The Relationship between Factor XI Coagulant and Factor XI Antigenic Activity in Cattle

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ABSTRACT

Factor XI protein, isolated from normal bovine plasma, was used to raise antiserum in rabbits. The antisera was partially purified and used in a neutralization-inhibition assay to investigate the relationship between factor XI coagulant activity and antigenic material in the plasma of normal cattle and cattle homozygous and heterozygous for factor XI deficiency. Factor XI antigen was reduced in both the homozygous and heterozygous animals to levels comparable to the factor XI coagulant activity. The reduction of immunologically cross-reactive material to normal factor XI suggests that the factor XI coagulation defect is associated with the absence of a normal protein.

Key words: Factor XI deficiency, cattle, antigen activity, coagulation activity.

RÉSUMÉ

Cette expérience consistait à isoler du plasma de bovins normaux la protéine du facteur XI et à l'utiliser pour produire un antisérum, chez le lapin. On fit subir une purification partielle à cet antisérum et on l'utilisa ensuite dans une épreuve d'inhibition de la neutralisation, pour étudier la relation entre l'activité coagulante du facteur XI et son matériel antigénique, dans le plasma de bovins tant normaux qu'homozygotes ou hétérozygotes, relativement à la déficience du facteur XI. L'antigène de ce facteur afficha, chez les sujets homozygotes et hétérozygotes, une réduction comparable à celle de son activité coagulante. La réduction du matériel qui donne une réaction immunologique croisée, avec le facteur XI normal, suggère que le défaut de coagulation attribuable au facteur XI résulte de l'absence d'une protéine normale.

Mots clés: déficience du facteur XI, bovins, activité antigénique, activité de coagulation.

INTRODUCTION

Factor XI is a plasma protein involved in the intrinsic or contact activation pathway of blood coagulation (1). An inherited hemorrhagic disorder, characterized by a deficiency in factor XI coagulant activity, has been identified in man, (2,3,4), dogs (5) and cattle (6,7). In the human populaton several forms of the defect have been recognized ranging from a clinically severe bleeding problem to an asymptomatic condition (8,9,10). The clinical severity of the disorder in cattle also appears to be quite variable (11,12).

In cattle factor XI deficiency is transmitted as an autosomal recessive gene (11) and the pattern of inheritance is similar in the human (2,3) and canine population (5). A heterozygous, or carrier, animal possesses one gene which is functionally normal for the factor XI protein and one gene which is abnormal and exhibits reduced plasma levels of factor XI activity. In a survey of 170 cattle it was found that the range of detectable factor XI activity was between 0.60 and 2.27 units per mL for normal animals and 0.26-0.58 units per mL for heterozygous or carrier animals (11). A homozygous, or deficient animal, which possess two recessive genes both incapable of producing functional factor XI protein, exhibits very low (<0.10 units per mL) plasma factor XI activity (11). Although a laboratory test for factor XI procoagulant activity can be used to reliably identify deficient animals, the test has proven to be less reliable when differentiating between normal and carrier animals, especially when plasma factor XI activity falls in the high carrier — low normal range. In certain difficult to categorize animals, selective test matings have been used to definitely identify animals as normal or heterozygous for the disorder. Not only is this method of identifying carriers time consuming and expensive but it is also impractical for screening large numbers of animals or for evaluating calves.

It has been shown that a relatively high frequency of the mutant gene can exist in some breeds of cattle (11). The increased use of artificial insemination and embryo transplants could greatly increase the frequency of the disorder if heterozygous animals were inadvertantly used. Thus, to be able to maintain the integrity of the breeds of cattle such as the Holstein-Friesian, it is important to be able to reliably and simply monitor animals for the genetic disorder. In human subjects with hereditary factor XI deficiency there is a distinct correlation between factor XI coagulant activity and factor XI antigen activity (13,14). The purpose of this investigation was twofold: (a) to develop a laboratory test which would detect bovine factor XI antigenic activity and (b) to evaluate the usefulness of the test as an additional screening procedure for the classification of cattle with respect to their factor XI status.

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Supported in part by the Holstein-Friesian Association and the Ontario Ministry of Agriculture and Food.

Submitted April 29, 1983.

MATERIALS AND METHODS

ANIMALS

The cattle used in the study were all of the Holstein-Friesian breed. As a representative sample of normal animals, blood samples were collected from 12 cows selected at random from the herd maintained at the Ontario Veterinary College. Six blood samples were also taken from animals known to be carriers of factor XI deficiency and nine samples from animals known to be factor XI deficient. These animals were from a small research herd being used to support the investigation of factor XI deficiency in cattle. Thirty two blood samples were obtained from calves, between two to six weeks of age, born during the 12 month period of the study in the Ontario Veterinary College and research herds. All animals were in good health at the time that the blood samples were collected.

