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THE PROLONGATION OF THE THROMBOTEST CLOTTING TIME IN NEWBORNS

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ABSTRACT

As judged from thrombotest dilution curves clotting inhibiting material was present in 67% of human umbilical cord plasma samples of healthy full term infants (n=40). The correlation coefficient (r-value) between the thrombotest clotting times and the prothrombin levels was -0.46. Using sensitive enzyme immuno assays for fibrin degradation products (FbDP) and fibrinogen degradation products (FgDP), we found that no degradation products could be demonstrated in the non-inhibited group whereas small amounts of these products were present in the inhibited group. These small amounts were undetectable using conventional assays. The most striking finding was the presence of fibrin and fibrinogen degradation products. The prolongation of the thrombotest clotting time could be imitated by adding fibrinogen fragment X to umbilical cord plasma in which no clotting inhibiting material was present in the thrombotest dilution curve. We conclude that the thrombotest clotting is of limited value in the assessment of the vitamin K-dependent coagulation factors in umbilical cord plasma. As utmost care was taken to avoid proteolytic breakdown in vitro, our findings most likely reflect an enhanced fibrinogenolytic activity in umbilical cord plasma in vivo.

INTRODUCTION

The vitamin K-dependent coagulation factors in full term, healthy newborns are at birth in the range of 20-60% of the values found in normal adults (1). A vitamin K deficiency can be held responsible for this in only a minority of the cases (2). Among the laboratory tests, often used in clinical practice, to obtain an impression of a deficiency of the coagulation factors II, VII and X, are Thrombotest^R and Normotest^R. These tests are variants of the classical prothrombin time, in the presence of excess factor V and fibrinogen. The exact composition of these reagents has not been published. Besides the amount of the coagulation factors II, VII and X, a number of other factors can also influence the thrombotest clotting time. Relatively short thrombotest clotting times are found, when the blood samples are stored for more than 2-4 hours in glass tubes at low

KEY WORDS: thrombotest clotting time, newborns, umbilical cord plasma, prothrombin levels, fibrin(ogen) degradation products, fibrinogen fragment X.

temperatures. This phenomenon is probably due to a factor XII dependent activation of factor VII (3) and is especially important in plasmas of women using oral contraceptive drugs (4). Relatively long thrombotest clotting times are found whenever one of the factors II, VII or X becomes rate limiting (congenitally or acquired deficiency, early in coumarin oral anticoagulant therapy), in the presence of a structurally abnormal factor IX (Haemophilia B_m) (5) or in the presence of other inhibitors such as heparin (6), descarboxy coagulation factor X (7), fibrin(ogen) degradation products (6) and in the case of excess citrate (8).

Inhibition of the thrombotest clotting time, defined as a difference of at least 5 seconds in the so called thrombotest dilution curve has been described in 67% of human umbilical cord plasma samples of normal full term healthy newborns by our group (6). This inhibition could not be attributed to discrepancies between the individual coagulation factors II, VII and X, the presence of descarboxy coagulation factors, an excess of citrate or the presence of fibrin(ogen) degradation products as assessed using conventional relatively insensitive assays. The presence of heparin-like activity has been postulated, but could not be confirmed (9). Thrombotest clotting times are widely used as a screening test for the amount of the vitamin K-dependent coagulation factors present and by some authors recommended as a diagnostic tool for the diagnosis of a vitamin K deficiency in newborns (10). The aim of this study was to assess the value of the thrombotest clotting time in defining the levels of the vitamin K-dependent coagulation factors in newborns and to see if any other factor(s) might contribute to the prolongation of the thrombotest clotting time described in normal healthy newborns at birth.

MATERIALS AND METHODS

We investigated 40 umbilical cord plasmas of healthy, full term newborns. No vitamin K had been administered to the mother or the newborns, nor any medication, known to influence the normal haemostatic mechanism. In all cases, the apgar score was above 7 (at 1 minute) and the plasma pH above 7.2; immediately after birth the umbilical cord was clamped and blood drawn by a clean puncture of the umbilical vein. After discarding the first 2 ml, 9 volumes of blood were mixed in a propylene tube with one volume of an anticoagulant mixture: 0.1 M trisodium citrate dihydrate, 15 mM sodium azide, 0.3 M hydroxyethylpiperazine ethane sulfonic acid (Hepes) and 1000 KIE Aprotinin (Trasylo1)/ml. Platelet poor plasma was obtained by centrifugation at 3000xg for 15 minutes and rendered platelet free by centrifugation at 20.000xg for 30 minutes at 4 °C. The samples were frozen immediately at -80 °C in aliquots of 1 ml until further use. Plasma of 20 adult donors was prepared in the same way to serve as a control normal adult pool.

