Studies on a Family with Combined Functional Deficiencies of Vitamin K-dependent Coagulation Factors

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ABSTRACT Two siblings with mild hemorrhagic symptoms had combined functional deficiencies of vitamin K-dependent clotting factors. Prothrombin (0.18–0.20 U/ml) and Stuart factor (Factor X, 0.18–0.20 U/ml) were most severely affected. Antigenic amounts of affected coagulation factors were normal and normal generation of thrombin activity occurred in the patients' plasmas after treatment with nonophysiologic activators that do not require calcium for prothrombin activation. Hepatobilary disease, malabsorptive disorders, and plasma warfarin were not present. Both parents had normal levels of all coagulation factors. The patients' plasmas contained prothrombin that reacted both with antibody directed against des-γ-carboxyprothrombin and native prothrombin. Crossed immunoelectrophoresis of patients' plasmas and studies of partially purified patient prothrombin suggested the presence of a relatively homogeneous species of dysfunctional prothrombin, distinct from the heterologous species found in the plasma of warfarin-treated persons. These studies are most consistent with a posttranslational defect in hepatic carboxylation of vitamin K-dependent factors. This kindred uniquely possesses an autosomal recessive disorder of vitamin K-dependent factor formation that causes production of an apparently homogeneous species of dysfunctional prothrombin; the functional deficiencies in clotting factors are totally corrected by oral or parenteral administration of vitamin K_1.

INTRODUCTION

The normal functioning of the blood coagulation factors prothrombin, Factor VII, Factor IX (Christmas factor), and Factor X (Stuart factor) has been shown to depend upon the posttranslational γ-carboxylation of selected glutamic acid residues of these proteins within the hepatocyte (1–9). In the normal circulating zymogen, these γ-carboxyglutamic acid (Gla)[1] residues appear to serve as binding sites for calcium ligands that attach to phospholipid and to proteolytic activators of the clotting factors (6–9). Vitamin K is necessary for the carboxylation reaction, which is coupled to oxidative conversion of vitamin K to a 2,3-epoxide derivative. Vitamin K epoxide is then sequentially reduced to regenerate the form necessary to support the linked carboxylation-oxidation reactions (10, 11). Administration of coumarinlike drugs impairs formation of Gla residues on the vitamin K-dependent factors, apparently by inhibition of the enzyme(s) responsible for reduction of the vitamin K epoxide metabolite (10).

Heritable deficiencies of individual vitamin K-dependent factors other than Factor IX are rare. The pattern of inheritance appears to be autosomal recessive, and the deficient coagulation factor activity may reflect either a true deficiency state or the presence of dysfunctional zymogen (12). In one family, deficiency of Factor X was sometimes associated with a deficiency in Factor VII (13). In other cases, a functional deficiency of prothrombin was associated with deficiencies of Factors VII or X (14, 15), and in one kindred deficient Factor VII and Factor IX activities were independently inherited (16).

Two previously reported cases of congenital, combined deficiencies of vitamin K-dependent factors have had characteristics indicative of defective Gla residue formation and/or function (17–19). An additional patient with deficiencies of all four vitamin

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[1] *Abbreviations used in this paper*: BCS, barbital-citrate-saline buffer; Gla, γ-carboxyglutamic acid; RVV, Russell's viper venom; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

K-dependent factors was less extensively characterized (20).

This report describes a kindred possessing an abnormality of vitamin K-dependent coagulation factors. The abnormality present in affected members of this family has clinical and biochemical features distinct from those of the two cases previously described.

METHODS

Case reports

The index patient (A.B.), a 20-yr-old woman in 1980, has a lifelong history of easy bruising, epistaxis, spontaneous gingival bleeding, and prolonged bleeding after dental extractions. She required transfusion for anemia resulting from pubertal menorrhagia at age 13. Between ages 18 and 20, she was hospitalized three times for severe vaginal hemorrhage despite hormonal regulation of menses. One of these episodes was complicated by intraperitoneal hemorrhage documented at laporatomy and was followed by repeated hemorrhage at the operative site. She also suffers intermittently from a polyarticular, nondeforming, seronegative arthritis.

Her brother (M.F., 18 yr old) had no history of abnormal bleeding except for hemorrhage requiring transfusion after dental extraction at age seven. He is otherwise well and has no symptoms of arthritis.

Between 1973 and 1980, prothrombin times on A.B. and M.F. were repeatedly abnormal, ranging from 21–31 s (control 11–14 s). On two occasions, reduced levels of Factor X (Stuart factor; 25% [A.B.], 12% and 25% [M.F.] and prothrombin [35%, M.F.]) were measured in another laboratory.

