Amidolytic Assay of Human Factor XI in Plasma: Comparison With a Coagulant Assay and a New Rapid Radioimmunoassay

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The traditional coagulant assay for plasma factor XI suffers from a relatively high coefficient of variation, the need for rare congenitally deficient plasma, and a poor correlation between precision and sensitivity. We have developed a simple functional amidolytic assay for factor XI in plasma using the chromogenic substrate PyrGlu-Pro-Arg-p-nitroanilide (S-2366). After inactivation of α-antitrypsin, Cl inhibitor, and other plasma protease inhibitors with CHCl₃, plasma was incubated with kaolin, in the absence of added calcium, which limited the enzymes formed to those dependent on contact activation. Soybean trypsin inhibitor was used to minimize the action of kallikrein on the substrate. Once the reaction was complete, corn trypsin inhibitor was used to inactive factor Xla, the enzyme generated by exposure of plasma to negatively charged surfaces, which had activated the factor XI. The assay is highly specific for factor XI, since plasma totally deficient in that zymogen yielded only 1%-3% of the enzymatic activity in normal plasma under identical conditions. The requirements for complete conversion of factor XI to Xla in plasma within 60 min were, respectively, factor XII, 0.6 U/ml, and high molecular weight kininogen, 0.2 U/ml. Prekallikrein was not an absolute requirement for complete activation but did accelerate the reaction. The intraassay coefficient of variation was 3.4%, and the mean of 35 normal plasmas was 1.00 U ± 0.24 SD. In addition, a new rapid radioimmunoassay was devised using staphylococcal protein A as the precipitating agent for a complex of factor XI antigen with monospecific rabbit antibody. The mean was 1.01 U ± 0.30 SD. The correlation coefficients for amidolytic versus coagulant and amidolytic versus radioimmunoassay were r = 0.95 for the former and 0.96 for the latter. Thus, a simple, accurate amidolytic assay and a radioimmunoassay have been devised for measuring factor XI in plasma that correlate well with the coagulant activity of factor XI, as determined in our laboratory.

Factor XI is one of four proteins comprising the contact activated system of blood coagulation, but it is the only component whose absence may result in a hemorrhagic disorder. Factor XI in plasma is activated in vitro by activated factor XII (factor XIIa). Factor XIIa arises from the interaction of factor XII zymogen with negatively charged surfaces, such as kaolin, ellagic acid, dextran sulfate, or sulfatides, and is amplified by kallikrein-mediated proteolysis in the presence of high molecular weight (HMW) kininogen, previously described as contact activation cofactor. The major plasma inhibitor of factor Xla is α-antitrypsin, which is responsible for 68% of plasma inhibitory activity. Antithrombin-III, in the presence or absence of heparin, is responsible for 16%, and Cl inhibitor and α₂-plasmin inhibitor account for the remainder of the plasma inhibitory activity. Unlike what is observed for factor XIIa and kallikrein, plant inhibitors, such as soybean, corn, and lima bean trypsin inhibitors, do not inhibit factor Xla substantially.

Traditionally, factor XI is assayed by a modified activated partial thromboplastin time, using plasma deficient in factor XI as the substrate. Several reagents currently used for the activation of the contact factors in coagulant assays include kaolin, celite, or ellagic acid. Commercially prepared reagents for use with automated equipment contain an activating agent and mixed phospholipids. The 1981 College of American Physicians Survey, which compiled statistical data from 466 laboratories, revealed that there is "poor correlation between precision and sensitivity" of most commercial systems, indicating the need for improvement of the factor XI assay. Furthermore, since factor XI deficiency is variable from patient to patient, consistent substrate is difficult to obtain. Coagulation assays, in general, have a high intraassay coefficient of variation (as high as 54%), probably due to the lability of several of the coagulant proteins. Moreover, coagulant assays require laborious, precise timing of both the activation step and the recalcification step. An immunochemical assay of factor XI has been described, but it is time-consuming, usually requiring 3 days for completion. Amidolytic assays for plasma prekallikrein and an important protease inhibitor of contact activation, Cl inhibitor, have recently been developed. However, no amidolytic assay has been previously described for plasma factor XI, probably due to lack of a specific synthetic substrate for factor Xla.