Thrombotest^R and Normotest^R reagents were obtained from Nyegaard, Oslo, Norway. The assays were performed exactly as indicated in the manufacturers prescription. Thrombotest dilution curves were made by plotting the thrombotest clotting times (Y-axis) against the corresponding dilution of plasma (X-axis). Plasmas were diluted in Michaelis buffer (0.15 M Na-Veronal, 0.1 M Na-acetate, 0.15 M NaCl) in plastic tubes immediately before they were tested. All measurements were made in duplicate. These curves have shown to be linear (11). At the intersection point in the Y-axis the concentration of the coagulation factors is "infinite high". If

inhibiting material is present, the degree of inhibition can be expressed in units as indicated in figure 1. The prothrombin levels (clotting activity) were measured with a one stage method using human thromboplastin and human prothrombin deficient plasma, using a computer program based on the clotting times of eight dilutions of adult normal pooled plasma (12). Thromborel (Boehringer Mannheim, GFR) was used as human thromboplastin. Human prothrombin deficient plasma (immunochemically depleted substrate plasma) was obtained from Diamed (Morat-Marten, Switzerland). Fibrin(ogen) degradation products in serum were determined using the conventional test kit of Wellcome (Thrombo-Wellco test kit, Wellcome Res. Lab Beckenham, England) and also in plasma using enzyme immuno assays (EIA's) based on monoclonal antibodies. Three different EIA's were used. In all three the capture monoclonal antibody is FDP-14 (13), which is specific for degradation products of fibrinogen (FgDP) and of fibrin (FbDP). The "Total degradation products" assay (TDP) measures the sum of FgDP and FbDP (14). Discrimination between the quantities of FgDP and FbDP is done by using different tagging antibodies, conjugated with horse radish peroxidase. In the FgDP-EIA, this is Y18 (15,16), whereas in the FbDP DD-13 is used (17). Purified fibrin(ogen) fragments were prepared as described previously for X (18), Y (19) and D-dimer (20).

RESULTS

In table I the mean values, range and SD are given of the Thrombotest clotting times, the Normotest clotting times and the prothrombin clotting activities (factor IIC) in cord plasmas from 40 healthy full term infants.

TABLE I

Assay	N	Mean value and SD	Range
Thrombotest Clotting Time(sec)	40	53.5" \pm 8.1	35.2 - 66.5
Normotest Clotting Time(sec)	40	32.8" \pm 4.7	25.3 - 44.1
Factor IIC (One Stage)(%)	40	50.6% \pm 12.2	28.8 - 73.7

The correlation coefficient (r-value) between the thrombotest clotting times and the prothrombin levels was -0.46, between the normotest clotting times and the prothrombin levels -0.70. This indicates that especially the thrombotest clotting time does not give reliable information on the level of the vitamin K-dependent coagulation factor II (prothrombin). This could be due to the presence of an inhibitor. We could confirm data published previously by our laboratory that in 67% of the umbilical cord plasma samples of normal infants an inhibitor is found as judged from thrombotest dilution curves (6). An example is given in figure 1.

Fibrin(ogen) degradation products could not be demonstrated in any of the serum samples, using the test kit of Wellcome. The sensitivity of this method, however, is not very high (detection limit: 10 μ g/ml). Moreover, some high molecular weight degradation products are thrombin clottable and will therefore not be measured in the conventional assay, which makes use of serum. With recently developed more sensitive EIA's that can be done in plasma we determined levels of FgDP, FbDP and TPD (FgDP+FbDP) in 6 umbili-

cal cord plasma with and in 6 without inhibiting material as judged from the thrombotest dilution curves. The results are summarized in Table II. The fibrin(ogen) degradation products are expressed as μg fibrinogen equivalent/ml. The large range of TDP detected in apparently normal babies must be regarded as interindividual variation, for instance due to the stress of the birth process.

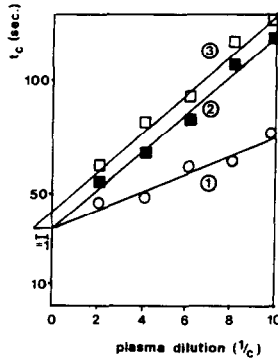


Figure 1:
Thrombotest Dilutioncurves
1. \circ - \circ adult normal pool
2. \blacksquare - \blacksquare non-inhibitory cord
plasma-pool
3. \square - \square inhibitory cord
plasma (1 Unit)

TABLE II

Sample	Degree of Inhibition (U)	TDP	FgDP	FbDP
1	0.4	1.9	0.7	<0.25
2	0.5	5.8	2.0	2.2
3	0.5	0.7	<0.25	<0.25
4	1.3	12.8	3.7	4.6
5	0.3	2.2	0.5	<0.25
6	0.3	1.8	<0.25	<0.25
Non inhibited cord plasma (n=6)	0	<0.25	<0.25	<0.25
adult plasma pool	0	<0.25	<0.25	<0.25

In the "inhibited samples small amounts of FbDP and FgDP were present, whereas they were below the detection limit ($0.25 \mu\text{g}$) in the non inhibited samples as well as in adult normal pool plasma. In order to see whether inhibition as observed in the thrombotest dilution curve could have been caused by fibrin(ogen) degradation products we added small amounts of purified degradation products X, Y or D-dimer to "non-inhibited" umbilical cord plasma and adult normal pooled plasma. In adults, the addition of a relatively high concentration of fragment X gave only a slight prolongation of the thrombotest clotting time (figure 2B). A more pronounced prolongation was found in the "non-inhibited" umbilical cord plasma pool (figure 2A). In adults as well as in newborns inhibition in the thrombotest dilution curves could be demonstrated by the addition of fragment X. Addition of the fragments Y and D-dimer in low concentrations did not affect the thrombotest-clotting times.