Both siblings had normal growth and development and were of normal height and weight. Neither had unusual diets or had taken antibiotics within several months of evaluation. There was no other family history of abnormal hemorrhage. Another sibling died at age 12 in an automobile accident. There were no historical or physical findings suggestive of malabsorption or liver disease in the affected siblings, and specific tests for these disorders in A.B. at the time of our initial studies of hemostasis demonstrated normal values of serum glutamic oxalacetic transaminase, glutamic pyruvic transaminase, lactate dehydrogenase, alkaline phosphatase, bilirubin, total protein and albumin, gamma glutamyl transpeptidase, cholesterol, triglyceride, serum carotene, postprandial serum bile acid, and D-xylose absorption. Radiologic examination of the upper gastrointestinal tract and small bowel was also normal.

Transfusion of fresh frozen plasma (seven units) shortened A.B.'s prothrombin time from 25 s to 17 s. On one occasion, parenteral administration of vitamin K_1 corrected the prothrombin time to normal with cessation of hemorrhage.

The abnormalities in the patients' plasma responded to treatment with large doses of oral vitamin K_1 (Table II). After daily ingestion of 10 mg Vitamin K_1 (Mephyton, Merck, Sharp & Dohme, West Point, PA) for 2 wk, plasma levels of prothrombin, Factor X, and Factor IX (77%) were within normal limits. Subsequent reduction in the daily dose of vitamin K_1 to 2.5–5.0 mg/d resulted in prolongation of the prothrombin time. No warfarin was detectable in the patient's plasma (21). A.B. also noted subjective improvement in frequency and severity of arthralgias while taking vitamin K_1 and exacerbation of symptoms with discontinuation or dose reduction.

Procedures

Citrated plasmas of normal individuals and of the subjects under study, plasmas of patients receiving warfarin, and plasmas deficient in single coagulation factors were prepared or obtained as previously described and used immediately or stored at −70° (22–25). Venepuncture was performed after obtaining informed consent, with the approval of the Committee on Human Experimentation, Case Western Reserve University.

Staphylocoagulase was the gift of Dr. Morris Tager, Emory University. *Echis carinatus* venom, Taipan viper venom and Russell's viper venom (RVV) were obtained from Sigma Chem. Co., St. Louis, MO. C-γ-alumina gel was purchased from Calbiochem-Behring Corp., American Holchst Corp., San Diego, CA. All chemicals not otherwise specified were reagent grade or better, obtained either from Fisher Scientific Co., Fairlawn, NJ or Mallinckrodt Inc., Science Products Div., St. Louis, MO.

Rabbit brain tissue thromboplastin (Permaplastin) was obtained from Alban Scientific, St. Louis, MO. Crude soybean phosphatides (Centrolex-P, the gift of Central Soya Co., Chicago, IL) was suspended at a concentration of 0.1% in 0.15 M sodium chloride. Kaolin-Centrolex was 10 mg kaolin (American Standard, Fisher Scientific Co.) suspended in 1 ml Centrolex-P. Centrolex-calcium mixture was equal volumes of Centrolex-P and 0.05 M calcium chloride.

Barbital-citrate-saline buffer (BCS) was 0.021 M barbital, 0.02 M sodium citrate, 0.11 sodium chloride, pH 7.5. Barbital-saline buffer was 0.025 M barbital sodium, 0.125 M sodium chloride, pH 7.5. Citrate-saline buffer was 0.02 M sodium citrate in 0.5 M sodium chloride, pH 6.0.

Rabbit antisera against human prothrombin and human Factor X were purchased from Calbiochem-Behring Corp., and were the gift of Dr. E. W. Davie, Seattle, respectively. Antiserum against prothrombin, after heat inactivation or isolation of a crude IgG fraction (26) selectively neutralized the prothrombin activity of normal pooled plasma without neutralizing other vitamin K-dependent clotting factors after incubation with an equal part of normal plasma for 2 h at 37°C. This antiserum formed a single band upon double immunodiffusion against normal, Factor X-deficient, Factor IX-deficient or Factor VII-deficient plasmas. After absorption with an equal volume of human Factor X-deficient plasma, a crude IgG fraction of antiserum against Factor X showed similar selective neutralization of Factor X activity in normal plasma.

Adsorption of vitamin K-dependent factors from normal and abnormal plasmas was performed for some assays by mixing with 1/10 vol C-γ-alumina for 10 min at room temperature followed by centrifugation at 30,000 *g* for 30 min at 2°C. For use in coagulation factor antigen assays and for purification of clotting factors, 1/10 vol 1 M $BaCl_2$ was added to plasma, then mixed and centrifuged in the same fashion. Supernatant solutions from the latter adsorption procedure were dialyzed against the appropriate buffer before assay.

