Characterization of the Catalytic Subunit of Factor XIII by Radioimmunoassay

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Plasma factor XIII is composed of two subunits, a and b, whereas platelet and other intracellular zymogens have only a-subunits. The catalytic subunit, a, is the same in all forms. In order to characterize the interactions of a- with b-chains in the plasma zymogen and a-chains with other molecules and to correlate factor XIII activity with a-protein, a specific, sensitive radioimmunoassay was developed. With the polyclonal antiseras used, the assay recognizes all molecular forms of a (zymogens, activation intermediates, enzyme) equally well. The assay can be used to determine a-chain concentration in plasma and serum and in purified test systems. Fibrinogen in high concentrations affects the assay, probably by interfering with the interactions of 125I-a with antibody. However, at the plasma dilutions used in the assay, there is no significant fibrinogen effect. With this assay, the a-chain concentration in normal plasma is approximately 15 μg/ml. This compares with 14 μg/ml b-chain in plasma and indicates that all of the a- and b-chains in plasma probably circulate in the form of an equimolar zymogen complex. The serum concentration of a-protein is about 6% of the plasma concentration. There is a high correlation between a-protein and factor XIII activity.

Plasma factor XIII is a blood proenzyme that is equally distributed in both plasma and platelet compartments.1 The plasma zymogen is composed of two nonidentical, immunochemically distinct subunits, a and b, which form a strong, noncovalent complex. Platelet factor XIII is composed of a-subunits only, which are identical to the plasma a-chains.2 An intracellular zymogen, identical to the platelet zymogen, has also been found in megakaryocytes, placenta, uterus, and prostate gland.3,4 A considerable amount of data, including sedimentation equilibrium, chemical crosslinking studies, and gel electrophoresis, support the proposal that the plasma zymogen is a tetramer, a2b2, while the intracellular zymogens are dimers, a2.3,5 Since the potential catalytic activity is an attribute of the a-chain only, both zymogens are converted to the same enzyme upon activation. Activation of factor XIII involves proteolysis of the amino-terminal segment of the a-chain by thrombin and conformational alteration by Ca2+ to expose the active center.6 During activation of the plasma zymogen, b-chains dissociate from the complex.7

The hemostatic function of factor XIIIa is to catalyze the formation of amide bonds between certain glutamine and lysine residues in fibrin. The crosslinking reaction is highly specific and yields γ-chain dimers and α-chain polymers of fibrin.8 Factor XIIIa also crosslinks fibronectin to fibrin9 and to collagen,10 and these reactions are also probably important for normal hemostasis and wound healing.10-12 The functional importance of the crosslinking reactions is underscored by observations that patients who are deficient in factor XIII or who have acquired inhibitors may have severe bleeding syndromes. Deficient patients frequently die from intracranial hemorrhage, and pregnancies in deficient women terminate with spontaneous abortion.13

Factor XIII activity can be measured accurately by incorporation of synthetic amines into casein.14,15 However, quantitative methods for determination of factor XIII proteins are not readily available, and this has hampered studies on factor XIII function. We have recently developed a radioimmunoassay (RIA) that is specific for the b-chain.16 In this report we describe an RIA that is specific for a-chain and can be used to measure "a" concentration in purified material and in plasma and serum. Combination of the two immunochemical assays with activity assays allows a correlation of factor XIII function with protein and provides a useful tool for studying the interaction of the subunit proteins.

MATERIALS AND METHODS

Purification of Human Factor XIII Proteins

Plasma zymogen was purified from cryoprecipitate-depleted Cohn I paste (Cutter Laboratories, Clayton, N.C.) as previously described,17 except that the Cohn paste was thawed in 0.05 M Tris, 0.5 M KCl, 1 mM EDTA (pH 7.0), filtered through cheese cloth, and heated for 15 min at 56°C. The a2b2 was purified from the supernatant by (NH4)2SO4 precipitation and chromatography on DEAE-cellulose. Purified b-chain was obtained from the thrombin-treated zymogen by affinity chromatography on organonumeric agarose.18

