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Factor XI Activity and Factor XI Antigen in Homozygous and Heterozygous Factor XI Deficiency

By Abraham Rimon, Sandra Schiffman, Donald I. Feinstein, and Samuel I. Rapaport

A relatively potent antiserum against highly purified, unactivated human factor XI antigen was raised in a rabbit. This antiserum, after concentration, neutralized 50% of the factor XI clotting activity of a standard normal plasma at an antiserum dilution of 1/900. The antiserum was used in a neutralization-inhibition assay to study the relation between factor XI clotting activity and factor XI antigen in plasma from ten unrelated patients with homozygous factor XI deficiency and from 12 heterozygous family members of these patients. No evidence of factor XI antigen significantly in excess of factor XI activity was found in either group. All data to date have been consistent with the hypothesis that hereditary factor XI deficiency represents a genetic disorder resulting from the absence of factor XI molecule. Severity of bleeding in factor XI deficiency could not be correlated with the level of factor XI activity or factor XI antigen.

THE HEREDITARY DISORDERS of blood coagulation are characterized by functional deficiencies of specific plasma clotting factor activities. Such functional deficiencies can stem from at least two types of molecular defects.[1] In one type, the patient's plasma will contain evidence of an abnormal molecule in the form of nonfunctional cross-reactive protein, which is detectable by its reaction with an antibody to the clotting factor. In the other type, the patient's plasma will lack such demonstrable cross-reactive material.

Studies of groups of patients with the same hereditary functional clotting factor deficiency have yielded evidence of both types of molecular defects. On occasion, genetic heterogeneity has been detected in studies involving only a small number of patients; for example, a study of four patients with hereditary factor VII deficiency revealed one patient whose plasma contained a high level of nonfunctional cross-reactive protein.[2] In contrast, study of a large number

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of patients was required to detect evidence of heterogeneity in hereditary factor VIII deficiency.[3]

The possibility of molecular heterogeneity in hereditary factor XI deficiency deserves particular consideration because patients with hereditary factor XI deficiency differ in their bleeding tendency. Whereas most such patients bleed abnormally after specific challenges such as tooth extraction, tonsillectomy, or other surgery, occasional patients with hereditary factor XI deficiency do not bleed abnormally after such challenges.[4,5] This clinical difference cannot be attributed to a difference in level of plasma factor XI clotting activity.

Factor XI deficiency is an uncommon coagulation disorder, and only one immunologic study of ten patients has been reported.[6] Genetic heterogeneity was not found; all ten patients lacked cross-reactive material in their plasma. Clinical histories were not given in this study.

We now report the analysis of 10 additional unrelated homozygous factor XI-deficient patients and 12 heterozygous patients for cross-reactive protein using a heterologous antibody, and these data are compared with clinical bleeding histories.

MATERIALS AND METHODS

Plasma Preparation

Nine parts of blood were drawn into plastic tubes containing one part citrate anticoagulant (0.06 *M* sodium citrate and 0.04 *M* citric acid) and centrifuged immediately at 12,000 *g* at 4°C for 10 min. The supernatant plasma was separated and centrifuged again. The resulting platelet-poor plasma was distributed in 1-ml portions in plastic vials and either tested immediately or frozen at −20°C. For analysis, frozen samples were thawed completely by a short incubation at 37°C, then kept at 4°C for the rest of the experiment. Each vial was thawed only once.

Standard Normal Plasma

Plasma from the same normal donor was used in each experiment as a working standard for preparing reference curves for quantitating both factor XI clotting activity and factor XI antigen levels. When compared on several occasions with a pooled normal plasma reference standard prepared from a minimum of 12 individuals, this plasma was found to contain 120% factor XI clotting activity and an average of 124% factor XI antigen. Consequently, all observed factor XI clotting and antigen activities were corrected by a factor of 1.2 to convert the values to per cent of pooled normal plasma activity.