PREPARATION OF PLASMA

Blood was collected by external jugular venepuncture using 18 gauge, one-inch disposable needles attached to 35 mL plastic syringes. The blood was immediately transferred to plastic tubes containing 3.8% trisodium citrate anticoagulant and mixed in the proportion nine parts blood to one part anticoagulant. After thorough mixing the samples were centrifuged at 2,500 x g for 20 min at 4° C to obtain platelet poor plasma. A portion of the plasma was stored at -20° C in sealed plastic tubes for the factor XI antigen assay and a portion of the plasma was used immediately for determining the factor XI coagulant activity.

DETERMINATION OF FACTOR XI COAGULANT ACTIVITY

Factor XI coagulant activity was measured using a modification of the activated partial thromboplastin time (APTT) method of Langdell *et al* (15) as previously described (16). The substrate plasma was a pool of samples obtained from five animals known to be congenitally deficient in factor XI coagulant activity. All samples were assayed in duplicate on two separate occasions. The specific factor XI coagulant activity of the plasma samples was calculated on a standard curve prepared using pooled plasma from at least ten animals normal for factor XI coagulant activity. The pooled normal plasma was designated as having 1.00 units factor XI activity per mL.

ISOLATION OF FACTOR XI PROTEIN

A modification of the methods of Wiggins *et al* (17) and Koide *et al* (18) was used to isolate factor XI from normal bovine plasma. To prevent activation of factor XI during the isolation procedure all buffers contained 1.0 mM ethylenediamine tetraacetic acid, 50 μg per mL polybrene, 1 mM benzamidine hydrochloride (Aldrich Chemical Co., Milwaukee, Wisconsin) and 0.02 percent sodium azide (Fisher Scientific Co., Toronto, Ontario) and all glassware and dialysis tubing was prerinsed with a polybrene solution (2 gm per L) followed by distilled water before use.

Blood was collected into plastic bags containing 0.1 vol of an anticoagulant solution (13.4 gm Na oxalate, 100 mg heparin [Sigma Chemical Co., St. Louis, Missouri] and 100 mg crude soybean trypsin inhibitor [Sigma Chemical Co., St. Louis, Missouri] per L) and after thorough mixing was centrifuged at 2,500 g for 30 min at 4° C. After the addition of inhibitors to the resulting plasma, the plasma was dialysed overnight at 4° C against 0.04 M Tris, 5 mM succinic acid buffer, pH 8.8. Six hundred mL of the dialysed plasma was applied to a 10 x 40 cm DEAE-sephadex (Pharmacia (Canada) Ltd., Dorval, Quebec) column equilibrated with the dialysis buffer. The factor XI activity was eluted using a linear gradient consisting of equal volumes of the dialysis buffer and 0.3 M Tris, 0.12 M succinic acid, 0.3 M NaCl, pH 7.75. Fractions containing factor XI activity were pooled and dialysed at 4° C overnight against 0.02 M sodium phosphate buffer pH 6.6 containing 0.07 M NaCl. CM sephadex C-50 (Pharmacia (Canada) Ltd., Dorval, Quebec) resin was added to the dialysed material stirred at 4° C for 60 min and a 2.6 x 40 cm column poured. The column was washed with 2 bed volumes of the dialysis buffer before a linear gradient consisting of 0.02 M sodium phosphate buffer, pH 6.6 containing 0.10 M NaCl and 0.55 NaCl was started to elute the factor XI activity. The fractions containing factor XI activity were pooled and concentrated tenfold (Amicon Diaflo Ultrafiltration, PM30 membrane, Amicon Canada Ltd., Oakville, Ontario)) before application to a 1.6 x 27 cm heparin-agarose (18) column equilibrated with 0.05 M sodium phosphate buffer containing 0.15 M NaCl, pH 6.6. The factor XI activity was eluted from the column with 0.05M sodium phosphate buffer pH 6.6 containing 0.20 M NaCl. The active fractions were pooled, concentrated sixfold and dialysed overnight at 4° C against 0.01 M sodium phosphate buffer containing 0.4 M NaCl, 0.02% sodium azide and 1 mM benzamidine. Four mL of protein solution were applied to a 1 x 3.5 cm column of concanavalin A sepharose (Pharmacia (Canada) Ltd., Dorval, Quebec) equilibrated with dialysis buffer. To elute factor XI activity from the column, 0.5 M α-0-methylglucoside (Sigma Chemical Co., St. Louis, Missouri) was added to the buffer.