Pure a-subunit was prepared from platelets19 and from placental concentrate (Fibrogammin, Behringwerke AG). One vial of Fibrogammin contained potential factor XIII activity equivalent to 1250 ml of plasma. Activated factor XIII was not present in the material...
used for purification. For the purification of a-subunit, 5 vials of Fibrogammin were suspended in 25 ml of buffer A (50 mM Tris, 1 mM EDTA, pH 7.4), dialyzed overnight, and then chromatographed on Blue-Sepharose (column volume 100 ml; flow rate 72 ml/hr). The first peak contained factor XIII protein. Fractions were pooled, dialyzed, and chromatographed two times on DEAE-cellulose. The columns were eluted with an exponential gradient of 0.1–0.25 M NaCl in buffer A. This procedure yielded pure a-chains, which were then concentrated by (NH₄)₂SO₄ precipitation. The material was stored at 4°C in buffer A containing 0.1 M NaCl and could be maintained for several months without degradation or loss of activity.

Antiserum Preparation

New Zealand male rabbits were injected at multiple subcutaneous sites at weekly intervals for 4 wk with pure a-subunit (100–150 µg a-protein per injection) in complete Freund's adjuvant. Booster injections (60 µg a-protein) were given every 3 wk until a sufficiently high antibody titer was obtained. Blood was collected into sodium citrate and centrifuged; serum was prepared by incubating plasma with thrombin and CaCl₂ overnight. Antiserum was treated with (NH₄)₂SO₄ to remove any remaining rabbit factor XIII activity. The fraction precipitating between 20% and 50% saturation was dissolved in buffer A plus 0.1 M NaCl, dialyzed, and used for all experiments. Antisera were monospecific for a-subunit on immunodiffusion versus purified a-chain or plasma. Antiserum to b-subunit was prepared similarly, except that it did not require any fractionation. Both types of antisera reacted with plasma zymogen, but anti-a did not react with b or anti-b with a on immunodiffusion.

125I-Labeling of a-Chain

Purified a-chain proved to be difficult to radiolabel. With both the chloramine-T procedure and the Iodo-gen (Pierce Chemical Co., Rockford, Ill.) method, extensive radiolytic damage to the protein occurred, as assessed by SDS gel electrophoresis and counting of 1-mm gel sections in a γ-spectrometer. However, successful labeling was obtained with the solid phase lactoperoxidase-glucose oxidase procedure (Enzymobead reagent, Bio-Rad Laboratories, Richmond, Ca.). Labeling was performed as suggested by the manufacturer; the reaction time was 15 min, and 30–40 µg protein were labeled. Separation of labeled protein from free iodine was achieved by gel filtration on Sephadex G-25 in 0.05 M Tris, 0.15 M NaCl, 3 mM NaN₃, 1% bovine serum albumin, pH 7.5 (RIA buffer). 125I-a-chain was more than 95% precipitable with 10% TCA. On polyacrylamide gel electrophoresis, one peak of radioactivity was found that comigrated with the protein band. The average specific radioactivity, determined from 10 labeling experiments, was 0.45 atoms 125I/a-chain.

RIA

A double antibody procedure was used for the a-chain RIA, and optimal conditions for the assay were determined experimentally. All reagents were diluted in RIA buffer. Labeled protein was diluted to give approximately 20,000 cpm/100 µl; nonimmune rabbit serum was diluted 1:640. Goat anti-rabbit IgG (125 U, CalbiochemBehring) was dissolved in 20 ml buffer. In the first phase of the assay, 100 µl test material were incubated with 100 µl 125I-a and 100 µl antiserum for 4 h at room temperature. In the second phase, 100 µl each of nonimmune rabbit serum and goat anti-rabbit IgG were added to each tube, and the samples were incubated at 4°C for 16 h. Each tube was then centrifuged at 1200 g for 20 min, the supernatant was removed, and the precipitate was counted in a γ-spectrometer. For each sample, three or more dilutions were assayed in triplicate. Fifty percent binding of labeled ligand was obtained with an antiserum dilution of 1:15,000. The maximum precipitability of labeled "a" was about 85% and nonspecific binding was less than 5%. Dose–response curves for standards and samples were converted to linear plots by the log-log transformation. Data were fitted by least-squares linear regression analysis.