Purified Native and Activated Factor XI

Highly purified native factor XI was prepared, as described previously, from fresh normal human plasma.[7] For unexplained reasons, the final product occasionally contained activated factor XI, as reflected by a short clotting time in the factor XI assay without surface activation by kaolin. The preparation containing activated factor XI (XIa) used in the experiments described below had a clotting time at a 1/80 dilution of 61 sec in the factor XI assay with kaolin and 76 sec in the absence of kaolin. By contrast, a preparation containing only native factor XI had a clotting time at a 1/50 dilution of 75 sec in the factor XI assay with kaolin and 235 sec in the absence of kaolin.

Factor XI Activity Assay With Kaolin

Factor XI was assayed in a one-stage partial thromboplastin time assay, as originally described by Rapaport et al.[8] The following reagents were added into a glass clotting tube in the order listed: 0.025 ml kaolin suspension (20 mg/ml saline), 0.025 ml cephalin emulsion[9] diluted to optimum concentration in barbital buffer (0.028 *M* sodium barbital plus 0.125 *M* NaCl adjusted

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to pH 7.35), 0.05 ml hereditary factor XI-deficient plasma from patient J.L. of Table 3, and 0.05 ml test substance. After 3 min incubation at 37°C, 0.05 ml of 40 m*M* $CaCl_2$ was added and the clotting time recorded. A standard curve was made from serial dilutions of standard normal plasma ranging from 1/5 to 1/80. The results were plotted on double logarithmic paper.

Factor XI Assay Without Kaolin

In this assay to detect activated factor XI, 0.05 ml cephalin emulsion diluted to optimum concentration in barbital buffer was incubated with 0.05 ml hereditary factor XI-deficient plasma in a plastic tube at 37°C for 3 min. Then 0.05 ml test substance was added, followed immediately by 0.05 ml 40 m*M* $CaCl_2$, and the clotting time was noted.

Fletcher Factor Activity Assay

Fletcher factor activity was measured in a system similar to the factor XI assay with kaolin in which Fletcher factor-deficient plasma (supplied through the generosity of Dr. Charles Abildgaard) was utilized as the substrate.

Preparation of Anti-Factor XI Antiserum

Rabbits were immunized with a solution of purified factor XI containing 800% of factor XI activity blended with an equal volume of complete Freund's adjuvant. Two milliliters of this mixture were administered intradermally at multiple sites into a rabbit weighing about 2.5 kg. A similar injection was given as a booster 12 days later and every 3–4 wk thereafter as required by the drop of antibody titer. Blood was withdrawn from the ear once a week, and the separated serum was kept at −20°C until analyzed. Prior to testing of antisera or antisera fractions in clotting systems, 0.2 ml citrate anticoagulant was added to each 1 ml of antiserum. The anticoagulated serum was adsorbed twice with 1/10 volume of 50% $Al(OH)_3$ gel (Cutter Laboratories, Berkeley, Calif.) and heated at 56°C for 2 hr to inactivate clotting factor activities.

Immunoadsorption of Antisera

Immunoadsorbent was prepared by coupling normal plasma, factor XI-deficient plasma, or Dicalite-adsorbed plasma ("exhausted plasma"[10]) to CNBr-activated Sepharose 4B.[11] For the adsorption of 10 ml antiserum, about 3–4 ml of adsorbant was packed into a 1 × 10-cm column and equilibrated with 0.5 *M* $NaHCO_3$, pH 8.0. The antiserum was first dialyzed overnight against ten volumes of the equilibration buffer, then chromatographed on the column using the same buffer. The fractions containing protein as detected by absorbance at 280 mμ were pooled, dialyzed against 0.15 *M* NaCl, and either frozen for future use or concentrated about sixfold over the original serum. Fractions were concentrated by adding $(NH_4)_2SO_4$ to 40% saturation, dissolving the precipitate in 0.15 *M* NaCl, and dialyzing against 0.15 *M* NaCl.

Determination of Antibody Titer

Antibody titers were defined as the dilution of antiserum or antiserum fraction that caused a 50% neutralization of factor XI activity in the standard plasma. These titers were determined by incubating 0.1 ml of this plasma with 0.1 ml of serially diluted antiserum for 30 min at 37°C. The mixture was centrifuged for 10 min at about 700 *g* to sediment any possible precipitate, diluted 1/5 in citrated saline (0.125 *M* sodium chloride, 0.017 *M* sodium citrate), and assayed for residual factor XI activity. This result was compared to the factor XI activity of a similarly diluted standard plasma sample without antiserum. The per cent factor XI neutralized was calculated as follows:

$$\frac{\text{Per Cent XI in diluted standard plasma} - \text{Per Cent residual XI in antiserum mixture}}{\text{Per Cent XI in diluted standard plasma}} \times 100.$$

A typical neutralization curve is shown in Fig. 1.