Protein concentration was determined by the method of Lowry (19) using bovine serum albumin (Sigma Chemical Co., St. Louis, Missouri) as standard.

PREPARATION OF ANTISERUM TO FACTOR XI

Before injection, the protein solution containing the purified factor XI protein, was mixed in equal portions with Freund's complete adjuvant (Difco Laboratories, Detroit, Michigan). Rabbits (2.0 kg body weight) were immunized by injecting 0.6 mg of the protein solution subcutaneously at multiple sites. Three booster injections containing 0.3 mg protein were administered at weekly intervals for the following three weeks. One week after the last injection the rabbits were exsanguinated and the blood allowed to clot and retract at 4° C overnight. The serum was treated with BaS04 (J.T. Baker Chem. Co., Phillipsburgh, New Jersey) (100 mg/mL) for 30 min at 22° C and centrifuged. Solid ammonium sulphate (J.T. Baker Chem. Co., Phillipsburg, New Jersey) was added to 35% saturation. The precipitate obtained after centrifugation was dissolved in half the original volume of 0.05 M Tris-HCl buffer, pH 7.4 containing 0.15 M NaCl and dialyzed against this buffer for 18 h at 4° C. The partially purified antiserum was stored

in small aliquots at -20° C. Before use the antisera was heat-treated at 56° C for 15 min.

Determination of antibody effectiveness — To determine whether the antibody preparation would neutralize factor XI coagulant activity, 0.05 mL of normal bovine pooled plasma was incubated with 0.05 mL antisera serially diluted with Tris buffered saline (0.05M Tris, 0.004 M NaCl, pH 7.4) for 30 min at 37° C. The samples were centrifuged for 10 min at 600 × g to remove any traces of precipitate and added to 0.4 mL citrated saline (0.125M sodium citrate, 0.017 M NaCl). The diluted samples were assayed for factor XI coagulant activity. The results were compared to the factor XI activity of a similarly diluted normal pooled plasma sample without antisera. The % factor XI coagulant activity neutralized was calculated as follows (14):

% XI in diluted standard plasma — % XI in antiserum mixture x 100, ÷ % in diluted standard plasma

Determination of factor XI antigen — Factor XI antigen was measured in a three-step neutralization-inhibition system essentially as described by Rimon *et al* (14). The concentration of antisera used in the assay was selected on the basis of the dilution of antisera which would neutralize at least 80% of the factor XI coagulant activity of normal pooled plasma. In the first stage of the assay 0.10 mL of test plasma was incubated at 37° C for 60 min with 0.01 mL of antisera. The second step involved the addition of 0.1 mL normal pooled bovine plasma and the continuation of the incubation at 37° for a further 30 min. The sample was centrifuged at 600 x g for 10 min to remove any traces of precipitate before the addition of 0.40 mL citrated saline to the mixture. The sample was then assayed for residual factor XI coagulant activity. The amount of factor XI in the sample was determined from a reference inhibition curve prepared for each assay. For the reference curve, serial dilutions of the normal pooled plasma were used in place of test plasma and the undiluted pooled plasma was designated as having 100% factor XI antigen activity.

TABLE I. Effect of Addition of Purified Factor XI Preparation to Specific Coagulation Factor Assays

Factor assay	Clotting times (sec)		
	Normal bovine plasma	Factor XI preparation	Control Tris-buffered saline
II	53	>360	>360
VII	22	54	52
VIII	39	93	90
IX	33	105	100
X	37	154	148
XI + Activator	109	107	—
XI - Activator	430	427	

RESULTS

Factor XI protein preparation — A 500-fold purification of the factor XI protein from normal bovine plasma was achieved. On the basis of specific coagulation factor assays, the preparation did not appear to contain other coagulation factors. Similar clotting times were obtained when either the factor XI preparation or Tris-buffered saline was added to either factor II, VII, VIII, IX or X deficient plasma in the specific factor assay systems (Table I). No fibrin strands could be detected in the preparation following the addition of thrombin indicating that it was not contaminated with fibrinogen. The factor XI protein appeared to be in its nonactivated form since the clotting times obtained in the factor XI assay system were significantly longer if an activator, such as celite, was omitted from the assay system (Table I).

Characterization of the Factor XI Antisera — Up to 95% of the factor XI coagulant activity was neutralized following incubation of normal bovine plasma with the partially purified, heat-treated antisera (Table II). The amount of factor XI activity neutralized was reduced as the dilution of antisera preparation was increased.

