function by activation of an inactive thromboplastinogen found in plasma. This substance would correspond to the antihemophilic factor and the mechanism of its action is probably somewhat different from the theory proposed by Quick. Ware, Fahey and Seegers fractionated platelet extracts with ammonium sulfate and found two different activities. One accelerated the conversion of purified prothrombin, in the presence of thromboplastin and calcium. Its action was said to be similar to that of serum Ac-globulin. It was called platelet factor 1 and later also platelet accelerator or platelet-AcG. The other showed fibrinplastic activity in hastening the interaction of thrombin and fibrinogen in purified systems as well as in oxalated plasma. This was designated platelet factor 2. van Creveld and Paulssen confirmed the existence of platelet factors 1 and 2, and demonstrated another one designated as platelet factor 3. This factor improved prothrombin consumption in platelet deficient plasma and had antiheparin activity. These properties were obtained in the phosphatide fraction of the platelets. Jürgens placed platelet extracts in the ultracentrifuge and found a fibrinplastic and an antiheparin activity in the supernatant, and in the precipitate a thromboplastic and an antithromboplastic activity.

This paper is concerned primarily with platelet factors 1 and 3. Their properties are adequately described to distinguish one from the other. Platelet factor 1 accelerates prothrombin activation in Ac-globulin deficient stored plasma. It also functions together with thromboplastin and calcium in the activation of Ac-globulin free purified prothrombin. Platelet factor 3 functions with platelet cofactor I, and plasma Ac-globulin in the activation of prothrombin. It may also function in the same way with histamine and certain anti-
bistamine compounds. The antiheparin activity of plasma is associated with a substance different from factor 3.

**Materials**

**Purified Prothrombin** was prepared by the method of Seegers and associates.4,5 and was, when necessary, freed of the last traces of Ac-globulin by dissolving in water and heating at minimal ionic concentration at 53°C for 2 hours.

**Platelet Cofactor 1** was prepared by a modification of the method of Lorand and Lakí.6 The residue of the plasma after Mg(OH)2 adsorption in the above described method for the preparation of purified prothrombin was used as starting material. It was brought to pH 5.9 with M/5 phosphate buffer and the factor was adsorbed on kaolin. The kaolin was washed once with M/20 borate buffer and then eluted with 0.1 per cent ammonium carbonate at room temperature. The eluate was neutralized and fractionated with ammonium sulfate. The precipitate between 35 and 50 per cent saturation was dissolved in distilled water and dialyzed against 0.1 per cent ammonium carbonate. After freeze-drying this preparation is stable for a long time. It restores the prothrombin utilization of hemophilic blood to normal.

**Platelet Suspension.** We were guided by the work of Johnson, Smathers and Schneider.7 Bovine platelets were obtained by differential centrifugation. They were washed thoroughly. Then the suspension was frozen, thawed, and washed again four times with saline. The platelet particles were suspended in saline and put through a mechanical homogenizer three times. Then the product was placed in a refrigerator at 4°C.

**Stored Oxalated Human Plasma** was prepared by the method of Deutsch and Schaden.8 Its one-stage prothrombin time was longer than 60 sec., and could be shortened, by addition of BaCO3 adsorbed bovine serum (Ac-globulin source) to a lower value than the normal value was before storage.

**Antiheparin Activity.** A simple qualitative test was set up. Bovine oxalated plasma was defibrinated by heating at 53°C for 3 min. Then a mixture of 0.3 ml. defibrinated plasma, plus 0.1 ml. fibrinogen solution, plus 0.1 ml. test material, plus 0.1 ml. thrombin was made. The thrombin was of such strength that clotting occurred in 15 sec. If the test material contained heparin the clotting time became longer in proportion to the amount of heparin it contained. Consequently if the test material previously had its heparin activity reduced, for example by platelet materials, the clotting time tended in the direction of 15 sec. from longer clotting times.

**Crude Lung Extract Thromboplastin** was prepared according to McClaughry and Seegers.9

**Thrombin determination** after Seegers and Smith.10

**EXPERIMENTAL RESULTS**

**Platelet Factor 1 of Platelet Suspension and Stored Human Plasma.** After aging of plasma its one stage prothrombin time was longer than 60 seconds, and was shortened to 14 seconds with BaCO3 adsorbed with serum. The latter serves as a source of serum Ac-globulin. With addition of fresh platelet suspensions to aged plasma the clotting time was reduced to 25-30 seconds. This property of the platelet suspensions was retained after two weeks of storage. Sometimes the accelerating effect was most effective on the second or third day of storage. In their study Mann, Hurn and Magath were the first to point out that platelets can restore the deficiency found in stored plasma and record a prothrombin time of 13 seconds. That represented complete restoration of the prothrombin time in their experiments. In our work we were only occasionally able to get near a complete restoration of the prothrombin time by testing the platelet extracts after 2 or 3 days of storage when their accelerating activity was at its peak. In the experiments of Mann, Hurn and Magath the platelets were not washed and might have been contaminated with appreciable amounts of plasma Ac-globulin. Despite these remarks about their technical details we are in general agreement with their conclusions, and add the observation that the accelerator factor or factor 1 is quite stable upon storage. We also found that factor 1 activity is in the supernatant solution after low speed centrifugation of platelet suspensions. By centrifuging for one hour at 100,000 g it is sedimented and can completely be recovered by resuspending the sediment in saline.