In this article, we describe the development of a new
functional amidolytic assay for plasma factor XI, using the synthetic substrate PyrGlu-Pro-Arg-pNA (S-2366). Although this substrate is not specific for factor Xla, our assay system allows only the activation and expression of factor Xla enzymatic activity. The methodology for inactivating inhibitors, activating factor XI, and preventing other enzymes from hydrolyzing the substrate is described. The specificity of the assay and the requirements for other proteins of the contact system are assessed in this report. In addition, we have developed a new, rapid radioimmunoassay (RIA) for factor XI that employs staphylococcal protein A as the second precipitating antibody. This RIA has been correlated with the coaguulant and amidolytic assays. The results of this study allow us to recommend a practical and reproducible amidolytic assay and a radioimmunoassay for the measurement of purified factor Xla and plasma factor XI.

MATERIALS AND METHODS

Reagents

Soybean trypsin inhibitor (SBTI), bovine serum albumin (crystalline), and tosyl-lys-chloromethylketone (TLCK) were purchased from Sigma Chemical Co., St. Louis, MO. Kaolin (acid washed) and chloroform were obtained from Fisher Scientific Chemical Co., (King of Prussia, PA), Inosithin (mixed soybean phospholipids) was purchased from American Concentrates, New York, NY.

S-2366 (PyrGlu-Pro-Arg-paranitroanilide·2HC1) was furnished to us as a gift by AB KABI Peptide Research, M{lindal, Sweden. It can be obtained in the United States from Helena Laboratories, Beaumont, TX. All other chemicals were reagent grade.

Plasma

Blood was obtained by clean venipuncture from normal healthy individuals after written, informed consent was obtained from each donor.

Blood for all three assays was collected into plastic syringes containing 3.8% (9:1 v/v) trisodium citrate. Blood was obtained by clean venipuncture from normal healthy individuals after written, informed consent was obtained from each donor.

Blood for all three assays was collected into plastic syringes containing 3.8% (9:1 v/v) trisodium citrate. Plasma was frozen in 1-ml aliquots at -70°C immediately after centrifugation. The prekallikrein-deficient plasma was kindly supplied by Dr. C. Abildgaard, Davis, CA. Plasma samples from two patients with severe liver disease were obtained from patients at Temple University Hospital. Reference plasma (pool of 20 normal donors) was purchased from George King Biomedicals, Overland Park, KS. Factor XI-deficient plasma and kininogen-deficient plasma were donated directly to us. Factor-XII-deficient plasma was kindly supplied by Dr. Margaret Johnson, Wilmington, DE. Additional samples of factor-XI-deficient plasma were generously donated by Mr. George King (George King Biomedicals).

Corn Trypsin Inhibitor

Corn trypsin inhibitor was a generous gift of Patrick McDevitt and Dr. Edward P. Kirby of this institution. The protein concentration was calculated using 1% E{lmax = 2.0.

Purified Factor Xla

This was prepared according to Scott et al. Nine hundred milliliters of fresh-frozen plasma containing 50 µg/ml hexadimethrine bromide, 1 mM EDTA, and 0.02% sodium azide, was mixed, sequentially, with 3 batches of QAE-Sephadex (900-ml slurry in 20 mM Tris·Cl, pH 8.1). The material, which did not adsorb, was mixed with 1,000 ml SP-Sephadex equilibrated with 20 mM Tris·Cl, pH 8.1 (conductivity adjusted to less than 2 mmho), mixed for 30 min, and filtered. The resin containing the adsorbed protein was poured into a column and eluted by a linear gradient between 0 and 0.13 M NaCl. The fractions containing factor XI coagulant activity were pooled, concentrated, and dialyzed in an Amicon concentrator (PM-30 membrane) versus 0.1 M sodium acetate, pH 5.3. The factor XI was eluted by a linear gradient from 0 to 0.35 M NaCl.