TABLE II. Relationship Between the Concentration of Antisera and Factor XI Neutralizing Activity

Reciprocal of antisera dilution	% factor XI activity neutralized
0	95.2
5	93.2
10	87.7
20	64.4
40	53.0
50	36.0
80	17.0
100	5.0

The incubation of undiluted antisera with normal bovine plasma failed to neutralize the coagulant activity of factors VII, VIII or IX in the sample. For all subsequent antigen level determinations, a tenfold dilution of the antisera preparation was used since this concentration would neutralize 88% of factor XI coagulant activity in normal bovine plasma (Table II).

Factor XI activity and antigen levels

(a) Mature Animals. The relative values for the factor XI coagulant activity and factor XI neutralizing activity obtained for the 27 plasma samples collected from mature animals are illustrated in Fig. 1. Animals which exhibited low levels of factor XI coagulant activity also had reduced levels of factor XI antigenic activity,

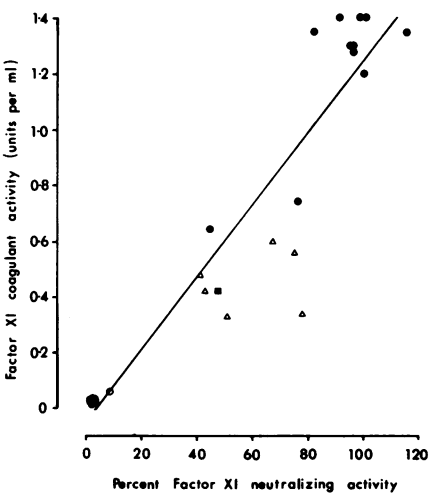

Fig. 1. The relationship between factor XI coagulant activity and factor XI neutralizing activity in mature cattle. Closed circles represent normal animals, triangles represent heterozygous animals and open circles represent homozygous animals. The closed square represents values from a cow from a normal herd but classified as heterozygous on the basis of the laboratory assays.

while animals with normal levels of factor XI coagulant activity also exhibited high levels of antigenic activity. The correlation coefficient for the coagulant and antigenic activity was 0.894 indicating that a strong positive correlation exists between these two parameters. One of the samples obtained from the herd presumed to be normal with respect to factor XI activity was found to have 0.43 units per mL coagulant activity and 57% factor XI neutralizing activity. This animal is represented by a solid square on Fig. 1 and has been classified as a carrier or heterozygous animal for purposes of computation of mean values and standard deviations (Table III).

(b) Calves. The distribution of factor XI coagulant and antigen neutralizing activities for the 32 calf plasma samples examined is shown in Fig. 2. In this group of animals the correlation coefficient between the two parameters was 0.786. On the basis of the factor XI status of the sire and the dam, 13 calves were expected to be either deficient or carrier animals. Four of these calves were clearly identifiable as homozygous for factor XI deficiency on the basis of both low factor XI coagulant and antigenic activity. Only one of the remaining nine calves in this group exhibited greater than 0.50 units per mL coagulant activity and greater than 65% antigen neutralizing activity. This animal would have been classified as a low-normal rather than a carrier on the basis of factor XI coagulant and antigenic activity. The plasma samples from the remaining 19 calves were found to have normal factor XI coagulant and antigenic activity as anticipated from the parentage classification. The mean values and standard deviations for the normal, carrier and deficient animals are shown in Table III.

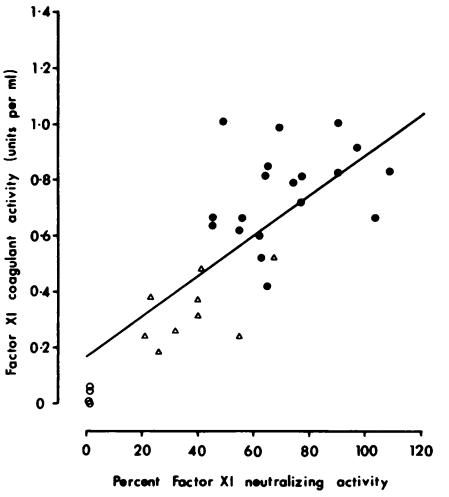

Fig. 2. The relationship between factor XI coagulant activity and factor XI neutralizing activity in calves. Closed circles represent normal animals, triangles represent heterozygous animals and open circles represent homozygous animals.