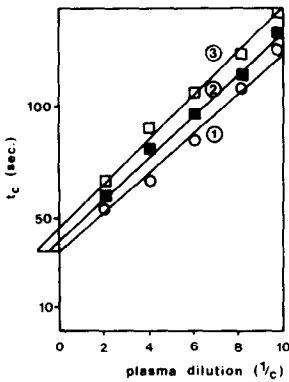


Figure 2A

The effect of adding fragment X to non-inhibitory cord plasma in the thrombotest dilution curve

1. ○—○: non inhibitory cord plasma pool
2. ■—■: 1 + 2.46 µg/ml fragment X
3. □—□: 1 + 24.6 µg/ml fragment X

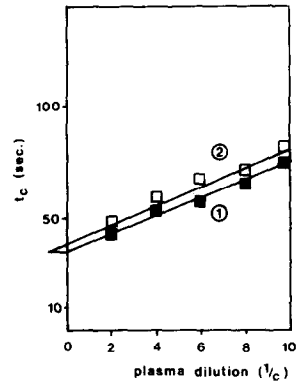


Figure 2B

The effect of adding fragment X to adult normal plasma in the thrombotest dilution curve

1. ■—■: adult normal pool plasma
2. □—□: 1 + 24.6 µg/ml fragment X

DISCUSSION

In this study, we have provided data, that the thrombotest clotting time is not a reliable tool to assess the vitamin K status of newborns. As has been found previously (6) this is probably due to the fact that an inhibitory factor of the thrombotest clotting time is present in 67% of normal healthy infants at birth. Using sensitive EIA's, we have demonstrated that degradation products both of fibrinogen and of fibrin are present in umbilical cord plasmas, which show an inhibitory factor as judged from the thrombotest dilution curves. Degradation products could not be detected in the samples of the group in which the inhibitory factor was absent. Using conventional serum assays we were unable to detect fibrin(ogen) degradation products in umbilical cord blood, which is in agreement with the literature (21). This can be due to the fact that the conventional method is not sensitive enough and/or the possibility that high molecular weight fibrin(ogen) degradation products are partially thrombin clottable and will therefore not contribute to the amount of fibrinogen related antigen recovered in serum after complete clotting induced by thrombin and thromboplastin in the presence of aprotinin (Trasylol). The most striking finding was the presence of fibrinogen degradation products. This is only found in disease states with primary fibrinogenolysis or with treatment with thrombolytic agents. As we took care to minimize proteolysis in vitro, we feel that our data reflect an enhanced fibrino(geno)lytic activity in umbilical cord blood in vivo at birth. We were also able to induce the prolongation of the thrombotest clotting time and inhibition in the thrombotest dilution curve by adding small amounts of the purified fibrinogen degradation product, fragment X to the "non-inhibited" umbilical cord plasma pool and adult normal pool plasma. The effect of added fragment X appeared to be more pronounced in umbilical cord plasma.

We conclude that the prolongation of the thrombotest clotting time in

newborns is only partially due to low levels of the vitamin K-dependent coagulation factors II, VII and X and that the results of this test should not be taken as conclusive evidence for a vitamin K-deficiency, as is recommended by some authors in the literature (10). Too many factors, besides the levels of the vitamin K-dependent coagulation factors influence the results of this screening coagulation assay. As shown here also fibrin(ogen) degradation products contribute to the prolongation of the thrombotest clotting time. Obviously, the limitation of the use of the thrombotest is even more pronounced, when the blood samples are not drawn carefully, and when adequate inhibitors of proteolysis in vitro are not present in the anticoagulant mixture in which the blood is collected. At present, the cause of the enhanced fibrin(ogen)olytic activity of normal newborns at birth is not known. Fibrin(ogen)olytic activity is the result of the relative activities of tissue plasminogen activator (tPA) and a fast acting tPA-inhibitor (22). Thus, increased fibrin(ogen)olytic activity may be the result of an increased secretion of tPA from endothelial cells and/or a decreased level of tPA-inhibitor. The latter may be caused by increased levels of activated protein C (APC), which is known to decrease the activity of tPA-inhibitor (22). The enhanced fibrin(ogen)olytic activity is probably one of the protective mechanisms against the development of thrombosis in the umbilical cord vessels.

It would be of interest to obtain our cord blood findings also in neonatal plasmas in the first week of life, because the nadir of the vitamin K-dependent coagulation factors is not usually at the time of birth but 2-3 days later. This would strengthen our claim that our findings are relevant for the assessment of neonatal haemostasis.

Unfortunately we are unable to obtain sufficient amounts of neonatal blood from healthy newborns, which is suited for haemostasis studies.

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