Cold-Induced Contact Surface Activation of the Prothrombin Time in Whole Blood

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Studies of the prothrombin time (PT) have revealed that contact with borosilicate or commercial siliconized borosilicate markedly shortens the PT. This shortening is related to the activation of the contact phase of blood coagulation. This shortening of the PT occurs in both normal whole blood and plasma when stored in borosilicate or siliconized borosilicate tubes at 4°C and at a lesser degree at room temperature. Studies indicated the importance of several coagulation factors in decreasing the PT. The PT did not change in blood deficient in factor XII or in plasma deficient in Fletcher factor or high molecular weight kininogen, while blood deficient in CI esterase inhibitor (CI INH) had the most profound shortening. Shortening of the PT correlated directly with increased levels of factor VII. When purified CI INH was added to normal blood, it markedly reduced the activation of factor VII and the shortening of the PT in a dose-related manner. These studies indicate the pivotal roles of the contact phase of coagulation in initiating activation of the PT and of CI INH in inhibiting the activation of the coagulation factor(s) responsible for the cold-promoted activation of factor VII.

MATERIALS AND METHODS

Whole Blood

Assays were performed on blood samples obtained from normal volunteers receiving no medications and patients with factor XII deficiency (coagulant activity <1%), hereditary angioedema (HAE), (antigenic CI INH = 8.5 mg/dl by Laurell electrophoresis, normal = 12.5-28), factor XI deficiency (coagulant activity <3%), and factor IX (coagulant activity <3%). Freshly collected blood samples were obtained via clean venipunctures using a 19-gauge butterfly needle and a polypropylene syringe. The whole blood from each patient was aliquoted randomly into: (A) borosilicate tubes (Becton-Dickinson, Rutherford, N.J., lot 9B131) with a 1:9 final v/v ratio of 3.8% citrate to whole blood; (B) siliconized borosilicate tubes (Becton-Dickinson, Rutherford, N.J., 6464, lot 9H056) with a 1:9 final v/v ratio of 3.2% citrate to whole blood; and (C) 17 x 100 mm polypropylene tubes (2029 Falcon, Oxnard, Calif.) to which 38% citrate had been previously added so that the final v/v ratio of citrate to whole blood was 1:99. The vacutainer tubes were filled through a 19-gauge needle via vacuum, and the polypropylene tubes were filled to a pretranscribed mark by manually expressing blood down the side of the tube. Each tube was then inverted five times and placed in an ice bath. In some experiments, after specimen collection, four of the borosilicate tubes and an identical number of the other tubes were kept at room temperature. The plasma was separated at various time intervals (immediately after blood collection, 1 hr, 2 hr, and 4 hr later). All tubes were centrifuged at 4000 rpm (2500 RCF) for 10 min at 4°C. The plasma was then transferred into a 12 x 17 mm polyethylene tube (2063 Falcon, Oxnard, Calif.) via a nonwettatable polypropylene pipette and kept uncapped in ice. Prothrombin times and plasma pH determinations were performed immediately after the plasma separation, and then the specimens were stored at –90°C for further studies.

Commercial Plasma

In other studies, because of the unavailability of whole blood from patients with certain congenital deficiencies, we used several frozen plasmas. This included normal plasma or plasma deficient in factor XII (<1%), prekallikrein (Fletcher factor <1%), or high molecular weight kininogen (Fitzgerald factor <1%) (George King Laboratories, Overland Park, Kans.). These samples were collected and...
stored in plastic containers. The thawed plasma samples were kept either on ice or at 23°C in a 12 x 75 mm borosilicate tube, and at various time intervals (immediately after transfer, 1 hr, 2 hr, and 4 hr) plasma was removed from each tube and the PTs were immediately performed. Pooled normal plasma from our laboratory and from George King Laboratories were studied for comparison. The studies were performed three times.

**Cl INH Purified**

Lyophilized high purity Cl INH, lot NFC #11-A, was a generous gift of Dr. M. Wickerhauser, National Red Cross Blood Research Center, Bethesda, Md. In some materials, trace amounts of ceruloplasmin and haptoglobin were detected by immunoelectrophoresis. The Cl INH was reconstituted with Tris-Saline buffer (0.05 M Tris, 0.1 M NaCl, pH 7.35) so that the concentrations were 50.0 PE/ml and 25.0 PE/ml, [1 plasma equivalent (PE) of Cl INH is defined as the equivalent amount of Cl INH in 1 ml of normal plasma as determined by electroimmunoassay electrophoresis]. Whole blood from 3 normal volunteers was collected and processed as was described above except that the borosilicate or siliconized borosilicate tubes had 100 μl of either Tris-saline buffer of Cl INH previously added via Ependorff pipettes. The tubes were then filled to a final volume of 5.0 ml and kept in an ice bath. Antigenic Cl INH levels were determined on frozen plasma by the Laurell immunoelectrophoresis technique. Antihuman Cl esterase inhibitor (rabbit) was obtained from Calbiochem-Behring Corp., La Jolla, Calif.