DISCUSSION

Congenital blood coagulation disorders may arise from either the absence of a normal clotting protein or the presence of a defective molecule (20). Factor XI deficiency has been recognized in the Holstein-Friesian breed of cattle for several years (6,7). Although the pattern of inheritance of the disorder has been determined (11), the biochemical nature of the defect has not been identified. In the present study, a heterologous antiserum, produced in rabbits with a partially purified bovine factor XI antigen, was used to investigate whether or not factor XI antigenic activity was present in the plasma of cattle known to be homozygous or heterozygous for factor XI deficiency. In the plasma of the 13 homozygous factor XI deficient animals included in this study, factor XI coagulant activity was reduced to between 1% and 6% of normal and factor XI antigen levels did not exceed 8% of normal (Figs. 1 and 2, Table III). This result indicates that factor XI deficiency in cattle is related to the absence of a normal clotting protein rather than to the presence of a defective protein. If a defective protein was present in plasma from a homozygous animal, the abnormal protein could be expected to have a sufficient number of common antigenic determinants with the normal factor XI molecule that some immunological cross-reactivity would have occurred with the antibody. Furthermore, heterozygous animals have comparable intermediate levels of factor XI coagulant and antigenic activity relative to normal and homozygous animals (Figs. 1 and 2, Table III). The ratio of factor XI coagulant and antigenic activity is constant in normal, heterozygous and homozygous animals.

The biochemical lesion in factor XI deficiency is similar in man and cattle. In two separate studies, involving a total of 32 human factor XI deficient individuals, immunologically cross-reactive material to human factor XI antisera was absent in homozygous and reduced in heterozygous individuals (13,14). The biochemical similarity of the disorder in man and cattle is compatible with the previously observed similarity in the mode of inheritance of the disorder in the two species (2,3,11).

The strong correlation between factor XI coagulant activity and antigenic neutralizing activity (0.894 and 0.786 respectively) found for both the mature and calf plasma samples indicates that the antigen assay may be useful for the identification of factor XI homozygous and heterozygous animals. However, the relatively high values for coagulant and antigenic activity (0.50 units per mL and 67% respectively) found in a known carrier animal indicates that in some instances selective test mating studies might still be necessary to uniquely identify a carrier animal from a low-normal. One advantage of being able to estimate both coagulant and antigenic activity

TABLE III. Factor XI Coagulant and Antigenic Activity in Normal, Carrier and Deficient Animals

Group (No. of animals)	Factor XI coagulant* activity (units per mL)	Factor XI neutralizing* activity (% of normal)
Mature animals		
Normal n = 11	1.32 ± 0.26^a	92.3 ± 21.5^a
Carrier n = 7	0.46 ± 0.12^b	59.1 ± 14.9^b
Deficient n = 9	0.03 ± 0.01^c	3.4 ± 1.8^c
Calves		
Normal n = 19	0.75 ± 0.17^a	72.4 ± 22.9^a
Carrier n = 9	0.34 ± 0.12^b	34.2 ± 10.9^b
Deficient n = 4	0.04 ± 0.03^c	1.1 ± 0.2^c

*Results are expressed as mean ± standard deviation
Within each column the values with different superscripts are significantly different ($p < 0.01$)

on a single blood sample is that, in most instances, the animal would have to be bled only once. In the few cases where the results were ambiguous, a repeat blood sampling would usually clarify the animal's factor XI status.

In both the normal and heterozygous calves the factor XI coagulant and antigenic activity were reduced compared to similar adult animals (Table III). Since the reference for both adult and calf samples was a pooled plasma sampled from mature animals, the apparently reduced levels in calves compared to adults, probably reflects age-related differences. Plasma coagulant activity of factor II, V, VII and X increases with age (21) and it has been our experience that factor XI activity also increases with age. Hence, when assessing the factor XI status of calves it is important to have age related control samples for comparison.

Although the screening of cattle for genetic biochemical defects has received little attention in the past, it may be important to the maintenance of the genetic integrity of breeds of cattle, such as the Holstein-Friesian, in the future. If a heterozygous factor XI deficient bull was used extensively for artificial insemination, or if a heterozygous factor XI deficient cow was utilized as a donor for superovulation and embryo transfer, the incidence of the genetic disorder could rapidly escalate in a breed (22). In some herds the incidence of heterozygous factor XI deficient animals may be as high as 16.9%. In the present study, the identification of one heterozygous animal from a group of 12 "normal" animals further indicates that unidentified heterozygous animals occur in the general population.

While the presence of a defective gene for factor XI is not a lethal condition, preliminary data from our small research herd indicate that an increased incidence of hemorrhagic incidences may occur in homozygous animals. The combination assessment of factor XI coagulant and antigenic activity should increase the efficiency and reliability of evaluating the factor XI status of an animal. This is a significant step towards the goal of achieving a breed free of the coagulation defect.

ACKNOWLEDGMENTS

The author is indebted to Dr. W. D. Black for helpful discussion and assistance with the preparation of the manuscript and wishes to thank Mrs. M. Cooper and Miss G. E. Templeton for excellent technical assistance.

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