Determination of Factor XI Antigen

Factor XI antigen was measured in a three-step neutralization-inhibition system modified from Goodnight et al.[2] In the first step, 0.1 ml of test plasma was incubated at 37°C with 0.01 ml of

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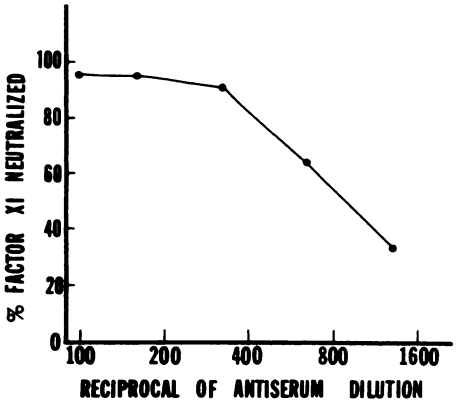

Fig. 1. A plot of per cent neutralization of factor XI activity obtained by incubating equal parts of normal plasma and serial dilutions of a concentrated rabbit antiserum to factor XI. The per cent factor XI neutralized was determined by comparing factor XI activity in comparably diluted mixtures with and without antiserum as described in the text.

diluted immunoadsorbed antiserum. The test plasma was added to the system either undiluted (samples with very low antigen) or diluted 1/2 in citrated saline. The same results were obtained with either 30 or 60 min incubation, and 30 min was selected as the standard incubation time. The final dilution of antiserum in the mixture was 1/400. This dilution of antiserum was chosen because it neutralized about 80% of the factor XI activity of normal plasma (see Fig. 1). In the second step, 0.1 ml of the standard normal plasma was added, and the incubation was continued for an additional 30 min. Although no precipitate was visible, the mixture was centrifuged at about 700 *g* for 10 min. In the third step, an aliquot of the supernatant was diluted 1/3 in citrated saline and tested for factor XI activity.

The amount of factor XI antigen in a test sample was determined from a reference inhibition curve (see Fig. 2), which was prepared for each experiment by using serial dilutions of the standard normal plasma as the test plasma in the above system. The antigen level of the undiluted standard plasma was called 100% factor XI antigen. As can be seen from Fig. 2, a linear relationship was found between clotting time and antigen concentration for values of antigen in plasma above 10%. Linearity was lost between 10% and 5% factor XI antigen. Below 5% antigen level, the data varied. A difference in clotting time between 5% factor XI antigen and control buffer could be demonstrated on some occasions (see Fig. 2) but not on others. Therefore, the test system could not be used to quantitate antigen levels below 5% of normal plasma factor XI antigen, and samples with clotting times equal to the buffer clotting time were reported as $< 5\%$. Samples with clotting times falling in the 5%–10% antigen range were reported as per cent antigen, but must be viewed as approximate values since the exact point at which the reference curve changed slope was unknown.

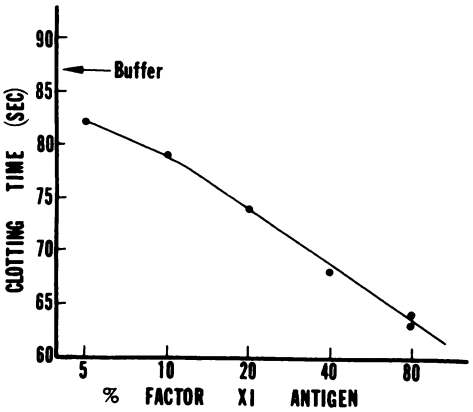

Fig. 2. A reference curve for the neutralization–inhibition assay made by plotting dilutions of the standard normal plasma (expressed as per cent factor XI antigen) against clotting times obtained when these dilutions were used as the test material in the netralization–inhibition assay. The clotting time obtained when control buffer was used as the test material is also shown.

