The blood coagulation mechanisms in thrombocytopenic blood*)

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The blood coagulation mechanisms in thrombocytopenia have received little attention in recent years although the therapeutic problem of replacement of platelets in transfusion has aroused much concern. The platelet factor, which at this moment seems to bear most the important relationship to the coagulation mechanisms is platelet factor 3 (1), although all of the many platelet factors previously described must certainly be considered. Platelet factor 3 has been studied extensively, in vitro, and two, main, rather diverse functions described. It has been known for more than a decade that platelets themselves activate prothrombin with the antihemophilic factor (= Factor VIII) (2). More recently it has been shown that platelets act with plasma thromboplastin component (Christmas factor — autoprothrombin II = Factor IX) (3) and with both the antihemophilic factor and plasma thromboplastin component in the same complex (4). The platelet factor specifically responsible for this is considered to be platelet factor 3.

However, a different function of platelet factor 3 has also been described. Platelet factor 3 in a certain environment can convert purified prothrombin to autoprothrombin I, an accelerator of the conversion of prothrombin to thrombin with tissue thromboplastin (5). Autoprothrombin I has many properties in common with factor VII, proconvertin and co-thromboplastin.

The one well-established phenomenon related to blood coagulation in thrombocytopenia is the observation that the prothrombin consumption is poor or incomplete. This was described by Quick, Shanberge and Stefanini (6) in 1949. These investigators stated that prothrombin was not consumed because the number of platelets was inadequate to activate thromboplastinogen (antihemophilic factor) to thromboplastin, and thereby convert the prothrombin to thrombin. Douglas (7), several years later in 1956, reported that the antihemophilic factor was consumed in coagulation in thrombocytopenia, and we have been able to confirm this.

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Alexander and de Vries (8) in 1949 reported that the sera from thrombocytopenic blood showed abnormally large residual prothrombic activity as measured by the one-stage test and smaller amounts than normal of proconvertin. The addition of platelets or thromboplastin corrected these abnormalities.

The authors have investigated this problem extensively using the cruder methods of one-stage type followed by the more refined two-stage tests. This paper is a report of some of these studies in which it was found consistently that the serum of the patient with thrombocytopenia contains by two-stage determination as little prothrombin as normal serum. The antihemophilic factor seems to disappear on the formation of serum just as in the normal serum. The amount and formation of autoprothrombin I, perhaps from prothrombin, is abnormally low. In, in vitro, experiments it has been found that this formation takes place in the presence of platelet factor 3 (5). Since few platelets are present in thrombocytopenia some change in the pathway of prothrombin conversion to autoprothrombin I might be anticipated. It is possible that the phenomenon of a short prothrombin time in the prothrombin consumption test could be due to the formation of yet another derivative of prothrombin which formed in lieu of autoprothrombin I. There have been several factors in serum described recently with chemical properties similar to prothrombin and the prothrombin derivative complex, and one of these may be related to this phenomenon of shortened prothrombin time in thrombocytopenic serum.

Materials and methods:

Purified prothrombin: This material was kindly supplied by Dr. H. O. Singher, Ortho Research Laboratories, for these studies. The method of production was essentially that of Seegers and associates (9). The specific activity of these products was around 20 000 units per mgm. tyrosine.

Platelets: Bovine platelets were supplied by Dr. W. H. Seegers. Human platelets were collected in our own laboratory. The method of collection was essentially the same in each case, and was that of Schneider, et al. (10).

Platelet Factor 3: This isolated platelet factor was prepared in the laboratory of Dr. W. H. Seegers from bovine platelets according to the method of Alkjaersig, Abe and Seegers (1).

Fibrinogen: Fibrinogen used in these studies came from two sources. Armour fibrinogen was made up in a solution 1.5 grams per cent in physiological saline containing 10 per cent imidazole. This preparation was kept in a deep freeze and thawed as needed. Fibrinogen from Warner-Chilcott Laboratories was used as directed in the prothrombin consumption test.

Thromboplastin: Rabbit brain thromboplastin from Difco Laboratories was used in the one-stage prothrombin time and in the prothrombin consumption test. This was prepared as directed. Bovine lung thromboplastin, Aplastin from Difco Laboratories was used in the two-stage prothrombin test.