The fractions containing factor XI coagulant activity were pooled and concentrated. Removal of IgG was accomplished by batch adsorption of 0.40 ml of factor XI (90 U/ml diluted 1:2 with H2O) for each 1 ml of anti-IgG-agarose that had been equilibrated in 0.1 M Tris·Cl, pH 8.0, containing 0.15 M NaCl. The sample was incubated for 5 min prior to centrifugation for 2 min at 12,000 g. Yields between 40% and 55% of the original plasma concentration of factor XI were repeatedly observed. No prekallikrein or factor XII activity could be detected by coagulation assay. No IgG could be detected by radial immunodiffusion. The specific activity was 203 U/mg. Activation of purified factor XI was accomplished by incubating 2 x 10{4 M factor XI (260 µg/ml) with 10{-8 M trypsin for 1.5 hr at 23°C. The trypsin was then inactivated by incubation with TLCK (10{-3 M) at 37°C for at least 3 hr. The half-life of trypsin amidolytic activity under these conditions was 4.5 min. Factor Xla was a single component of mol wt = 150,000 on gel filtration and a single band on SDS gel electrophoresis of mol wt = 160,000 in the absence of a reducing agent. On reduction, two polypeptides of mol wt 50,000 and 30,000 were found, indicating complete activation to factor Xla.

Purified Factor XI

Human factor XI for the radioimmunoassay and for antiserum production was purified to homogeneity using the procedure of Bouma and Griffin. On SDS gel electrophoresis, the unreduced protein migrated as a single band at 160,000 daltons; whereas upon reduction, a single band appeared at 80,000 daltons. Purified factor Xla was radiolabeled with 125I, using the procedure of Bolton and Hunter. The 125I-labeled factor XI was separated from the other iotination products by gel filtration using Sephadex G-25. The specific radioactivity of 125I-factor-XI was approximately 500 cpm/ng.

Antiserum Preparation

Monospecific antibody to purified human factor XI was raised in rabbits by 3 weekly subcutaneous injections of purified human factor XI, 80-100 µg, in a 1:1 emulsion with complete Freund's adjuvant followed by a booster injection of the same amount of antigen with incomplete Freund's adjuvant 7-10 days before bleeding. The antiserum formed a single precipitin band with purified factor XI, and at a dilution of 1:100, and abolished the clotting activity of factor Xla in a normal pooled plasma sample diluted 1:10. Preimmune rabbit serum was negative on all double immunodiffusion tests and did not inhibit factor Xla clotting activity in normal pooled plasma.

Kinetic Studies of Purified Factor Xla on the Chromogenic Substrate S-2366

The K_m for S-2366 was determined by using the substrate at various concentrations between 0.2 and 1.6 mM and factor Xla at a final concentration of 0.6 nM. The buffer was 0.1 M Na phosphate, pH 7.6, containing 0.15 M NaCl and 1 mM EDTA. The change in...
absorbance at 405 nm was continuously recorded on a Gilford 240 spectrometer with a Gilford 6015 chart recorder, and the initial rate measured at 37°C. The molar extinction coefficient for pNA at A405 is 9,800. The K_m for the reaction between factor XIa and S-2366 was 0.36 mM and the V_max (0.75 U/ml purified factor XIa) was 0.143 absorbance units/min or 0.24 nmole/sec/ml. The V_{max}/K_m ratio was 0.36 mM and the K_m ratio was therefore 5.5 x 10^6 M^{-1} sec^{-1}; if we assume two active sites for factor XIa.

Amidolytic Assay of Plasma Factor XIa

Activation

One hundred and fifty microliters of plasma plus 150 µl CHCl₃, to inactivate plasma protease inhibitors, were mixed for 3 min in a polypropylene microcentrifuge tube at 23°C, followed by centrifugation at 12,000 g for 5 min. One hundred microliters of the plasma (top layer) was then transferred to a 0.4-ml plastic microcentrifuge tube containing 10 µl of 50 µM soybean trypsin inhibitor, to prevent kallikrein action on the substrate. Ten microliters of kaolin* (20 mg/ml of 0.1 M Na phosphate, pH 7.6, containing 0.15 M NaCl and 1 mM EDTA) was then added. The sample was then intermittently mixed during the times indicated. Thirty microliters of the activated sample was then transferred to another 0.4-ml microcentrifuge tube containing 7.5 µl of corrin trypsin inhibitor (0.5 mg/ml) to inactivate factor XIIa and thereby prevent its action on the substrate.

Assay

Ten microliters of the activated plasma mixture was added to a prewarmed (37°C) cuvette containing 270 µl 0.1 M Na phosphate, pH 7.6, 0.15 M NaCl, 1 mM EDTA, and 30 µl of S-2366 (11.5 mM). The final substrate concentration was 1.15 mM. The change in absorbance was recorded at 405 nm (at a sensitivity of 0.1–0.2 full scale and a chart speed of 2 cm/min).