Analytical Procedures

Factor XIII activity was measured by incorporation of monodansylcadaverine into casein. Protein concentration of pure plasma factor XIII was determined spectrophotometrically by using E₂₈₀ = 13.8. Protein concentration of the purified subunits was determined with the protein assay from Bio-Rad, which uses bovine IgG as standard. Polyclamidamide gel electrophoresis in sodium dodecyl sulfate was carried out in 5% gels under reducing and nonreducing conditions. Ouchterlony double immunodiffusion was performed in small agar plates (Hyland, Costa Mesa, Ca.).

Blood Samples

Blood samples were collected from healthy adult men and women, who had given informed consent, into 1/10 volume of 3.8% sodium citrate and centrifuged twice. Plasma samples were stored at −80°C. Serum was prepared from plasma by incubation with thrombin and CaCl₂ or from blood by recalcification.

RESULTS

Characteristics of the RIA

The most successful method for radiolabeling a-subunit was found to be with the solid phase lactoperoxidase-glucose oxidase method. This procedure yielded 125I-a that had >95% precipitability with 10% TCA and >85% precipitability with antiserum. Gel electrophoresis showed only one peak of radioactivity, which was coincident with the protein peak of a-chain (mol wt = 75,000). Several attempts to label a-chain with various procedures using chloramine-T yielded products that showed reduced antigenicity. With these methods the maximum precipitability of labeled ligand with antiserum was 40%. This is in contrast to the b-subunit of factor XIII, which can be easily labeled with chloramine-T. The Iodo-gen procedure resulted in significant radiolytic damage to a-chain. With the lactoperoxidase procedure, diluted 125I-a was stable for 2 wk, after which time loss of binding affinity occurred. Undiluted labeled ligand remained stable for 4 wk at 4°C and could be used in the RIA for 2 wk after being diluted.

A typical antibody binding curve is shown in Fig. 1. Nonspecific activity represented <5% of the total counts. The concentration of 125I-a used for assay was normally 30–40 ng/ml (20,000 cpm/100 µl). However, the same antiserum dilution curve was obtained when the amount of labeled ligand was decreased to 8 ng/ml. The same curve was also obtained in the presence of 10 mM CaCl₂, indicating that with this polyclonal antiserum essentially the same antigenic
determinants were detected in the presence and absence of calcium ions. The order of addition of reactants did not affect the assay. The same results were also obtained when the incubation time of ligand and specific (first) antibody was varied from 1 to 5.5 hr.

Standard curves were constructed using purified intracellular zymogen (a2) and plasma zymogen (a2b2) as competing antigens. The logit-log transformation was used to analyze the data. For all experiments in this study, the following molecular weights were used: a-chain, 75,000; b-chain, 80,000–88,000; a2b2, 320,000; a2, 146,000. When a2b2 was used as the standard, it was assumed that “a” represented 48% of the total protein. This assumption was verified by constructing standard curves in assays with a, b, and a2b2. With each standard curve the values of the components were found to be within 15% of the expected value.

Typical standard curves were constructed in the range of 50–800 ng/ml a-chain (Fig. 2). Variations were observed in purified systems but not in assays of pooled normal plasma. With a2 as the competing ligand, the mean values for the standard curve (n = 15) were slope = −2.85 ± 0.45 and ED50 = 448 ± 178 ng/ml; for a2b2 (n = 27), slope = −2.45 ± 0.41 and ED50 = 364 ± 132 ng/ml. In some experiments the assay was tested with concentrations of purified a2 or a2b2 up to 40,000 ng/ml of “a” and was found to be linear throughout the entire range. Addition of purified b-chain to a test system containing only “a” did not have any effect on the assay for a-subunit.

Measurement of Nonactivated and Activated “a”

Factor XIII zymogens, their thrombin-cleaved intermediates, and the active enzyme were tested in the a-subunit RIA. Figure 3 shows that, within experimental error, all forms of a-chain were recognized equally well, since with an equal amount of a-chains in the test sample the same degree of binding was observed in the RIA. This means that with the polyclonal antisera used, the assay is valid for measuring a-chain regardless of whether or not it is complexed to “b” or thrombin cleaved or conformationally altered by calcium ions.

Fig. 1. Antibody binding curve for a-chain RIA under optimal assay conditions.

Fig. 2. Standard curves for a-chain assay (logit-log transformation). With purified a2 [○], slope = −2.45 and ED50 = 248 ng/ml. With purified a2b2 [△], slope = −2.48 and ED50 = 262 ng/ml. For a2b2, a protein was assumed to equal 48% of the total protein. Logit y = ln [(B/B0)/(1 − B/B0)] with counts corrected for nonspecific binding.