Incubation Mixture: This was prepared according to the method of Ware and Seegers (11) using Dextran 6 per cent in place of Acacia as suggested by Owen, Hurn and Mann (12).

Imidazole Buffer: This was made up according to the method of Mertz and Owen (13).

Adsorbed bovine serum: This preparation was used in the two-stage prothrombin determination and was purchased from Difco Laboratories.

Thrombin: Further purification of topical thrombin, purchased from Parke-Davis and Company, was done according to Johnson, Caldwell and Priest (14). The thrombin had a specific activity of 11 000 units per milligram tyrosine.

The one-stage prothrombin time: This test was described by Quick 1938 (15).

The modified two-stage prothrombin determination: This assay procedure was carried out according to the method of Ware and Seegers (11). The unmodified two-stage prothrombin of Warner, et al. (16) was also used.

The cofactor assays. The cofactor assays for plasma and serum were carried out according to Johnson and Seegers (17) using the thrombin assay of Seegers and Smith (18).

The prothrombin consumption test: This test was carried out according to the method described by Sussman, Cohen and Gittler (19).

The tests for autoprothrombin I: One-stage co-thromboplastin test: This was described by Mann and Hurn (20).

It is carried out by mixing 0.1 milliliters of brain thromboplastin (Difco) with 0.1 milliliters of 0.025 M $CaCl_2$ and 0.1 milliliters of material to be tested. After three minutes incubation at room temperature 0.1 milliliters of Marcumar plasma was blown in and the clotting time determined. The test is not specific for co-thromboplastin for any factor that is reduced by anticoagulants and which affects the one-stage prothrombin time may shorten the clotting time. Any test based on the correction of the prothrombin time of adsorbed plasma is subject to exactly the same criticism.

Two-stage co-thromboplastin assay: This was carried out according to the method of Johnson and Seegers. Bovine lung extract thromboplastin was diluted 1 : 10 with saline and the thrombin formed measured as purified prothrombin, lung extracted thromboplastin so diluted and calcium were incubated together. Serum from thrombocytopenic and normal individuals or prepared extracts were added to this incubation mixture and the formation of thrombin followed by the method of Seegers and Smith (18).

Russell Viper Venom Assay: This was carried out according to the method of Seegers, Penner and Johnson (21). Russell viper venom was incubated with purified prothrombin, calcium and defibrinated plasma or serum and the thrombin formed measured quantitatively by the method of Seegers and Smith (18).

Purified prothrombin 3000 u/ml.	0.5 ml.
Diluted lung thromboplastin 1 : 10 or Russell viper venom 0.001 per cent	0.25 ml.
Calcium chloride 0.625 M in imidazole	0.25 ml.
Plasma (defibrinated) or serum	0.25 ml.
Physiological saline	0.25 ml.

Test for Fibrinoplastin Activity: The thrombin assay of Seegers and Smith (18) was used here. This consisted of measuring the clotting time of purified fibrinogen by adding diluted purified thrombin. Instead of adding 0.1 milliliters of thrombin to 0.3 milliliters of thrombin diluent, and then adding 0.1 milliliters of purified fibrinogen, 0.2 milliliters of thrombin diluent and 0.1 milliliters of either normal serum or thrombocytopenic serum were added.

Two-stage Ac-globulin test: This test was carried out according to the method of Ware and Seegers (22) by Dr. Frederic Johnson.

Results

Prothrombin Consumption: When the serum of the thrombocytopenic patient was tested in the prothrombin consumption test using added fibrinogen a

short clotting time was observed varying from 18.0 to 9.0 seconds while the clotting time of normal serum varied from 40 to 90 seconds in the same procedure. Below 20.0 seconds is considered to be abnormal and above 30.0 seconds normal. When purified bovine platelet factor 3 from Dr. Seegers' laboratory was added to the whole blood from a thrombocytopenic patient before coagulation had taken place prothrombin consumption within the normal range was observed. In this way dilutions of platelet factor 3 could be added, and the activity of the preparation determined. Stefanini and Campbell (23) reported this same type of assay for their platelet fraction (P.T.F.) in 1954.

Several preparations of purified platelet co-thromboplastin (24) from Dr. Seegers' laboratory were also added to the whole blood of the thrombocytopenic patient and no increase in the prothrombin consumption was observed.