Blood coagulation factors were assayed in one-stage tests (22, 27). Assays of the prothrombin time, (PT), activated partial thromboplastin time (aPTT), fibrinogen concentration, and platelet function were performed by previously described techniques (28). Prothrombin assay with staphylocoagulase (29) was performed in 10 × 75-mm glass tubes by incubating 0.1 ml substrate (human oxalated serum-bovine absorbed plasma (27)) with 0.1 ml test plasma, diluted in BCS buffer, for 1 min at 37°C, then adding 0.1 ml prewarmed staphylocoagulase (5 mg/ml in the same buffer), and tilting until a visible clot was observed. Percent normal activity was obtained from a curve of clotting times observed

with serial dilutions of normal pooled plasma in BCS buffer; this curve corresponded to that obtained from plasma composed of appropriate ratios of normal pooled plasma and alumina gel-adsorbed normal plasma. Assay of prothrombin by venom from *Echis carinatus* (18) was performed in the same way, using 0.1 ml prewarmed *E. carinatus* venom (10 mg/ml BCS buffer). Activation of prothrombin by venom of *Oxyuranus Scutellatus scutellatus* (Taipan viper venom) (30) was performed in a similar fashion, except that test plasma dilutions were in barbital-saline buffer, and 0.2 ml of an equal mixture of Taipan viper venom, 0.1 mg/ml, and 0.025 M $CaCl_2$ was added before assay. Assay for activation of Factor X by RVV (31), was performed in the presence of calcium by preincubating 0.1 ml substrate (Factor X-deficient) plasma, for 1 min at 37°C with 0.1 ml test or normal pooled plasma, serially diluted in barbital-saline buffer, and then adding, in order, 0.1 ml RVV (0.1 mg/ml) and 0.1 ml Centrolex-$CaCl_2$ mixture.

Antigenic material reacting with heterologous antibody to either human prothrombin or human Factor X were assayed both by radial immunodiffusion and, more quantitatively, by antibody neutralization using the same heterologous antibody. For the latter assay, equal volumes of diluted heat-inactivated antiserum and varying dilutions of test or control plasma were incubated for 2 h at 37°C, additionally incubated at 58°C for 60 min, and then centrifuged at 2,000 *g* for 20 min. The supernatant solutions were then incubated at 37°C with diluted pooled normal plasma for 60 min and assayed for residual prothrombin activity. The percent prothrombin antigen was estimated from a log-log curve of the clotting times obtained after incubation of the antibody with serial dilutions of normal pooled plasma. This curve corresponded to a curve obtained with equivalent dilutions of normal pooled plasma in alumina-adsorbed normal plasma; duplicate test samples varied from each other by ≤ 10 % over a range of prothrombin antigen equivalent to 12.5–100% the amount present in normal pooled plasma.

Radial immunodiffusion and double immunodiffusion were carried out in 1% agarose in barbital-saline buffer, pH 7.4 (22). Crossed immunoelectrophoresis was performed with 1 % agarose in 0.075 M sodium barbital (pH 8.6) containing 2 mM calcium lactate at room temperature (18, 32). Anodal migration in the first dimension was quantified by addition of Evan's blue dye (0.5%, General Diagnostics, Warner Lambert Company, Morris Plains, NJ), 2 μl, to 50 μl plasma before sample application and measurement of the distance from the well center to the leading edge of dye after electrophoresis. The relative migrations of different plasmas in the presence of this marker did not differ from those seen in its absence.

Measurements of (*a*) abnormal (des-γ-carboxy) prothrombin, (*b*) normal prothrombin, and (*c*) total prothrombin in patient and normal plasmas were performed using immunoassays described elsewhere (33).[2] In brief, the antibodies used were directed at (a) a prothrombin species deficient in Gla residues (des-γ-carboxy-prothrombin), (b) a fully carboxylated prothrombin species (normal prothrombin), and (c) the carboxy-terminal antigenic determinants common to both abnormal prothrombin and prothrombin (total prothrombin) (33).[2]

Partial purification of normal and abnormal prothrombin was performed by the method described by Mann (34). 500 ml of normal plasma or 100 ml of patient plasma were adsorbed to barium citrate by addition of 1 M $BaCl_2$ to plasma after adjustment of plasma pH to 8.6. The precipitates were two times dissolved in citrate-saline buffer and reprecipitated with the $BaCl_2$ solution. The precipitate was resuspended in 0.2 M EDTA pH 7.4, extensively dialyzed successively against citrate-saline buffer containing 0.02 M EDTA and citrate-saline buffer, and then reprecipitated between 40% and 60% saturation with neutralized saturated ammonium sulfate at 4°C. This fraction, after treatment with diisopropylphosphofluoridate (1 mM final concentration) and dialysis against 0.025 M sodium citrate buffer (pH 6.0), was applied to DEAE-cellulose (Whatman Inc., Springfield, MO) equilibrated with the same buffer. The column was successively washed with the same buffer, and buffer containing 0.1 M NaCl. Prothrombin was eluted with buffer containing 0.15 M NaCl.