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RESULTS

Characterization of the Antiserum

An antiserum with a maximum factor XI neutralization titer of 1/120 to 1/180 was obtained after three booster injections of antigen. The titer decreased rapidly within a few weeks of the last booster injection. Complete neutralization of factor XI activity in normal plasma could not be obtained with our antiserum; the overall neutralization obtained with low dilutions of antiserum ranged between 80% and 95% activity. The antiserum did not neutralize Fletcher factor activity (a known contaminant of less purified factor XI preparations) in normal plasma.

Gel diffusion and immunoelectrophoresis tests of the antiserum showed two precipitin lines. Both could be removed by passing the antiserum through an immunoadsorbant column under the conditions described above without impairing the factor XI-neutralizing capacity of the antiserum. The same results were obtained when hereditary factor XI-deficient plasma, normal plasma adsorbed with Dicalite, or normal unadsorbed plasma was used as the immunoadsorbant. Therefore, normal plasma was used routinely for this purpose. Immunoadsorbed antiserum that was concentrated about sixfold had a neutralization titer of 1/900. It produced no precipitin lines on gel diffusion against either normal plasma or the autologous antigen (at 800% factor XI activity). It was used at a 1/400 dilution in the factor XI antigen assay, as described above. At a concentration of 1/400, the antiserum produced a very slight shortening of the factor XI assay equivalent to about 0.4% factor XI activity.

The antiserum neutralized factor XI activity in a purified preparation of factor XI. With the neutralization–inhibition technique, it was possible to show that the antibody also reacted with heat-denatured factor XI. As shown in Table 1, factor XI activity decreased when purified factor XI was incubated at 56°C, whereas the amount of factor XI antigen detected by the antiserum remained essentially unchanged. The antiserum also neutralized activated factor XI, as shown in Table 2.

Factor XI Activity and Antigen Levels in Individuals With Homozygous or Heterozygous Factor XI Deficiency

The relation between factor XI activity and antigen was examined in plasma from normal subjects, in plasma from patients with severe hereditary factor XI deficiency (homozygotes), and in the plasma of parents and children of these patients (heterozygotes). In 13 samples from 10 normal individuals, mean factor

Table 1. Reaction of Factor XI Antiserum With Native and Heat-denatured Purified Factor XI

Test Material	Factor XI (%)	
	Activity	Antigen
Untreated	40	41
56°C, 30 min	18	34
56°C, 60 min	15	39

A diluted purified factor XI preparation with 40% factor XI activity was tested for activity and antigen before and after heating for the times listed.

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Table 2. Effect of Factor XI Antiserum on Activated Factor XI

	Clotting Time–XI Assay (sec)	
	Kaolin	Plastic
XI_a + antiserum 1/80	75	118
XI_a + antiserum 1/160	64	93
XI_a + citrated saline	61	77
Citrated saline	109	> 480

To 0.1 ml factor XI_a in a plastic tube was added 0.01 ml of diluted, adsorbed antiserum or citrated saline. After 30 min incubation at 37°C, 0.1 ml citrated saline was added, and 0.05-ml samples were tested in the factor XI assay with and without kaolin.

XI activity was 113% (range, 88%–156%), and mean factor XI antigen was 115% (range, 68%–168%).

The results of activity and antigen determinations on the individuals with homozygous or heterozygous factor XI deficiency are listed in Table 3. Ten homozygous factor XI deficiency patients from different families had between 4% and 13% factor XI activity, and factor XI antigen between < 5% and 9%. Hence, there was no evidence that any of these patients had an abnormal circulating factor XI molecule.