**Platelet Factor 1 of Platelet Suspension and the Activation of Purified Prothrombin.** For these experiments the purified prothrombin was heated to remove the last traces of plasma Ac-globulin. Then calcium + platelet suspension + lung thromboplastin yielded thrombin rapidly (fig. 1). Next it was found that calcium + platelet suspension + rabbit brain thromboplastin yielded thrombin less rapidly and there was not a full yield. The results were, however, very dependent upon the way in which the brain thromboplastin was prepared.
Fig. 1. Activation of Ac-globulin free purified prothrombin. With lung thromboplastin and platelet factor 1 as found in fresh platelets, in 6 day old platelets, and in 6 day old supernatant. With brain thromboplastin and platelet factor 1 as found in fresh platelets, in 6 day old platelets, and in 6 day old supernatant. The controls were with brain thromboplastin or lung thromboplastin and calcium alone. Platelet factor 1 functions with either lung extract thromboplastin or with brain thromboplastin. It is stable. It substitutes for Ac-globulin.

For example: crude brain thromboplastin was obtained from rabbit brain according to Quick[16]. Two different extracts were then made. The dried brain powder was suspended in physiological saline solution, homogenized and used with the particles in it. This extract gave the results recorded on fig. 1. In another experiment 0.5 gm. brain powder were extracted with 5 ml. saline 30 minutes at 37 C. The extract was centrifuged at a low speed for 5 minutes to remove the particles. Together with platelet suspension this extract almost equals that of lung thromboplastin. It seems likely, therefore, that platelet factor 1 can substitute for Ac-globulin under appropriate conditions.

A good activation rate could be obtained not only with fresh platelet suspensions but also with those stored up to 6 to 9 days. Moreover, the activity remained in the supernatant solutions upon light centrifugation of fresh or stored platelet suspensions. Thus we may conclude that platelet factor 1 is relatively stable at 4 C.

Storage of Platelet Suspension and Platelet Factor 3 Activity. When the platelet suspension was stored in the refrigerator platelet factor 3 activity began to decrease on the third day of storage in the refrigerator (fig. 2) and disappeared almost completely after 6 to 8 days. As indicated above the activity of platelet factor 1 was not significantly changed under the same conditions. Factor 3 activity was associated with the platelet particles, since the supernatant obtained by light centrifugation of the platelet suspensions had only a very small amount of activity. Whether any is found in the supernatant is very much de-
dependent upon the speed of centrifugation. Even centrifugation at 2000 g reduces its concentration greatly.

Platelet Factor 3 Functions with Linadryl. Murray, Johnson and Seegers discovered that many purified prothrombin preparations activate with calcium, Ac-globulin, platelets and certain antihistamine compounds. The activation takes place rapidly if the reaction is started with a small amount of thrombin. We confirmed their observations and tested various platelet preparations in combination with Linadryl (4)-2-(benzhydryloxy)ethylmorpholine. The purpose was to see whether the factor in platelets which functions with Linadryl is platelet factor 3 or another factor. We found a fast rate of activation and a maximum yield of thrombin, under the conditions of the test, with platelet suspensions. Upon light centrifugation of platelet suspensions it was found that the activity remained with the particles and not so much with the supernatant. In this respect, the results followed the same pattern with Linadryl as with the respective platelet preparations and platelet cofactor I of plasma.

Importance of Thrombin. It can be shown that thrombin is important for the activation of prothrombin with, platelet factor 3, plus diluted plasma. To demonstrate this (fig. 3) a large amount of prothrombin was used as a substrate. As source of platelet cofactor I plasma (defibrinated, and ether treated to reduce antithrombin-III) was diluted 1:12. Six day old platelet suspensions were used as a source of platelet factor 3. Without adding thrombin only 600 units of thrombin were obtained. With 8 units/ml of thrombin added to start the interactions the thrombin yield was 1800 units. This yield is 100 per cent in terms of two-stage prothrombin analysis and equals the yield obtained when thrombin and Linadryl, serve as activators.

Antiheparin Activity of Platelet Preparations. With the use of methods described above an antiheparin activity could be detected in the platelet suspensions, and in the supernatant after light centrifugation of platelet suspensions. When platelet suspensions are washed much antiheparin activity is found in the first wash solutions. The 8 day old platelet suspension still showed a fairly high activity, but after 14 days of storage the activity had disappeared. The distribution of the antiheparin activity does not parallel that of the other two factors, and particularly not that of factor 3. It can be considered that the antiheparin activity is not associated with factor 3, and that it is a separate factor.
DISCUSSION