Protein determinations were performed by the method of Lowry et al. (35). Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed with and without prior treatment with β-mercaptoethanol (36, 37).

RESULTS

The index patient and her brother had significant prolongation of both the PT and aPTT (Table I); these abnormalities were corrected by the addition of an equal volume of normal plasma. Individual factor assays showed significant deficiency of Stuart factor (Factor X) and prothrombin and variable, mild deficiency in Christmas factor (Factor IX). Factor VII levels were normal. Assays for fibrin stabilizing factor (Factor XIII) and platelet aggregation in response to ADP, collagen, and ristocetin were normal. The patient's mother and father had normal levels of all vitamin K-dependent factors (Table I).

The titer of immunoreactive prothrombin, as measured by an antibody reactive only with prothrombin among the vitamin K-dependent coagulation factors, exceeded the prothrombin titer obtained in coagulation assays (Table II) in both affected siblings' plasmas. A similar discrepancy between coagulant activity and antigen was found with Factor X. Monospecific antibodies for Factors IX and VII were not available. Both parents possessed near equivalant ratios of coagulant activity to antigen.

Assays using prothrombin activators not dependent upon calcium binding for their activity indicated that the prothrombin present in the affected individuals' plasma possessed an active site for enzymatic activity (Table II). The prothrombin titer measured by staphylocoagulase and by the procoagulant in *E. carinatus* venom was equivalent to immunoreactive prothrombin in the patients' plasmas. In contrast, prothrombin activation by Taipan viper venom and Factor X activation by RVV, both calcium dependent, gave results similar to those obtained when tissue thromboplastin was used.

Additional studies designed to detect differences

[2] Blanchard, R. A., M. Jorgensen, B. C. Furie, G. Waneck, and B. Furie. Quantitation of human abnormal (des-γ-carboxy) prothrombin and prothrombin in plasma with specific radioimmunoassays. Submitted for publication.

TABLE I
Coagulation Studies on Patient A.B. and Family

	A.B.	M.F.	Mother	Father	Normal*
Prothrombin time (*s*)	17.8–20.3	22.1	13.8	14.1	14 (12–16)
aPPT (*s*)	59.5–64.2	65.2	45.2	49.1	48 (34.1–57.3)
Platelet count ($\times 10^9$/liter)	308	205			272 (152–342)
Bleeding time (*min*)	4.5	4.0			4.5 (1.8–7.2)
Inhibitor assay	neg.	neg.			neg.
Fibrinogen (*mg/ml*)	3.06	2.92	3.82		2.72 (1.52–3.92)
Prothrombin (*% nl*)	20	18	100	140	100 (65–150)
Factor X (*% nl*)	20–22	18	98	90	100 (65–185)
Factor IX (*% nl*)	48–71	56	92	162	100 (50–180)
Factor VII (*% nl*)	74–117	88	128	135	100 (55–185)
Factor V (*% nl*)	83–92	62	86	80	100 (45–165)
Factor VIII (*% nl*)	95		82		100 (50–250)
Factor XII (*% nl*)	179		154		100 (50–220)

* Mean±2 SD.

between calcium binding sites in patient and normal prothrombin were performed. Treatment of both normal and patient citated plasma with 1/10 vol 1 M barium chloride removed ≥90% of immunoreactive prothrombin. In contrast, such treatment of plasma from a patient receiving warfarin, which possessed 27% prothrombin activity, similar to that of patient plasma, removed only 60% of immunoreactive plasma prothrombin.

Patient, normal, and a warfarin-treated patient's plasma were subjected to crossed immunoelectrophoresis with antiprothrombin antiserum in the presence of 2 mM calcium lactate. The patient's plasma regularly formed a single symmetrical peak located anodal with respect to that of normal plasma and cathodal with respect to the second, anodal peak observed with plasma from a patient treated with warfarin (Fig. 1). This result was obtained in seven different electrophoreses using varying conditions of buffer, ionic strength, voltage, and duration of electrophoresis. The anodal "shoulder" seen with the normal plasma sample was a constant finding with both pooled and single donor normal plasmas electrophoresed under the conditions described. To supplement visual inspection of

TABLE II
*Prothrombin and Factor X. Coagulant and Antigenic Assays**

Assay	A.B. % N	M.F. % N	A.B. after vitamin K_1 % N	Mother % N	Father % N
Prothrombin (coagulant)‡	20	18	90–100	100	140
Prothrombin by					
Staphylocoagulase‡	78				
E. carinatus‡	85				
Taipan viper venom‡	32	35	86		
Antigenic prothrombin§	80–90	84	80–94	124	114
Factor X (coagulant)‡	20	18	76		
Factor X by RVV‡	12	13	60		
Antigenic Factor X§	85–91	93			

* Data represent percent nanoliters.
‡ Represent means of duplicate determinations at two different dilutions.
§ Antibody neutralization assays.
N, normal value.