It has been possible to adsorb the thrombocytopenic serum with washed $BaCO_3$ and to thereby lengthen the prothrombin time in the prothrombin consumption test from 15 seconds to 50 seconds, a value well-within the normal range. It has been also possible to make an eluate with one molar sodium citrate from this $BaCO_3$ and after dialysis to remove the citrate to place this eluate in normal whole blood and reduce the prothrombin consumption value from 50 seconds in the normal range to 19 seconds in the abnormal range.

Prothrombin in serum of the thrombocytopenic patient: When the prothrombin concentration of the serum of the thrombocytopenic patient was determined by the modified two-stage method of Ware and Seegers practically no prothrombin was found. As in the case of normal serum prothrombin values of between 30—0 units per milliliter were found. The plasma values of prothrombin of the thrombocytopenic patients were about the same as those of normal individuals varying from 198 to 260 units per milliliter. The control prothrombin for normal plasma in this laboratory is about 235 units per milliliter. The $BaCO_3$ eluate prepared from thrombocytopenic serum contains less than 5 units per milliliter of prothrombin.

Ac-globulin in the thrombocytopenic patients plasma: Since Ac-globulin was known to be related to the one-stage prothrombin time and consequently the prothrombin consumption test, the amount of ac-globulin in thrombocytopenic plasma was measured by the difference between the modified and unmodified prothrombin two-stage tests. In normal plasma the difference in prothrombin units in these two determinations is about 20 units, that is adding diluted $BaCO_3$ adsorbed bovine serum to provide ample ac-globulin increases the apparent prothrombin-value by this amount. However, if ac-globulin is decreased in the plasma, in vivo, this difference between the two tests becomes greater. In our study of thrombocytopenic plasma we found the difference in prothrombin about the same as in normal plasma. For example in a typical case we found a modified two-stage prothrombin test of 210 units per milliliter and an unmodi-

fied one of 192 units per milliliter. It was possible to conclude, therefore, that the short prothrombin consumption value of the thrombocytopenic serum was not due to increased quantities of ac-globulin in plasma.

By the two-stage ac-globulin test, the thrombocytopenic serum was found to contain as little ac-globulin as normal serum. This is evidence that the short prothrombin consumption value of thrombocytopenic blood is not due to a large amount of residual ac-globulin in this serum.

The concentration of the antihemophilic factor in thrombocytopenic serum: When the cofactor assays of Johnson and Seegers were carried out on thrombocytopenic plasma and serum the cofactor activity was approximately the same as on normal plasma and serum (fig. 1). Thrombocytopenic plasma and normal plasma with platelets activate purified prothrombin at approximately the same rate and a full yield of thrombin was obtained. However, when thrombocytopenic serum and normal serum with platelets activated purified prothrombin less thrombin was formed than when either plasma was used, but the thrombocytopenic blood and normal blood gave about the same values in