Table 3. Factor XI Activity and Antigen Levels in the Plasma of Homozygous Factor XI-deficient Individuals and Their Relatives

Factor XI Deficients	Sex	Activity (%)*	Antigen (%)	Bleeding†	Bruising‡
S.B.§	F	4	<5	Yes	Yes
M.E.§	M	6	<5	Yes	No
D.G.§	F	5	<5	Yes	Yes
E.G.§	F	4	5	Yes	Yes
S.K.	F	5	<5	Yes	Yes
J.Ky.	M	7	<5	No	No
J.L.§	M	5	8	Yes	No
F.M.§	M	13	9	Yes	No
M.M.§	F	11	9	Yes	Yes
S.Sm.§	M	9	4¶	No	No
Parents and/or children of factor XI deficients					
H.B.-4	F	60	54		
H.B.-6	F	46	41		
H.B.-9	M	66	67		
A.G.§	F	53	42		
D.H.§	F	48	40		
M.H.§	F	61	72		
S.H.§	M	47	59		
C.K.	F	50	53		
D.K.	F	66	68		
P.K.	M	47	52		
S.S.§	F	24	10		
T.S.§	M	60	71		

*All samples were tested in duplicate.

†Surgery and/or dental extraction.

‡By history.

§Results are averages of two or more separate determinations on different days.

¶Average of two determinations of 7% and < 5%.

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As noted above, immunoadsorption with normal plasma in the preparation of the antiserum did not measurably decrease its factor XI-neutralizing activity, and immunoadsorbed antiserum was used routinely. However, the remote possibility existed that immunoadsorption preferentially removed from the whole rabbit antiserum an antibody which could react with an abnormal factor XI. Consequently, plasma from eight of the ten factor XI-deficient patients was also tested for factor XI antigen using a nonimmunoadsorbed antiserum. Again, no evidence of an abnormal factor XI molecule was found. The antigen levels ranged from < 5% to 7% (M.M. of Table 3).

The 12 heterozygous parents and children had factor XI activity levels between 24% and 66%. In 11 individuals, the antigen level approximated the measured factor XI activity. One individual (S.S.) on repeated determinations had activity levels two to three times greater than antigen level. (The highest ratio of activity:antigen observed among the normals tested was 1.4:1) S.S. was not studied further.

Clinical Evaluation

Each subject was questioned carefully for a history of abnormal bleeding or bruising. Eight of the ten homozygous factor XI-deficient subjects had bled abnormally from tooth extraction, tonsillectomy, or both procedures. However, one presumably homozygous subject (S.Sm.), who had 9% factor XI clotting activity and antigen levels of 7% and < 5% on two determinations, had not bled abnormally from either tooth extraction or tonsillectomy. Another homozygous patient (J.Ky.) who had 7% factor XI clotting activity and an antigen level below 5%, had incurred multiple serious injuries (see Discussion) without abnormal bleeding and did not bleed excessively following surgery for a pilonidal cyst. A third homozygous factor XI-deficient subject (F.M.) required seven transfusions following a tooth extraction but had undergone three eye operations without abnormal bleeding. No male homozygous subject had noted "spontaneous bruising," whereas all five of the homozygous women with factor XI deficiency reported spontaneous bruising. Episodes of spontaneous bruising were sometimes associated with episodes of heavy menstrual bleeding, which could last for up to 2 wk.

A history suggestive of abnormal hemostasis was obtained from 1 of the 12 heterozygous factor XI-deficient subjects. The subject (D.H.), who had 48% factor XI clotting activity and an antigen level of 40%, stated that she had bled slowly for 2 wk following tooth extraction and had noted spontaneous bruising.

DISCUSSION

Studies utilizing antibodies to clotting factors have demonstrated that hereditary coagulation disorders may stem from either the absence of a normal clotting factor or the presence of a defective molecule.[1] In the present study, a heterologous antiserum produced in a rabbit with a purified unactivated human factor XI antigen was utilized to look for a factor XI molecule in plasma from patients with homozygous factor XI deficiency or heterozygous members of their families. We assumed that a defective molecule in the patient's plasma, if it existed, would have enough common antigenic determinants with normal factor

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XI protein to cross-react immunologically with our antibody. This assumption was supported by the observation that neither the alteration associated with activation of factor XI nor the conformational changes induced by heat denaturation of purified factor XI prevented our antiserum from reacting with the factor XI molecule.

None of the ten homozygous factor XI-deficient patients whom we studied had evidence of a circulating abnormal factor XI molecule. Factor XI activity was reduced to between 4% and 13% of normal, and antigen levels did not exceed 9%. Absence of a nonreactive circulating factor XI molecule in factor XI deficiency was further supported by the results obtained from the 11 individuals with heterozygous factor XI deficiency who were found to possess comparable intermediate levels of clotting activity and antigen.