Fig. 3. Quantitation of a-protein in the various molecular forms of factor XIII by RIA. All points fall on a single line (r² = 0.98 by linear regression analysis). Intracellular forms: zymogen, a2 [○]; thrombin-cleaved intermediate, a2 (△); enzyme (thrombin + CaCl2), a2 [◆]. Plasma forms: zymogen, a2b2 [□]; thrombin intermediate, a2b2 (Δ); enzyme, a2 + b2 [θ].
Effect of Fibrinogen on the a-Chain Assay

Varying amounts of fibrinogen and fibronectin were added to purified factor XIII to determine their effect on the specificity of the RIA for "a." Fibronectin, at a concentration of 0.3 mg/ml had no effect on the assay. However, the apparent binding of radioligand to antibody was affected by the addition of fibrinogen. Table 1 shows that in a purified system with increasing amounts of fibrinogen added to the test system, there was a decrease in the binding of labeled "a." The percentage decrease in binding was determined by the amount of fibrinogen in the test system and was the same at two concentrations of a-chain. The observed effect did not become significant until the fibrinogen concentration in the test system reached 100 μg/ml and could be accounted for by interference of fibrinogen with radiolabeled ligand binding to antibody. In order to determine if the observed effect was due to antibody recognition of fibrinogen, antibody was incubated with fibrinogen at three concentrations (Fig. 4). This mixture was then reacted with antifibrinogen and the supernatant tested in an antiserum dilution assay for a-chain. The results showed that the a-chain antibody did not react with fibrinogen and that after removal of fibrinogen the antibody behaved normally. Removal of fibrinogen from the antibody was also confirmed by immunodiffusion. These results eliminate the possibility of a-chain antibody binding to fibrinogen and confine the effect of fibrinogen to interference with radioligand binding to antibody or to interference with precipitation of the 125I-a-antibody complex with secondary antibody. Since the observed effect appears to be rather specific for fibrinogen, the first possibility seems more likely.

Concentration of a-Chain in Normal Plasma

Any effect of fibrinogen on the a-chain RIA in a purified system was not noticeable in the plasma test system. This was demonstrated by the linearity of results obtained in the RIA when plasma was diluted over a range corresponding to 15-120 μg/ml fibrinogen (Fig. 5), which would not be expected if fibrinogen were having a significant effect on the apparent concentration of a-protein in plasma. If fibrinogen were interfering with binding, the error in a-chain

![Graph](https://via.placeholder.com/150)

**Table 1. Effect of Fibrinogen on Ligand Binding in Purified Systems**

<table>
<thead>
<tr>
<th>Fibrinogen (μg/ml)</th>
<th>Fraction of (1)</th>
<th>125I-a Bound* (2)</th>
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<tr>
<td>4,000</td>
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<td>0.09</td>
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*Corrected for nonspecific binding.

(1) Test system contained constant amounts of "a" (50 μl at 613 ng/ml), 125I-a, anti-a, and anti-IgG with variable amounts of fibrinogen.
(2) These system did not have any unlabeled "a."
ATP DEPENDENT REACTIVATION OF GLUCOCORTICOID RECEPTORS IN L CELLS. Juliane J. Sando* and William B. Pratt. Univ. of Michigan, Ann Arbor, MI. 48109

Specific glucocorticoid binding capacity in 100,000 x g supernatants of L929 mouse fibroblasts (L cells) prepared with hypertonic buffer is inactivated with a half-life of about 3 hours at 25°C. This inactivation can be prevented with 10^{-2} M molybdate and markedly slowed by other phosphatase inhibitors, like fluoride and glucose-1-phosphate (Nielsen et al., J Biol Chem. 252: 7586, 1977). After being extensively inactivating the receptor by preincubating the supernatant for 4 hours, addition of molybdate prevents further inactivation and addition of molybdate and ATP results in reactivation of the steroid binding capacity. Maximal reactivation of 40-70% is achieved with 1 to 10 mM ATP. ATP and GTP are ineffective, and preliminary results indicate no effect of cyclic nucleotide. Added magnesium (0.02-2 mM) has no effect on the ATP-stimulated reactivation, and higher concentrations inhibit binding capacity in vivo. Glucocorticoid receptor reactivation in L cells therefore requires ATP and is best demonstrated when receptor inactivation is blocked by molybdate. (Supported by NIH grants CA-16041 and AM-15740)