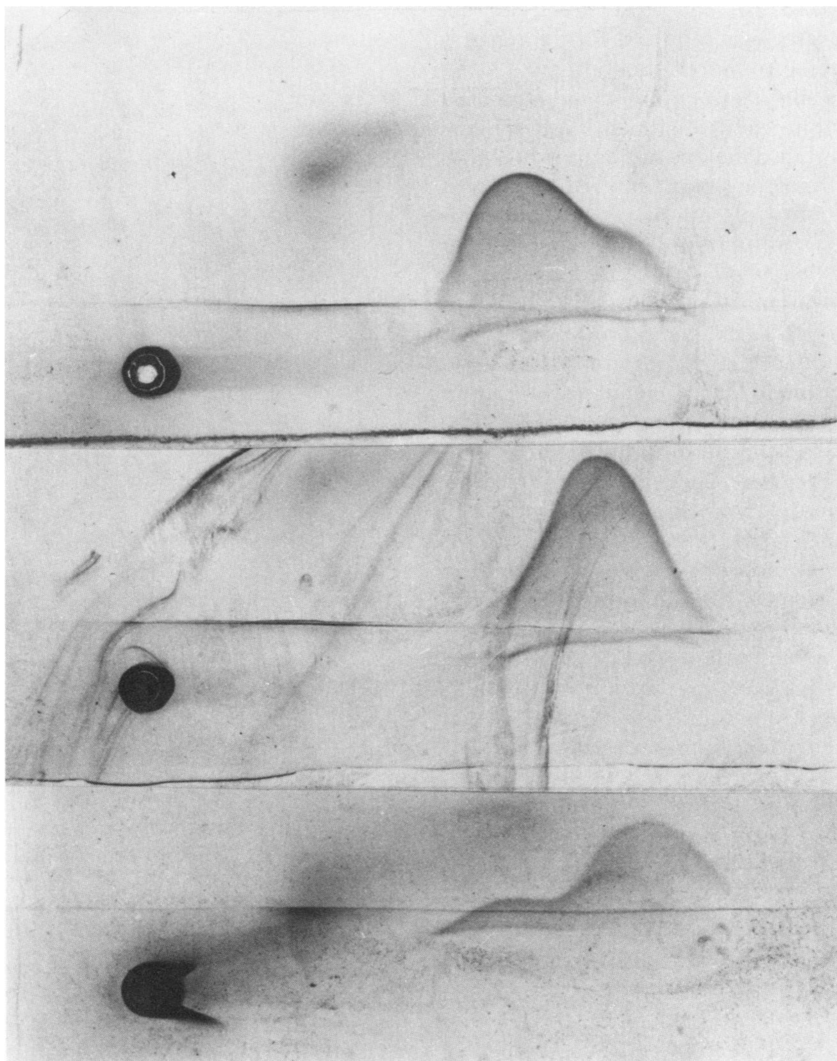

FIGURE 1 Crossed immunoelectrophoresis. Top, normal plasma; Middle, A.B. plasma; Bottom, plasma from patient on warfarin therapy. The first dimension was run for 6.5 h at 20 V/cm, and the second dimension for 17 h at 20 V/cm. Both dimensions were run in 1% agarose in 0.075 M sodium barbital containing 2 mM calcium lactate, pH 8.6. The second dimension contained specific antiprothrombin antibody.

prothrombin mobility in normal and abnormal plasmas, the observed mobility in the first dimension was compared with the mobility, relative to normal plasma, expected by the ratio: albumin migration (normal plasma)/albumin migration (abnormal plasma) = prothrombin migration (normal)/prothrombin migration (abnormal).

Under the electrophoresis conditions used, the prothrombin mobility in patient plasma was 6–7 mm anodal to that predicted for normal prothrombin by this formula. Prothrombin from patients receiving warfarin separated into two apparent populations migrating 0–2 mm and 9–11 mm anodal to the mobility of normal prothrombin. The height and staining intensity of the more anodal peak was roughly proportional to the degree of prolongation of the prothrombin time. The warfarin-treatment plasma illustrated (Fig. 1) is that of a patient with approximately the same plasma prothrombin activity (27%) as that observed in A.B. and M.F. Patient plasma, obtained after treatment with oral vitamin K_1 for 2 wk, formed a single peak with mobility identical to that of normal plasma.