**Performance of Prothrombin Times**

All PTs were performed with rabbit brain thromboplastin (Ortho Laboratories, Raritan, N.J., lot 10T863A) using fibrometers. Duplicate determinations were performed on each specimen. The mean of the duplicates was recorded as the PT. The mean and standard deviations from 10 normal volunteers who were tested 22 times were calculated.

**Coagulation Factor Assay**

Factors V, VII, X, IX, XI, XII, and prekallikrein (Fletcher factor) were determined by one-stage assays with congenitaly deficient plasmas.

**Calculations**

The percentage change of the PT and factors was calculated by subtracting the value at any time from that at 0 time for each type of tube. The percent change was calculated by the following formula:

\[
\text{Percentage} = \frac{(0 \text{ time}) \text{ sec} - (X \text{ time}) \text{ sec}}{(0 \text{ time}) \text{ sec}} \times 100
\]

where X time is the value at any time other than 0 time, and 0 time is the value when done immediately after the collection of blood and the separation of plasma. (All values are compared to the 0 time for each type of contact surface collection tube.)

**RESULTS**

We previously had found that the exposure of whole blood to borosilicate or commercial siliconized borosilicate resulted in the most pronounced shortening of the PT at 4°C. Contact of whole blood with borosilicate or commercial siliconized borosilicate at 23°C resulted in a less pronounced shortening of the PT, while the PT of blood collected in polypropylene did not change (Fig. 1).

Studies of the influence of specific intrinsic coagulation factors on the PT showed that the PT of blood deficient in factor XII was not affected. The percentage shortening at 4 hr was less than 3%, while the normal was over 21% (Fig. 2). On the other hand, the PT of blood deficient in factor XI had a time course of shortening similar to normal, while the PT of factor-IX-deficient blood had a shortening intermediate between normal blood and factor-XII-deficient blood (factor IX shortening at 4 hr was 10.9%, normal 21.3%, and factor XII was 2.7%). The most pronounced cold activation was in the blood of the patient deficient in Cl IH (36.6%).

Although the preparation of plasma could not be optimized because of the unavailability of patients deficient in high molecular weight kininogen or prekallikrein, the commercial plasmas deficient in these factors (and factor XII used as controls) involved in the contact phase of coagulation did not result in significant cold-induced shortening of the prothrombin time (Fig. 3). The PT of the normal...
Fig. 2. Effect of borosilicate contact surfaces stored at 4°C in whole blood deficient in: Cl INH (O), factor XI (Δ), factor IX (●), and factor XII (○). One SEM for normals (□) is indicated by bars. The percentage decrease of the PTs at 4 hr is given in parentheses.

Fig. 3. Effect of borosilicate contact surfaces stored at 4°C when commercial plasma deficient in factor XII (■), prekallikrein (Fletcher factor, △), and high molecular weight kininogen (Fitzgerald factor, ▲) was used. Pooled normal plasma (PNP) was stored at 4°C (▲) and room temperature (○). The values are given as the mean from experiments done on 3 separate days.

Fig. 4. The percentage change of coagulation factors VII (>-160%, ■), V (+15%–20%, ●), and IX (-10%–15%, ○) and the percentage decrease of the PT (□) is given when normal whole blood is collected in borosilicate tubes and stored at 4°C. There were no changes in factor X, factor XII, or prekallikrein (Fletcher factor). The changes with commercial siliconized tubes were similar to those shown but were minimal with polypropylene tubes.

Fig. 5. The effect of CI INH and/or various contact surfaces on the percentage decreases of the PT and increases of factor VII at 4 hr are shown for normal whole blood stored at 4°C. The addition of CI INH inhibits the percent decrease of the PT in a concentration-dependent manner. Polypropylene contact surfaces have the greatest inhibitory effect on the percent decrease of the PT. Commercial siliconized borosilicate tubes are slightly more efficient than borosilicate tubes when CI INH is added.
plasma(s) shortened to a similar percentage as in previous studies.

Analyses of the coagulation factors over time during the in vitro cold activation of normal whole blood revealed that factor VII is increased 160% over baseline in borosilicate tubes. Factor V increased by 15%–20%, while factor IX decreased 10%–15%. No changes in factor XII, prekallikrein (Fletcher factor), or factor X were found (Fig. 4). In commercial siliconized borosilicate tubes the changes (not shown) in the factor levels were almost identical. In polypropylene tubes, changes in coagulation factors were less than 10% of the baseline values.

The addition of purified Cl INH to normal whole blood in both borosilicate and commercial siliconized borosilicate tubes at final concentrations of 0.5 and 1.0 PE/ml resulted in a dose-dependent reduction of the in vitro activation of the PT and a significant reduction in the activation of factor VII (Fig. 5). There was no change in the antigenic Cl INH levels over time in any of the tubes reported in this study.

The shortening of the PT is directly related to the percentage activation of factor VII. After 4 hr incubation at 4°C, there was less shortening of the PT and less increases of factor VII when the activation of the contact phase of coagulation was inhibited by either polypropylene or Cl INH (Fig. 5).