Our series of 22 individuals brings to 32 the total number of reported factor XI-deficient individuals who have been tested for cross-reactive material using heterologous antibodies raised in rabbits. Twenty of these individuals who were apparently unrelated were homozygotes for factor XI deficiency. All 32 individuals lacked cross-reactive material significantly in excess of observed factor XI activity[6] (Table 3).

Two different antisera were used in the screening of these patients. Both our antiserum and that of Forbes and Ratnoff[6] were raised in rabbits with purified human factor XI. The antigens differed in that our factor XI was not activated, whereas that of Forbes and Ratnoff was. However, the physical modification of factor XI during activation appears to be relatively small, since the size of factor XI on gel filtration appears unchanged on activation with trypsin.[12,13] Moreover, our antiserum was demonstrated to neutralize both native and activated factor XI. Hence, one may strongly suspect that the antigenic sites are very similar to the two forms of factor XI and that our antiserum and the antiserum of Forbes and Ratnoff[6] would recognize, or fail to recognize, the same types of molecules. Presumably, therefore, all 32 individuals with homozygous or heterozygous factor XI deficiency studied to date represent examples of the same genetic molecular defect. It seems reasonable to generalize that the usual patient with factor XI deficiency does not possess a circulating factor XI-like molecule with antigenic determinants similar enough to normal factor XI to be detectable by a heterologous rabbit antiserum to normal factor XI.

However, the possibility still exists that a second type of factor XI deficiency, with a detectable abnormal molecule, will be found when more patents are tested. In a study from this laboratory of 54 patients with hereditary factor VIII deficiency, only two patients were discovered who possessed a defective factor VIII molecule reacting with a human factor VIII antibody.[3] A data base of similar magnitude will be more difficult to obtain in factor XI deficiency and will probably require additional studies of small groups of patients from several laboratories.

It seemed possible that the rare patient with hereditary factor XI deficiency who withstood significant hemostatic challenges without abnormal bleeding might synthesize an altered factor XI molecule, demonstrable by its ability to cross-react with a factor XI antibody, which was inactive in vitro but which functioned normally in vivo. Our data did not support this hypothesis. One presumably homozygous factor XI-deficient man in our series (S.Sm.) had 9%

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factor XI activity. His factor XI deficiency was discovered accidentally when he donated blood for use in preparing a normal pooled plasma for a reference standard. He gave a completely negative bleeding history and had not bled excessively after tooth extraction or tonsillectomy. His plasma had 7% factor XI antigen on one determination and less than 5% antigen on a second determination.

A second patient (J. Ky.) was first seen at age 5 yr because of a long clotting time discovered prior to a contemplated tonsillectomy. The tonsillectomy was canceled when study of the patient and his family (family K. of reference 4) established that he had homozygous factor XI deficiency with 3% factor XI activity. He was studied again at age 22 years as part of the present investigation. He has never bled or bruised abnormally despite multiple injuries as follows: a scalp laceration requiring four sutures; a broken nose received while playing basketball; a broken nose a second time in a surfboard accident; repeated minor injuries including cuts of his tongue incurred during 1 yr of participation in karate; a blow to the mouth which broke his front tooth; a motorcycle accident with cuts on his legs; and an accident in which a grinding wheel scraped his face producing an extensive abrasion of his upper lip and cheek. He did not bleed excessively from recent surgery for a pilonidal cyst in which a large surgical wound was left open to heal by secondary intention. In the present study this patient had 7% factor XI activity and less than 5% factor XI antigen.

Thus, neither of the two patients in this series who have not bled abnormally despite major hemostatic challenges have evidence of excess factor XI antigen in their blood which might function in vivo but not in vitro. Apparently, this mechanism cannot explain the absence of clinical bleeding in the subset of patients with marked factor XI deficiency who appear to have normal hemostatic function. Presumably, another activity or activities, which remain to be identified, can compensate for lack of factor XI activity in the hemostatic function of such patients.

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