Coagulant Assay of Factor XI and XIa

The coagulant activity of factor XI was determined by a modified activated partial thromboplastin time assay with factor-XI-deficient plasma as the substrate. One hundred microliters of factor-XI-deficient plasma was incubated with 100 µl kaolin (5 mg/ml in saline), 100 µl 0.2% isothiobarbituric acid (pH 7.4) + 0.15 M NaCl, and 10 µl of plasma + 90 µl of the abovementioned buffer for 5 min at 37°C. Then, 100 µl of 30 mM CaCl₂ was added to initiate clot formation. Factor XIa was assayed by substituting saline for kaolin and decreasing the incubation time, prior to recalcification, to 1 min. One unit of factor XI is defined as the amount in 1 ml of normal pooled plasma.

Radioimmunoassay

Radioimmunoassay of factor XI antigen was performed using the rabbit anti-factor-XI antibody as the primary antibody and protein A bacterial adsorbent as the secondary precipitating agent. Staphylocococcus protein A (Staph A) can interact specifically with the Fc region of most mammalian immunoglobulins, including all rabbit IgG subclasses. A typical assay mixture, containing 25 µl of a 1:20,000 dilution of rabbit anti-factor-XI antibody, 25 µl of purified factor XI or unknown sample, and 25 µl of 125I-factor XI, was incubated in an Eppendorf microcentrifuge tube with narrow bore extended tip, for 1.5 hr at 37°C. Thirty microliters of a 10% suspension of Staph A was added to the tube, mixed thoroughly, and incubated another 30 min at 37°C. After the samples were centrifuged, the tips of the tubes containing the pellets were amputated and counted in a CG 4000 Intertechnique gamma counter.

RESULTS

Correlation of Purified Factor XIa Amidolytic and Coagulant Activity

Ten microliters of purified factor XIa, at various dilutions, was assayed for amidolytic activity utilizing the substrate S-2366. The same dilutions were also assayed for factor XIa coagulant activity, in the presence and absence of kaolin (Fig. 1). (No detectable differences were found between the two coagulant assays.) The correlation coefficient (r) was equal to 0.98. One unit factor XIa was found to hydrolyze 0.49 µmole S-2366/min/ml.

Effect of Plasma Protease Inhibitors on Purified Factor XIa Amidolytic Activity in the Presence and Absence of Soybean Trypsin Inhibitor (SBTI)

Since plasma protease inhibitors clearly have the potential to affect factor XI activation and factor XIa activity, we explored methods to obviate the problem. Purified factor XIa lost only 8% of its amidolytic activity in 60 min (Fig. 2, curve a) when incubated in buffer. However, when purified factor XIa was incubated with normal native plasma, where inhibitors were present at a ratio of >1,000:1 (Fig. 2, curve b), 65% of the factor XIa activity was inhibited within 10 min, and the initial inactivation obeyed pseudo-first-order kinetics. However, when plasma was treated with CHCl₃ prior to incubation with purified factor XIa (Fig. 2, curve c), only 10% of the amidolytic activity was lost at 10 min, indicating that most of the factor XIa plasma inhibitors were inactivated by

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*Micronized kaolin, prepared by Dr. James Brown of Helena Laboratories, Beaumont TX, can be used at a final concentration of 0.45 mg/ml. Its two main advantages over kaolin are that it remains in suspension for a longer period of time and it causes less optical interference.
AMIDOLYTIC ASSAY OF PLASMA FACTOR XI

Fig. 2. Effect of plasma protease inhibitors on purified factor XIa amidolytic activity. Purified factor XIa (2.5 nM) was incubated at 23°C with either 0.1 M Na phosphate, 0.15 M NaCl, 1 mM EDTA, and 0.1% polyethylene glycol 6000 (curve a), native plasma (curve b), native plasma and 4.5 μM SBTI (curve d), CHCl₃-treated plasma (curve c), or CHCl₃-treated plasma and 4.5 μM SBTI (curve e). At the times indicated, 10 μl was assayed for residual amidolytic activity (see Materials and Methods). The data are expressed as percent original factor XIa activity.