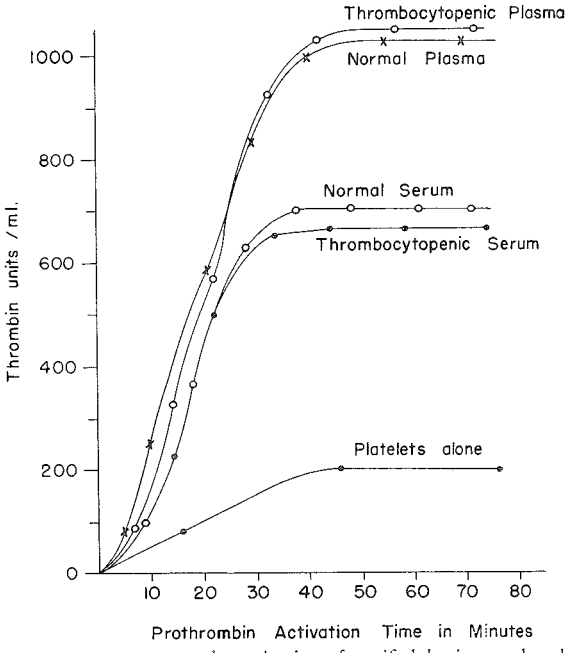

Fig. 1: The top two curves represent the activation of purified bovine prothrombin 0.5 milliliters of 3150 units per milliliter, by bovine platelets 0.25 milliliters, calcium and imidazole 0.25 milliliters and physiological saline 0.25 milliliters and either defibrinated ether-treated normal or thrombocytopenic plasma 0.25 milliliters. The two middle curves represent the activation of purified bovine prothrombin 0.5 milliliters of 3150 units per milliliter, by bovine platelets 0.25 milliliters, calcium and imidazole buffer 0.25 milliliters and physiological saline 0.25 milliliters and either ether-treated normal or thrombocytopenic serum 0.25 milliliters. The lower curve is that found when purified bovine prothrombin 0.5 milliliters of 3150 units per milliliter was added to bovine platelets 0.25 milliliters, calcium and imidazole 0.25 millimeters and physiological saline 0.50 milliliters.

this respect. The concentration of the cofactor activity believed by us to be the antihemophilic activity disappeared from plasma leaving only the serum cofactor activity which is probably plasma thromboplastin component in the serum in each case (11).

Autoprothrombin I concentration of thrombocytopenic serum: Thrombocytopenic serum does not increase the activation of purified prothrombin in the presence of diluted lung extract thromboplastin and calcium (fig. 2). In fact, only 24 units per milliliter of thrombin were formed when thrombocytopenic serum was added while alone diluted thromboplastin and calcium activated 120 units per milliliter of thrombin. This represents an inhibition of thrombin formation by thrombocytopenic serum. Normal serum, diluted 1 : 100 on the other hand, added to diluted lung extract thromboplastin and calcium produced 500 units per milliliter of thrombin from a substrate of 840 units per milliliter of

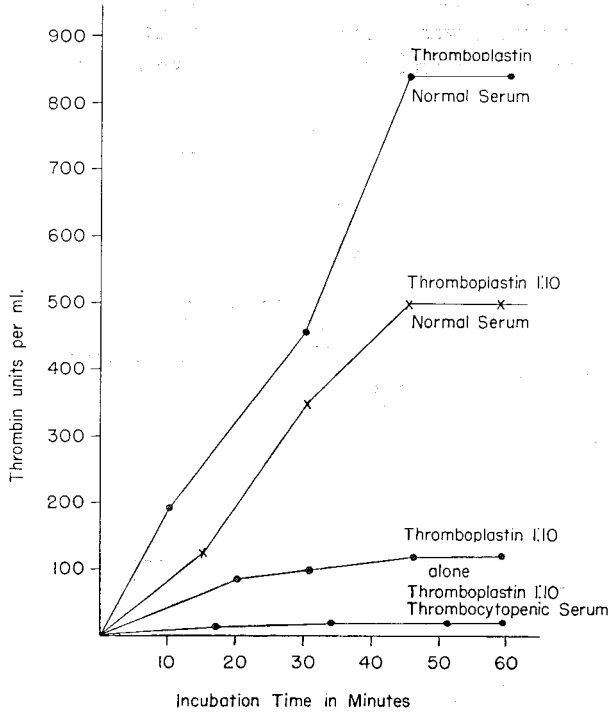

Fig. 2: The top curve represents the activation of purified bovine prothrombin 2550 units per milliliter in a volume of 0.5 milliliters, by lung extract thromboplastin 0.25 milliliters, calcium and imidazole 0.25 milliliters, physiological saline 0.25 milliliters, and normal serum 0.25 milliliters. When the thromboplastin was diluted 1 : 10 and normal serum 1 : 100 about 500 units of thrombin formed from the purified prothrombin, 750 units per milliliter as can be seen in the next curve. The diluted thromboplastin 1 : 10 in volume 0.25 milliliters, alone with calcium and imidazole 0.25 milliliters and physiological saline 0.50 milliliters formed only 120 units of thrombin. The addition of thrombocytopenic serum 0.25 milliliters to diluted thromboplastin 1 : 10 in volume 0.25 milliliters, calcium and imidazole 0.25 milliliters and physiological saline 0.25 milliliters permitted the formation of only 24 units of thrombin when added to purified bovine prothrombin 0.5 milliliters even though the resulting concentration of purified prothrombin in the incubation tube was 850 units per milliliter.

purified prothrombin. This co-thromboplastin activity of serum is attributed to autoprothrombin I and was the activity measured when purified autoprothrombin I was produced from purified prothrombin (5). Not only does thrombocytopenic serum lack this activity, but an antithromboplastin activity seems to be present as well.