**DISCUSSION**

The observation that significant shortening of the PT occurs when whole blood from normal volunteers and patients receiving coumadin is stored in borosilicate or commercial siliconized borosilicate collection tubes at 4°C has not been adequately emphasized in the literature. We found that the cold-promoted activation of the contact phase of coagulation, primarily factor XII, prekallikrein, and high molecular weight kininogen, was required for the activation of factor VII and the subsequent shortening of the PT. Factor IX appears to contribute to approximately 40%–50% of the factor VII activation in our whole blood system, similar to the results previously reported. Cl INH acts as an effective inhibitor to the cold-promoted activation of factor VII and shortening of the PT by the contact phase of coagulation.

The contact surface to which both whole blood and plasma are exposed is an important variable in the activation of the PT. The use of polypropylene surfaces did not result in significant shortening of the PT; however, borosilicate and commercial siliconized borosilicate surfaces resulted in significant temperature-dependent activation of the PT. Personal communication with the manufacturer has revealed that the siliconization is not complete with the current process, and this most likely accounts for the disappointing results with this collection tube. The pivotal role of the contact phase of coagulation is further emphasized by the lack of cold-promoted activation of the PT when either whole blood deficient in factor XII or plasma deficient in factor XII (Hageman factor), or prekallikrein (Fletcher factor) or high molecular weight kininogen (Fitzgerald factor) are used.

Similar shortening of the thrombotest and PT using normal plasma has been previously reported by others. The mechanism appears to be related to activation of factor VII either directly or indirectly by one of several substances (activated Hageman factor, Hageman factor fragments, kallikrein, plasmin, or activated factor IX) and appears to involve at least one of the plasma inhibitors of coagulation, the plasma inhibitor of the first component of complement (Cl INH). Factor XIIa and Hageman factor fragments have been shown to activate factor VII. The activation of factor VII is directly proportional to the concentration of factor XII fragments, and after an initial rapid activation of factor VII, a plateau is reached. The cause for this plateau is unclear, but in plasma systems it may be related to the effect of plasma inhibitors such as Cl INH or the depletion of the proenzyme factor VII molecule. In purified systems this effect is probably related to depletion of the proenzyme factor VII molecule. Factor XII fragments or factor XIIa are also potent activators of the conversion of prekallikrein to kallikrein, which then in turn catalyzes the activation of factor XII. Plasma from patients who lack prekallikrein did not demonstrate a pronounced shortening of the thrombotest nor was there enhanced factor VII activity. However, in purified systems kallikrein does not directly activate factor VII, but does activate factor IX, which in turn activates factor VII.

Despite using several dilutions of test plasma and variations of incubation times of the plasma with and without kaolin activation, we were unable to demonstrate an increase in the coagulant factor XII levels. The explanation for this is unclear but may be related to a conformational change in the factor XII molecule by the negatively charged borosilicate surface rather than a cleavage of the molecule with the resultant exposure of an active site. Alternatively, this lack of change in factor XII activity may reflect the low procoagulant activity of β-XIIa.

Likewise, there was no significant change in prekallikrein coagulant activity at 4 hr. Others have shown that prekallikrein is cleaved after 4 hr when cold-promoted activation of normal plasma occurs.
had prolonged our incubation times at 4°C to longer than 4 hr, then changes in coagulant prekallikrein levels may have been observed.

The importance of the natural occurring inhibitor Cl INH is emphasized by the pronounced shortening of the PT in the patient with HAE (either at room temperature or 4°C) and by the temperature dependence of the borosilicate activation of normal whole blood when Cl INH is added to the collection tube. We found that Cl INH resulted in a concentration-dependent inhibition of the borosilicate cold activation of the PT and factor VII. This confirms the importance of Cl INH in inhibiting the contact phase of coagulation and subsequently the activation of the PT. Others have shown that Cl INH inhibits factor XII fragments, plasmin, kallikrein, and the esterase activation of the complement system.14,16,17,23 Furthermore, both cold and the kallikrein--Cl-INH combination inhibit or inactivate the Cl INH.14 Thus, our findings and the findings of others suggest that when Cl INH is functionally active, the activation of factor XII by kallikrein and the activation of factor VII by factor XII fragments are inhibited. Furthermore, cold inactivation of functional Cl INH can be overcome by adding purified Cl INH into the system. Studies using other inhibitors to the contact phase of coagulation are underway.

Our findings that factor IX pathways contribute approximately 40%–50% to the conversion of factor VII to VIIa are consistent with the role of factor IX reported by others.5,6 Cleavage of factor IX by factor VII has been reported in both purified and plasma systems24 and factor IX has been shown to activate factor VII in a purified system.5 We had found that factor IX levels decrease slightly during the cold activation of factor VII in whole blood. Others have suggested that kallikrein activates factor IX, which in turn activates factor VII, and this results in the shortening of the PT or thrombostest. An alternative and/or coexisting explanation in whole blood or plasma systems would be that activated factor VII may also be directly cleaving factor IX, which then amplifies the further activation of factor VII.

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REFERENCES

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