Activation of Plasma Factor XI in Native and Chloroform-Treated Plasma in the Presence of Soybean Trypsin Inhibitor

We then assessed the effect of plasma protease inhibitors on the activation of factor XI in plasma in the presence of SBTI. When plasma was incubated with kaolin in the presence of SBTI to inhibit kallikrein (Fig. 3, curve a), an initial increase in amidolytic activity was observed over the first 10 min, with maximal activity attained at 35 min, followed by a decrease in activity of about 20% by 80 min. When the plasma was treated with CHCl₃, prior to incubation with kaolin and SBTI, a more complete activation was observed, which was maximal by 20 min, without a subsequent decrease in activity for at least 80 min (Fig. 3, curve b). If SBTI was omitted from the CHCl₃-treated plasma, prior to incubation with kaolin, twice as much amidolytic activity resulted (data not shown). This activity corresponded to 0.35 U/ml kallikrein, when assayed with the amidolytic substrate S-2366. Therefore, since kallikrein can hydrolyze S-2366, SBTI must be included in the incubation mixture in order to prevent substantial amounts of kallikrein in the assay. The actual contribution of kallikrein toward the amidolytic activity of S-2366, after including SBTI, ranged from 2% to 5% of the total (data not shown). Therefore, in all subsequent experiments, plasma protease inhibitors were inactivated with CHCl₃ prior to activation of factor XI, and SBTI was included to stabilize the factor XIa as well as to minimize the contribution of kallikrein.

To ascertain the specificity of this assay, we applied it to several samples of plasma from individuals with severe factor XI deficiency (<10% of normal). Only 2%–12% of normal pooled plasma amidolytic activity was formed, which correlated well with the coagulant activity of the sample (0%–9% of normal). To further
Radioimmunoassay for Factor XI

A representative standard curve for the assay shows that bound 125I-factor-XI is inversely proportional to the logarithm of the concentration of normal pooled plasma added (Fig. 5). This relationship is linear between 0.005 U/ml and 0.05 U/ml. Test materials were diluted as necessary to give values that fell within this range. Similar standard curves were obtained when purified factor XI was assayed instead of normal pooled plasma. In contrast, the addition of plasma severely deficient in factor XI did not appreciably decrease binding of radiolabeled factor XI to the antibody.

To further validate the assay, various amounts of normal pooled plasma or purified factor XI were added to factor-XI-deficient plasma and then assayed both by coagulant and radioimmunoassay (Fig. 6). The titers of factor XI coagulant activity were plotted against those of factor XI antigen, and the regression line was calculated by the method of least squares. The values for reconstitution of factor-XI-deficient plasma with purified factor XI and the factor XI in normal pooled plasma were colinear with $r = 0.99$.

**Activation Requirements for Factor XI Amidolytic Activity**

**Factor XII**

Since factor XIIa is the enzyme that cleaves factor XI to XIa, it is obvious that decreases in factor XII could influence this assay. To determine the requirement for this enzyme, factor-XII-deficient plasma and factor-XI-deficient plasma were mixed, in various ratios, subsequent to chloroform treatment. Each mixture was activated with kaolin (Fig. 7A), as in Materials and Methods. At various times, a portion of the mixture was transferred to a tube containing corn trypsin inhibitor to stop the reaction. The samples were assayed as in Materials and Methods, and the values were corrected for dilution of the factor XI by the factor-XI-deficient plasma. Factor-XI-deficient plasma was used as the diluent in order to maintain normal concentrations of all other plasma proteins. Activation of factor XI did not occur in the absence of factor XII. At least 0.6 U/ml of factor XII was
Fig. 7. Activation requirements for factor XIa amidolytic activity. (A) Factor-XI-deficient plasma was mixed with factor-XI-deficient plasma at various ratios and activated, as in Materials and Methods, in order to assay the contribution of factor XII toward the activation of factor XI. (B) Factor-XI-deficient plasma mixed with HMW kininogen-deficient plasma. (C) Factor-XI-deficient plasma mixed with prekallikrein-deficient plasma. The data are expressed as percent of activatable factor XI. (A) 0.06 U/ml, (B) 0.4 U/ml, (X) 0.2 U/ml, (O) 0.1 U/ml, (C) factor in incubation mixture.

required in order to ensure full activation of the factor XI in plasma if a 60-min incubation time was employed. However, with as little as 0.1 U/ml factor XII, 75% of the maximal factor XIa activity evolved during that time.