A similar assay procedure was used to measure the co-thromboplastin activity of autoprothrombin I by replacing bovine lung extract thromboplastin with Russell viper venom 0.001 per cent. Normal serum, diluted Russell viper venom and calcium with purified prothrombin 1000 units per milliliter produced 150 units of thrombin per milliliter. Thrombocytopenic serum produced 30 units of thrombin under similar conditions. Russell's viper venom with calcium alone produced 5 units per milliliter.

By using the one-stage co-thromboplastin assay of Mann, which is based on a correction of the prothrombin time of anticoagulant (Marcumar) plasma, thrombocytopenic serum corrected clotting time of the plasma as completely as normal serum. This may mean that the component responsible for the short prothrombin consumption test of thrombocytopenic serum is also reduced in Marcumar plasma.

Fibrinoplastic activity of thrombocytopenic serum: Thrombocytopenic serum did not enhance the clotting time of fibrinogen by thrombin when added to this system. In fact the clotting time of the fibrinogen was prolonged from 25 seconds to 50 seconds when either normal or thrombocytopenic serum was added to the thrombin diluent.

Discussion

Many authors have indicated that the accelerator co-thromboplastin (autoprothrombin I, factor VII) is present in higher concentrations in serum than in plasma. This increase has puzzled the authors since the description of platelet co-thromboplastin (24) for it was difficult to decide whether the increase was due to a formation of serum co-thromboplastin from a precursor, such as prothrombin or due to the disintegration of platelets thus releasing the platelet co-thromboplastin. The activity of these two components is difficult to separate although the platelet cothromboplastin has been found to be inactive in the one-stage prothrombin time. The addition of the platelet co-thromboplastin to the blood before clotting did not alter the prothrombin consumption or the two-stage co-thromboplastin values, so in this experiment, at least, this platelet factor did not seem to alter the serum of thrombocytopenia.

The disappearance of the antihemophilic activity as coagulation takes place in the thrombocytopenic patient is in complete agreement with previous in vitro studies. Johnson and Seegers found that the activity of their antihemo-

(25) and in purified systems (26) when latelet factor 3 was never required for the postulate that an inhibitor conjugates with ctivity consequently disappears as a little s.

est is essentially a one-stage prothrombin s supplied. A shortened prothrombin con- a factor which affects the prothrombin mbin, ac-globulin, autoprothrombin I, a n factor or some combination of inhibitors rombin determination in twenty cases of s (leukemia, lymphoma, and hypoplastic en 50 000 and 12 000 platelets per cubic d practically disappeared and was found tion as in normal serum. Since the short rom 15 seconds to the normal value of CO_3, it can be suggested that the factor n time is readily absorbed on $BaCO_3$. As it it may be autoprothrombin I or some perties (a) shortening the one-stage pro- ion on $BaCO_3$, the possibility of a fibrino- t.

which many were tried give us the follow- measured by two-stage determinations is thrombocytopenic serum than in normal easuring a co-thromboplastin activity of . The findings are in agreement with those (8) who used their one-stage procedure. oplastin test based on correction of anti- nbocytopenic serum contains as much of utoprothrombin I is present in thrombo- nounts than in normal serum some yet for the short prothrombin time in the

iques shows that in addition to the form- form derivatives. Prothrombin and these in common. Platelet factor 3 was shown in the formation of autoprothrombin I. prothrombin time. However, one might derivative in the absence of platelets. The ould possibly form since the prothrombin

disappears and a little thrombin is known to convert prothrombin to autoprothrombin II (27). However, this factor is considered to be inactive in the one-stage prothrombin time.

In our efforts to make an extract containing this thrombocytopenic factor by treating the serum with washed $BaCO_3$ and thus lengthening the prothrombin consumption an inhibitory activity was also concentrated. High concentrations of this dialysed eluate added to normal blood, instead of shortening the prothrombin consumption test rendered the blood incoagulable. Fibrinogen and prothrombin remained in this incoagulable blood.