As noted previously (18, 19), the presence of calcium in electrophoresis buffer was required for demonstration of differential electrophoretic mobility.

The patients' plasma also contained an increased amount of prothrombin reactive with antibody specific for human prothrombin deficient in Gla residues (33) (Table III). In addition, a significant reduction was observed in the patients' plasma of prothrombin immunoreactive in a radioimmunoassay specific for prothrombin containing a full complement of Gla residues. Their father's plasma also contained a small, but significant amount of Gla-deficient prothrombin antigen. Appreciable abnormal prothrombin remained in patient A.B.'s plasma after treatment with vitamin K_1 had resulted in normal prothrombin activity. The assay of abnormal prothrombin uses antiabnormal prothrombin antibodies that likely vary in their affinity for different abnormal prothrombin species (33). Accordingly, the measured level of abnormal prothrombin must be considered a lower limit of the amount of abnormal prothrombin present in A.B.'s plasma.

Partial purification of patient M.F.'s prothrombin was achieved by $BaCl_2$ adsorption, elution with EDTA, and anion-exchange chromatography identical to that used for isolation of normal human prothrombin (34)

TABLE III
*Radioimmunoassay of Normal and Abnormal Prothrombin**

	Prothrombin		
	Abnormal‡	Normal§	Total‖
		μg/ml	
A.B.	26±1.5	34±3	112±14
M.F.	23±4.2	34±3	94±12
A.B. after vit. K_1	1.2±0.5	94±3	140±27
Mother	0.0	164±17	158±10
Father	0.3	155±19	207±31
Normal controls			
a.	0.0	110±18	121±6
b.	0.0	157±34	170±11
c.	0.0	163±33	129±11

* Data represents mean±SD.

‡ Abnormal prothrombin, quantitated with anti-abnormal prothrombin specific antibody, includes acarboxy- and descarboxy-forms of prothrombin.

§ Normal prothrombin, quantitated with antibodies specific for the calcium-stabilized prothrombin conformer, is the fully carboxylated prothrombin species.

‖ Total prothrombin, quantitated using antiprethrombin I antibodies, is specific for the COOH-terminal domain, and includes all prothrombin species.

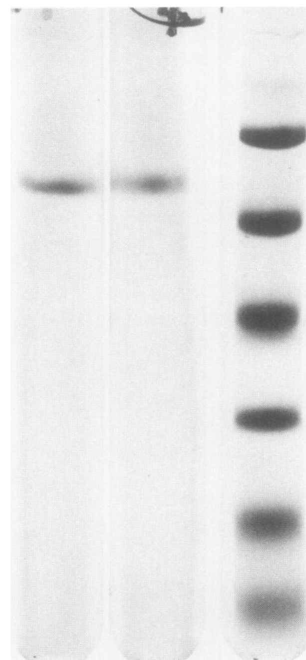

FIGURE 2 SDS-PAGE of normal and patient M.F.'s prothrombin. Left, normal prothrombin without, and with prior treatment with β-mercaptoethanol and markers. Right, patient without and with β-mercaptoethanol and markers as for normal prothrombin previously. In both sets the top two marker bands are 94,000 and 67,000 daltons, respectively. Normal prothrombin contained 9.5 units antigenic prothrombin/mg, 7.2 units prothrombin activity/milligram with activity/antigen ratio 0.76. Patient prothrombin contained 8.8 units antigenic prothrombin/milligram, 2.4 units prothrombin activity/mg with activity/antigen ratio 0.27.

(Fig. 2). Pooled fractions of both normal and the patient's prothrombin showed a single band of ~80,000 mol wt on SDS-PAGE both in the presence and absence of β-mercaptoethanol. While the prothrombin antigen content of the two preparations corresponded well, the ratio of activity to antigen showed the same discrepancy found in studies of whole plasma (Table II).

DISCUSSION

The presence of multiple abnormalities of vitamin K-dependent coagulation factors in siblings, coupled with the history of correction of the prothrombin time of the propositus by parenteral vitamin K_1, suggested the presence of an abnormality in vitamin-K dependent posttranslational modification of these proteins. Determination of the ratios of activity to antigen for the affected coagulation factors and the demonstration of normal thrombin generation in the patients' plasmas after treatment with nonphysiologic activators that do

not require the presence of calcium for prothrombin activation offered additional support for the hypothesis that the plasmas in this kindred contained a prothrombin species similar to that which results from vitamin K deficiency states or treatment with vitamin K antagonists (8, 10, 11). Specific radioimmunoassay for normal and des-α-carboxy prothrombin also indicated the presence in the patients' plasmas of prothrombin deficient in Gla residues.