**HMW Kininogen**

When HMW kininogen-deficient plasma was incubated under the same conditions as in Fig. 7A, activation of factor XI did not occur (Fig. 7B), as previously reported.23 HMW kininogen-deficient plasma was mixed with factor-XI-deficient plasma in various ratios. At least 0.2 U/ml HMW kininogen was required in order to ensure full activation of factor XI in 60 min, but even 0.1 U/ml gave 85% of the maximal activation at 60 min.

**Prekallikrein**

Kallikrein accelerates the activation of factor XII on a negatively charged surface by a feedback reaction and, thus, indirectly increases the rate of formation of factor XIa during contact activation. Therefore, the rate of activation of factor XII in prekallikrein-deficient plasma is slow. Prekallikrein-deficient plasma was mixed with factor-XI-deficient plasma under the same conditions as Fig. 7A. Even in the absence of prekallikrein, 89% of the factor XI was activated in 60 min (Fig. 7C). Thus, unlike factor XII or HMW kininogen, prekallikrein was not an absolute requirement for generating factor XIa amidolytic activity, as expected. However, the presence of prekallikrein had an accelerating effect on the rate of activation of factor XI due to the feedback-amplification of factor XII activation.22 This result implied that kallikrein activity was still expressed in the presence of SBTI. In separate determinations, however, the concentration of kallikrein, as determined by amidolytic assay, was 0.008–0.048 U/ml, corresponding to the 0.1–0.6 U/ml added prekallikrein (Fig. 7C), which generated less than 5% of the possible amidolytic activity.

**Correlation of Factor XI Amidolytic and Coagulant Activity in Plasma**

Factor XI amidolytic activity was determined in 35 normal plasma samples using normal pooled plasma
(20 donors) as a reference. Factor XIa activity ranged from 0.62 to 1.39 U/ml, with a mean of $1.00 \pm 0.24$ SD. The coagulant activity, determined on the same aliquot of each plasma, showed a range of 0.60–1.45 and a mean of $0.99 \pm 0.30$. In addition, determinations were performed on plasma samples from two patients with liver disease and as well as five patients with factor XI deficiency (Fig. 8A). The correlation coefficient between the amidolytic activity and the coagulant activity was $r = 0.95$. Based on multiple determinations on the pooled normal plasma on at least 5 separate occasions, the intraassay coefficient of variation for the amidolytic assay was 3.4%.

The plasma from normal individuals assayed for factor XI antigen showed a range of 0.56–1.61 U/ml (mean 1.01 ± 0.30). When the same samples from Fig. 8A were assayed for antigenic reactivity (Fig. 8B) and compared with the values obtained for amidolytic activity, the correlation coefficient ($r$) was equal to 0.96, similar to that of coagulant activity and amidolytic activity.

**DISCUSSION**

We present two new assays for factor XI in plasma, one functional, utilizing a tripeptide amide as substrate, and the other antigenic, a rapid (1-day) RIA using Staph A as the second precipitating agent. Both of these assays correlate closely with each other as well as with the conventional coagulant assay, as performed in our laboratory.

S-2366, when used with factor XIa, displays a $K_{cat}$ to $K_m$ ratio of $5.5 \times 10^6 M^{-1} sec^{-1}$, making this chromogenic substrate very suitable for human factor XIa. This ratio is higher by an order of magnitude than the two substrates we have used previously for assay of purified factor XIa. Furthermore, the $K_{cat}$ (2,000 sec$^{-1}$) is higher than any chromogenic substrate known. Under the conditions used, normal plasma factor XI was fully activated in 20 min, and this activity remained stable for at least 80 min (Fig. 2, curve b), due to prior inactivation of plasma protease inhibitors by CHCl$_3$ and partial protection by SBTI. This stability should facilitate automation, which is a major advantage of this type of assay.