By way of speculation the authors would like to put forth the following ideas. In the absence of platelet factor 3 the conversion of prothrombin to autoprothrombin I is changed and less autoprothrombin I is formed. Since prothrombin has disappeared from thrombocytopenic serum either more of it has gone in the direction of thrombin or in the direction of a still undescribed derivative. Because the prothrombin consumption test is much shorter than normal it is possible that prothrombin has been converted into another derivative or one in larger quantities than in normal serum. We do know that that which shortens the prothrombin consumption test in thrombocytopenic serum is readily adsorbed on $BaCO_3$, and that it will correct the defect in anticoagulant plasma. Both of these properties are associated with the other prothrombin derivatives. Since the formation of autoprothrombin I from prothrombin and the reversal of this reaction has been carried out in vitro, it is possible that the prothrombin molecule undergoes changes easily and in the absence of platelet factor 3 a slight change in the molecule takes place resulting in such a derivative.

Table 1

One-stage co-thromboplastin test of Man

Dilution	*Normal Serum* Seconds	*Thrombocytopenic Serum* Seconds
1 : 10	11.2	10.0
1 : 50	14.6	14.4
1 : 100	16.4	15.6
1 : 250	26.4	24.4
1 : 500	25.8	25.2
1 : 1000	29.8	27.0

Control with saline was 31 seconds.
Control plasma from a patient on marcumar.
Marcumar plasma has been shown to contain: (14)
(Prothrombin below 50 units per milliliter. Autoprothrombin I or Factor VII very low Autoprothrombin II or Christmas factor in normal amounts.

A number of serum factors have been postulated recently by Biggs (28), and while the authors find little comfort in the description of new factors to explain phenomena in blood coagulation it is possible that this thrombocytopenic factor is similar to either factor X (29) or the Stuart factor (30).

Summary

Thrombocytopenic serum supports a short prothrombin consumption time, however, the same serum contains as little prothrombin as normal serum. The prothrombin consumption test of thrombocytopenic serum can be prolonged by adsorption on $BaCO_3$ or reduced again by the addition of the sodium citrate eluate. The antihemophilic activity of thrombocytopenic blood disappears when the blood clots as in normal blood and autoprothrombin I is present in much smaller amounts than in normal serum. The authors suggest that a new factor, a possible derivative of prothrombin, is responsible for the short prothrombin consumption value in thrombocytopenic blood.

Résumé

Les sérums thrombocytopéniques montrent un temps de consommation de la prothrombine plus court que la normale; néanmoins le même sérum contient aussi peu de prothrombine qu'un sérum normal. Le temps de consommation de la prothrombine peut être prolongé jusqu' à la normale par adsorption sur $BaCO_3$ et de nouveau réduit par addition de l'éluat au citrate de sodium (obtenu à partir du $BaCO_3$). L'activité antihémophilique du sang thrombocytopénique disparaît quand le sang coagule comme pour le sang normal. Par contre l'autoprothrombine I (Facteur VII) est présente en concentration beaucoup plus faible que dans le sérum normal. Les auteurs suggèrent qu'un nouveau facteur, éventuellement un dérivé de la prothrombine, est responsable des courtes valeurs obtenues pour la consommation de la prothrombine dans le sang thrombocytopénique.

Zusammenfassung

Thrombopenisches Serum weist eine kurze Prothrombin-Konsumptions-Zeit auf, obwohl dieses Serum ebensowenig Prothrombin enthält wie Normalserum. Der Prothrombin-Verbrauchs-Test des thrombopenischen Serums kann durch Adsorption mit $BaCO_3$ auf normale Werte verlängert und durch Zufügen des Zitrat-Eluats wieder verkürzt werden. Die antihämophile Aktivität (Faktor-

VIII-Aktivität) verschwindet aus thrombopenischem Blut. Autoprothrombin I (Faktor VII) ist in thrombopenischem Serum in geringerer Menge enthalten als in normalem Serum. Die Autoren vermuten, daß ein neuer Faktor — wahrscheinlich ein Derivat des Prothrombins — für die kurzen Prothrombin-Verbrauchs-Werte in thrombopenischem Serum verantwortlich ist.

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