SEPARATION OF ENKEPHALINS BY HIGH PRESSURE LIQUID CHROMATOGRAPHY. Dan H. Morris* and John H. Stewart. Dept. of Biochem., U. Colo., Boulder, CO 80309

The sulfide and sulfuryl derivatives of methionine-enkephalin (Met-Enk) were resolved by reverse phase high pressure liquid chromatography on a 0.46 x 25 cm Partisil-10 ODS-2 column under ambient temperature conditions. Retention times for Met(Og)-Enk, Met(O)-Enk and Met-Enk were 1.7, 2.1 and 2.3 min respectively. Better separation of Met-Enk from its oxidized forms was achieved using a methanol:ammonium acetate (0.01M, pH 5.0), 1:1 system where sulfide and sulfuryl derivatives co-elute, Met(Og)-Enk, Met(O)-Enk, Met-Enk and (Met=O)-Met-Enk eluted at 1.9, 2.8, and 6.2 min respectively. With a methanol:ammonium acetate (0.01M, pH 5.0) 4:6 mixture, retention times for Met(Og)-Enk, Met(O)-Enk, Met-Enk and (Met=O)-Met-Enk were 1.7, 2.7, 5.1 and 8.9 min respectively. The methanol:ammonium acetate solvent systems can be used to resolve Met-Enk analogs in general, whereas, a straight methanol system can be employed to determine the species of oxidized sulfur in Met-Enk when present. (Aided by a grant from Pennwalt Corp.)

IN VITRO ACTIVATION OF CORTICOSTERONE BY LACTATING RAT MAMMARY ACINI AND NUCLEAR LOCALIZATION OF THE ACTIVATED GLUCOCORTICOID. M. H. Hampel*, L. H. Pengh, M. S. L. Pearlman* and W. H. Pearllman. Dept. of Pharmacology, School of Medicine, Univ. of North Carolina, Chapel Hill, N.C. 27514

Mammary alveolar cells isolated as intact acini from the mammary glands of lactating rats (Katz et al, 1974) offer certain experimental advantages over whole mammary gland preparations for the study of steroid hormone uptake and metabolism. The acini were accordingly incubated with [1n, 2n-3H] corticosterone in Hank's buffer, pH 7.4, at 37°C; the gas phase was 95% O2:5% CO2. The glucocorticoid was extensively metabolized to a less polar radiomethanetol which appears to be a 21-acetyl derivative of corticosterone when isolated and perdeuterated oxidation and furnished 3p-corticosterone on mild alkaline hydrolysis. The precise nature of the 21-acetyl group is under investigation. The acetylated corticosterone appeared in the nuclear fraction in amounts larger than those of 3p-corticosterone. The cytosol fraction contained, however, only 3p-corticosterone. The nuclear localization of the activated corticosterone suggests that this metabolite actively participates in the mechanism of hormone action, but further study is required. The extensive acetylation of these steroids may be linked to the high lipogenic activity of the mammary gland during lactation. (Supported by USPHS, NCI grant NO 1-CA-53905 and NIGMS grant T32-GM07040)

ESTRAGE ACTION INDEPENDENT OF NUCLEAR ACCEPTOR BINDING. Thomas G. Muldoon. Med. Coll. of Georgia, Augusta, GA 30902