The long history of a bleeding disorder in the proband, the detection of a prolonged prothrombin time when no medications were being taken, the presence of the same abnormality in her sibling, and the absence of detectable warfarin in their plasmas appears to rule out the presence of an exogenous inhibitor of normal prothrombin synthesis. Careful evaluation excluded malabsorptive or maldigestive disorders. These studies, then, suggest that the affected siblings had either a selective defect in vitamin K absorption or a hepatic abnormality in vitamin K metabolism. Since the titer of Factor VII was normal, and that of Factor IX only marginally decreased, a selective defect in the absorption of vitamin K from the gut or impaired transport of vitamin K into the hepatocyte seems unlikely. Impaired utilization of the vitamin within these cells better explains the data obtained.

Crossed immunoelectrophoresis in a calcium-containing buffer suggested the presence in the patients' plasma of prothrombin molecules exhibiting a larger net negative charge than normal prothrombin when saturated with calcium ions. The crossed-immunoelectrophoretic pattern of this prothrombin was distinct from that seen with the heterogeneous prothrombin species found in plasma of patients treated with warfarin. The patients' single symmetrical peak also differed from the patterns obtained in studies of similar cases (18, 19). Consistent with these results, patient M.F.'s prothrombin, partially purified from plasma by techniques identical to those used for partial purification of normal prothrombin, retained the excess of antigenic material to procoagulant activity found in studies of whole plasma.

This kindred appears to have an abnormality of vitamin K-dependent coagulation factors distinct from those previously described, both clinically and biochemically. The patient reported by McMillan et al. (17) and subsequently further evaluated by Chung et al. (18), had a severe (<1% activity) deficiency of all evaluated vitamin K-dependent factors; the patient reported by Johnson et al. (19) had mild combined deficiencies of vitamin-K dependent factors analogous to the levels found in our family of patients. Both of these patients had prothrombin with sufficiently variable electrophoretic mobility to produce two peaks on crossed immunoelectrophoresis. Also in contrast to our kindred, only partial (18) and no (19) correction of coagulation factor deficiencies occurred after treatment with vitamin K_1.

Our data are most consistent with a defect in the posttranslational vitamin K-dependent formation of Gla residues that resulted in a relatively homogeneous population of dysfunctional prothrombin, as compared with the heterogeneously carboxylated prothrombins that characterize the plasma of patients receiving prolonged oral anticoagulant therapy. Pending direct analysis of the other affected coagulation factors, it seems likely that this hereditary defect is responsible for an identical abnormality of posttranslational carboxylation. Normal functional levels of the involved factors in both parents with equally affected siblings, of both sexes, suggests an autosomal recessive inheritance pattern of expression for this defect.

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REFERENCERS

1. Stenflo, J., P. Fernlund, W. Egan, and P. Roepstorff. 1974. Vitamin K-dependent modification of glutamic acid residues in prothrombin. *Proc. Natl. Acad. Sci. U. S. A.* **71:** 2730–2733.
2. Nelsestuen, G. L., T. H. Zytkovicz, and J. B. Howard. 1974. The mode of action of vitamin K: identification of γ-carboxyglutamic acid as a component of prothrombin. *J. Biol. Chem.* **249:** 6347–6350.
3. Magnusson, S., L. Sottrup-Jensen, T. E. Petersen, H. R. Morris, and A. Dell. 1974. Primary structure of the vitamin K dependent part of prothrombin. *FEBS. (Fed. Eur. Biochem. Soc.) Lett.* **44:** 189–193.
4. Shah, B. V., and J. W. Suttie. 1974. The vitamin K-dependent *in vitro* synthesis of prothrombin. *Biochem. Biophys. Res. Commun.* **60:** 1397–1402.
5. Esmon, C. T., J. A. Sadowski, and J. W. Suttie. 1975. A new carboxylation reaction: the vitamin K-dependent incorporation of $H^{14}CO_3$-into prothrombin. *J. Biol. Chem.* **250:** 4744–4748.
6. Sperling, R., B. C. Furie, M. Blumenstein, B. Keyt, and B. Furie. 1978. Metal binding properties of γ-carboxyglutamic acid. Implications for the vitamin K-dependent blood coagulation proteins. *J. Biol. Chem.* **253:** 3898–3906.
7. Furie, B. C., M. Blumenstein, and B. Furie. 1979. Metal binding sites of a γ-carboxyglutamic acid-rich fragment of bovine prothrombin. *J. Biol. Chem.* **254:** 12521–12530.
8. Esmon, C. T., J. W. Suttie, and C. M. Jackson. 1975. The

functional significance of Vitamin K action: difference in phospholipid binding between unusual and abnormal prothrombin. *J. Biol. Chem.* **250:** 4095–4099.