The platelets are of great importance for the coagulation of blood. They have the potential of function in the clotting process at different points: They participate in the activation of prothrombin, when in combination with platelet cofactor I or with the plasma thromboplastin component (PTC)\textsuperscript{17, 18, 19}; they accelerate the activation of prothrombin and the interaction of thrombin and fibrinogen, they inhibit heparin and fibrinolysin\textsuperscript{21} and contain a substance that can be clotted with thrombin. Moreover, they are necessary for clot retraction, and exert a vasoconstrictor action. There is as yet not enough information to use as a basis for defining all these properties in terms of different substances, but it is evident that the factor 1 and 3 functions are associated with separate platelet substances. Therefore, it seems convenient to speak about these in terms of definite platelet factors. Platelet factor 1 is much more stable when stored at 4°C than is factor 3. The latter has largely lost its activity after 6 to 8 days, while at that time the platelet suspensions still have a high factor 1 activity. Platelet factor 1 is very easily extracted with water from a platelet homogenate, while factor 3 activity remains with the platelet particles. A small amount of factor 3 activity which may sometimes be found in these extracts, is easily sedimented at 2000 to 3000 g. Factor 3 is associated with a phosphatide fraction of the platelets. Factor 1 can largely correct the one-stage prothrombin time of aged oxalated human plasma and would, therefore, represent the factor, whose activity Mann, Hum and Magath were measuring. In that respect it functions largely like Ac-globulin. From the kinetics of prothrombin activation Ware, Fahey and Seegers\textsuperscript{4} observed that the activity corresponds more nearly to that of serum Ac-globulin than plasma Ac-globulin. Our work amplifies these findings. Platelet factor 1 activates Ac-globulin free purified prothrombin in conjunction with lung or certain brain extract thromboplastins. This may be indicated as follows:

\begin{align*}
\text{Ca}^{++} & \quad \text{Thromboplastin} \\
\text{Platelet factor 1} & \quad \text{Prothrombin} \\
\end{align*}

For these reasons factor 1 can also be referred to as platelet accelerator, or platelet Ac-G. The chemical differences between Ac-globulin (a plasma factor) and platelet factor 1 are so marked that they can hardly be one and the same chemical substance.

It is evident that these observations may be used for the development of quantitative assay procedures. In fact two possibilities are presented. The activity of platelet factor 1 may be tested on stored oxalated human plasma or by its ability to activate purified prothrombin with lung or brain thromboplastin and calcium.

Factor 3 and platelet cofactor I together are able to substitute for tissue thromboplastin in the activation of purified prothrombin in the presence of Ac-globulin and calcium. Following the suggestion of Murray, Johnson and Seegers\textsuperscript{18} the activity represented by the combined action of platelet factor 3 and platelet cofactor I is called threone activity. It is not proposed that platelet factor 3 and platelet cofactor I of plasma combine to form a new compound. The importance of thrombin for threone activity is ascribed to details related to the change of plasma Ac-globulin to serum Ac-globulin. These concepts may be represented by the following equations:

\begin{align*}
(1) \quad \text{Plasma Ac-globulin} & \quad \xrightarrow{\text{Serum Ac-globulin}} \\
(2) \quad \text{Platelet Factor 3 + Platelet Cofactor I} & \quad \text{Threone} \\
(3) \quad \text{Prothrombin} & \quad \xrightarrow{\text{Thrombin}} \\
\end{align*}

In considering equation 3 above from the viewpoint of the quantitative determination of either platelet factor 3 or platelet cofactor I the following rule applies: When cofactor I is in excess limited amounts of platelet factor 3 yield limited amounts of thrombin and thus the quantity of thrombin formed may be taken as a measure of the quantity of platelet factor 3 (cf. fig. 2); and, conversely as observed by Johnson, Smathers and Schneider\textsuperscript{11}, when platelet factor 3 is in excess limited amounts of platelet cofactor I yield limited amounts of thrombin. Thus the quantity of thrombin formed may be taken as a measure
of the quantity of platelet cofactor I. Any platelet cofactor II would first be removed from plasma by adsorption on BaSO₄ or BaCO₃. Jürgens⁵, ⁶ has recently come to the conclusion that the antiheparin activity of platelets can be ascribed to a factor distinctly different from factor 3. This distinction was not made in the work of van Creveld and Paulssen⁷, but could be confirmed in several experiments performed by us. For example, the first wash solution of platelet suspension has a very high antiheparin activity (factor 4), while the third and fourth wash solutions are without activity. After high speed centrifugation of the first wash solution the total antiheparin activity is obtained in the lower layer, which shows practically no factor 3 activity. The latter is found in the sediment.

SUMMARY

Platelet factor 1 accelerates the activation of Ac-globulin free prothrombin preparations in the presence of thromboplastin and calcium. It remains stable when platelet “extracts” are stored at 4°C, is extracted from the platelet particles with water, and is easily sedimented by ultracentrifugation. It can almost restore the prothrombin time of oxalated stored human plasma to normal.

Platelet factor 3 functions with platelet cofactor I to give thromone activity, and the latter can replace tissue thromboplastin in the activation of purified prothrombin. It is not extracted with water, remains in the platelet fragments and is easily sedimented by centrifugation at medium speed. With storage this factor is not stable. It can function together with certain antihistamine compounds in the activation of purified prothrombin.

Platelet factor 4 has antiheparin activity. It is found in the bottom layer after high speed centrifugation of platelet suspensions.

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