This amidolytic assay appears to be specific for factor XI by the following criteria: (1) purified factor XIa showed similar activity to that in normal plasma; (2) mixtures of normal plasma with factor-XI-deficient plasma revealed a precise linear correlation of amidolytic with coagulant activity, dependent on the contribution of factor XI from normal plasma (Fig. 3); (3) factor-XI-deficient plasma showed virtually no activity, despite normal prekallikrein, HMW kininogen, and factor XII levels; and (4) a close correlation was found between the amidolytic activity, the coagulant assay, and the antigenic determination of factor XI. Consideration of the details of the assay help account for its usefulness. Plasma protease inhibitors destroy ~75% of the factor XIa activity in 15 min. However, SBTI, which initially inhibited 21% of the activity, appeared to slow down subsequent inhibition, since only ~40% of the factor XIa was destroyed in 15 min. Chloroform treatment inactivated most of the plasma protease inhibitors of factor XIa (less than 15% of factor XIa activity inhibited in 15 min). Destruction of plasma protease inhibitors by CHCl$_3$ permits unopposed formation of factor XIa. In addition to the protective effect of SBTI on factor XIa activity, it was necessary to include SBTI in the incubation mixture to inactivate most of the kallikrein that would have formed, although the small amount of residual kallikrein had an accelerating effect. The dual role of SBTI in this assay allowed the development of an activation procedure whereby the formed factor XIa was stable for long periods of time. This protective effect of SBTI has also been noted for thrombin and enzymes not inhibited by this plant inhibitor.

The time of 60 min for the activation step was selected to ensure full activation of the factor XI. Since 0.6 U/ml factor XII and 0.2 U/ml HMW kininogen were required for full activation of factor XI in 60 min (Fig. 7), a low value for factor XI could reflect a deficiency of one of these contact coagulation proteins. Therefore, it is recommended that the finding of decreased factor XI amidolytic activity be further evaluated by coagulant assays for factor XII and high molecular weight kininogen. Alternatively, addition of normal plasma to the patient plasma in equal amounts prior to the assay would provide these proteins. This step would also allow detection of naturally occurring antibody to factor XI, such as that recently reported.

The amidolytic assay that we have developed is easy to perform, requires a small amount of sample, and can be adapted to an automated system. Since the activity during the activation process plateaus between 20 and at least 120 min (data not shown), many samples can be activated at the same time without the need for laborious, precise timing of the activation step, as is required for the coagulant assay, allowing the activated factor XI to be assayed spectrophotometrically, at leisure. Furthermore, this substrate can be employed for purified factor XIa or partially purified factor XI. In the case of partially purified material, however, adequate amounts of contact activation factors must be included, and inhibitors must be included to inactivate any unwanted proteases.

The amidolytic activity in the 35 normal plasma samples approximated a normal distribution, with a
range from 0.62 to 1.39 U/ml of the normal reference plasma, similar to the values observed for both coagulant and antigenic activity. The assay detected low concentrations of factor XI in two patients with liver disease. These results agreed well with values reported previously using an immunoassay. The antigenic activity was also commensurately reduced. Excellent correlation \((r = 0.95 - 0.96)\) between coagulant, amidolytic activity, and antigenic levels reinforces our confidence in both new assays.

The assays of coagulation factor XI previously available include functional coagulant assays or immunological determinations. The coagulant assays require either plasma genetically deficient in factor XI or normal plasma that has been adsorbed with celite. The latter may not be specific, since the procedure variably removes concentrations of factor XII and HMW kininogen. The former assay has a disadvantage, since it utilizes rare plasma with various degrees of factor XI deficiency. An assay employing a synthetic chromogenic substrate has intrinsic advantages, since these substrates are more homogeneous as well as stable. The interlaboratory coefficient of variation for coagulant assays for plasma factor XI ranges from 27.9% to 101.4%, while the intraassay coefficient of variation for our assays is 3.4%. The previously described RIA is time-consuming, requiring 3 days for completion of each set. Our RIA, which employs Staph A as the second precipitate, only requires 1 day to complete a set of 10 triplicate determinations. With our assay system, a low amidolytic value could be assayed the same day for antigenic reactivity of factor XI in order to distinguish an abnormal protein or an inhibitor of factor XI from a true deficiency. Since patients with mild deficiencies rarely have spontaneous hemorrhage, they are primarily at potential risk during or after surgical procedures. A preoperative assay, which can reproducibly detect 30% or less of normal activity, could minimize the risk. These two new assays, alone or combined, should prove valuable in detecting and evaluating both genetic and acquired deficiencies of plasma factor XI.

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