The molecular basis for the action of 4-mercuri-17a-estradiol (HgE), previously shown in this laboratory to be an affinity-labeling agent possessing appreciable estrogenic activity, has been further examined. [3H]HgE interacts with immature rat uterine cytosol estrogen receptors (t~ 30 min) to form a stable precipitable-protein complex which sediments as an essentially monomer insulin surroge gradient ultracentrifugation pattern and forms to a 55% form upon incubation for 1 hr at 25°C. Addition of 0.3 M KCl effects a reversible transition of the 55% complex to a 45% form. Uptake of the transformed cytosol estrogen complexes by purified uterine nuclei occurs very slowly at 0°C, but can be accomplished by 60 min incubation at 25°C. Intracellular interactions of the steroid-receptor complex are weak and can be disrupted readily by washing with buffer; no evidence of specific chromatin binding is demonstrable. Moreover, the presence of intranuclear HgE-receptor complex does not reduce subsequent salt-extractable binding of transformed 17a-estradiol-receptor complex by the same nuclei. Subcellular fractionation analyses over a discrete time interval following introduction of [3H]HgE indicate early preferential accumulation of HgE-receptor complex in the microsomal fraction. The data suggest that HgE estrogrenicity is manifested by a mechanism distinct from that generally associated with nuclear estrogen acceptor involvement at the level of the genomic apparatus. (Supported by USPHS Grant #AM 17650 from the NIAID, NIH.)

A SENSITIVE AMIDASE ASSAY FOR HUMAN URINARY KALLIKREIN. Alfred Chung*, James W. Ryan, Guillermo Pena* and Narendra B. Desai. Dept. of Med., Univ. of Miami School of Med., Miami, Fl. 33136

Human urinary kallikrein (HUK) is commonly measured by its ability to form kinins from standard amounts of kinogen or to hydrolyze substrate such as Nω-tosyl-arginyl-methyl ester (TAME). However, the esterase assays are not specific and TAME undergoes spontaneous hydrolysis at slightly alkaline pH. The kinin-generating assays are tedious and subject to large experimental errors. We have developed a sensitive, highly specific assay that uses Nω-tosyl-L-arginyl-N-ω-aminocaproic acid (HUK-substrate) for HUK. The amino acid sequence is that of the C-terminal tripeptide of bradykinin. The substrate is synthesized by reaction fragment condensation of 1-arginine and L-phenylalanine, and synthesized 3p-arginine was used to define reaction kinetics. In brief, the reaction proceeds optimally in 0.1 M Tris-HCl buffer, pH 9.5. As little as 5 ng of HUK can be measured on a 15 min incubation time of 15 min (37°C). As a routine, we measure HUK using 50 µl of dialyzed urine. Reactions are terminated by adding 0.1 N NaOH and then reaction product (Nω-aminine) is separated from substrate by precipitation with an equal volume of cation. Nω-arginine is quantified by liquid scintillation counting. To our knowledge, this is the first synthetic amide substrate known to be hydrolyzed by HUK. (Supported in part by HL15631, HL22087, and the John A. Hartford Foundation.)
cannot be conclusively ruled out (Fig. 4). Although Fig. 5 shows that the effect of fibrinogen on the a-chain RIA is not due to cross-reactivity with the antibody, the effect appears to be specific since fibrinogen did not affect the b-chain RIA, and fibronectin, another substrate for factor XIIIa, had no effect on either assay. Fibrinogen interfered with labeled “a” binding to a greater extent than with nonlabeled “a,” and the effects were dependent on the fibrinogen concentration (Table 1). The fibrinogen used in these experiments was prepared by ammonium sulfate precipitation of normal pooled human plasma. It did not react with the antibody to a-chain.12

Under the test conditions used, a-protein was essentially completely masked when the weight ratio of fibrinogen to a-chain exceeded 330:1. The mechanism for fibrinogen interference is not clear. Although a-chain (zymogen) interaction with fibrinogen is normally weak, it may be that at high fibrinogen concentrations this is sufficient to compete with a-chain–anti-a interaction. Since labeled “a” has an apparently higher affinity for fibrinogen than untreated “a,” possibly due to damage during labeling, any interaction of the radioligand with fibrinogen may be magnified in the test system.

No significant effect of fibrinogen was observed at a concentration below 100 μg/ml. This means that fibrinogen would have little or no effect on determination of the plasma concentration of a-chain, since at the plasma dilutions normally used in the assay, the fibrinogen concentration would be ≤60 μg/ml. The maximum effect of fibrinogen would be to overestimate the plasma a-chain concentration by about 10%. The basic assumptions for the determination of a-protein concentration by RIA are also validated by the high correlation between activity and protein concentration and between a- and b-protein levels (Fig. 6). In the future, ratios of a:b and a:activity may be used to discriminate factor XIII abnormalities, whether due to congenital or acquired defects, in patients.

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Characterization of the catalytic subunit of factor XIII by radioimmunoassay

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