9. Nelsestuen, G. L., and T. K. Lim. 1977. Equilibria involved in prothrombin—and blood-clotting factor X-membrane binding. *Biochemistry.* **16:** 4164–4170.
10. Olsen, R. E., and J. W. Suttie. 1978. Vitamin K and γ-carboxyglutamate biosynthesis. *Vitam. Horm.* **35:** 59–108.
11. Gallop, P. M., J. B. Lian, and P. V. Hauschka. 1980. Carboxylated calcium-binding proteins and vitamin K. *N. Engl. J. Med.* **302:** 1460–1466.
12. Ratnoff, O. D. 1978. Hereditary disorders of hemostasis. *In* The Metabolic Basis of Inherited Disease. J. B. Stanbury, J. B. Wyngaarden, and D. S. Frederickson, editors. 4th edition. McGraw-Hill Book Co., Inc., New York. 1755–1791.
13. Kroll, A. J., B. Alexander, F. Cochias, and L. Pechet. 1964. Hereditary deficiencies of clotting factors VII and X associated with carotid-body tumors. *N. Engl. J. Med.* **270:** 6–13.
14. Pool, J. G., R. Desai, and M. Kropatkin. 1962. Severe congenital hypoprothrombinemia in a Negro boy. *Thromb. Diath. Haemorrh.* **8:** 235–240.
15. Girolami, A. 1971. The hereditary transmission of congenital true hypoprothrombinemia. *Br. J. Haematol.* **21:** 695–703.
16. Hall, C. A., A. R. London, A. C. Moyinhan, and W. J. Dodds. 1975. Hereditary factor VII and IX differences in a large kindred. *Br. J. Haematol.* **29:** 319–328.
17. McMillan, C. W., and H. R. Roberts. 1966. Congenital combined deficiency of coagulation factors II, VII, IX, and X. *N. Engl. J. Med.* **274:** 1313–1315.
18. Chung, K. S., A. Berzeaud, J. C. Goldsmith, C. W. McMillan, D. Ménaché, and H. R. Roberts. 1979. Congenital deficiency of blood clotting factors II, VII, IX, and X. *Blood.* **53:** 776–787.
19. Johnson, C. A., K. S. Chung, K. M. McGrath, P. E. Bean, and H. R. Roberts. 1980. Characterization of a variant prothrombin in a patient congenitally deficient in factors II, VII, IX, and X. *Br. J. Haematol.* **44:** 461–469.
20. Fischer, M., and E. Zweymüller. 1966. Kongenitaler Kombiniester Mangel der Faktoren II, VII, and X. *Z. Kinderheilkd.* **95:** 309–323.
21. Forman, W. B., and J. Schlaes. 1978. Comparison of high-performance liquid chromatography and a spectrophotometric technique for determining plasma warfarin. *J. Chromatogr. (Amsterdam).* **146:** 522–526.
22. Saito, H., O. D. Ratnoff, J. S. Marshall, and J. Pensky. 1973. Partial purification of plasma thromboplastin antecedent (Factor XI) and its activation by trypsin. *J. Clin. Invest.* **52:** 850–861.
23. Saito, H., O. D. Ratnoff, and V. H. Donaldson. 1974. Defective activation of clotting, fibrinolysis, and permeability enhancing systems in human Fletcher trait plasma. *Circ. Res.* **34:** 641–651.
24. Saito, H., G. H. Goldsmith, and O. D. Ratnoff. 1974. Fletcher factor activity in plasma of various species. *Proc. Soc. Exp. Biol. Med.* **147:** 519–523.
25. Donaldson, V. H., H. Saito, and O. D. Ratnoff. 1974. Defective esterase and kinin-forming activity in human Fletcher trait plasma: a fraction rich in kallikrein-like activity. *Circ. Res.* **34:** 652–662.
26. Poon, M-C., and O. D. Ratnoff. 1977. Immunologic evidence that the Factor VIII-like material in hemophilic plasma possesses a nonfunctional low molecular weight subcomponent. *Blood.* **50:** 367–376.
27. Alexander, B. 1955. One-stage method for specific prothrombin determination. *In* The Coagulation of Blood. Methods of Study. L. M. Tocantins, editor. Grune & Stratton, Inc., New York. 94–97.
28. Crum, E. D., J. R. Shainoff, R. C. Graham, and O. D. Ratnoff. 1974. Fibrinogen Cleveland II: an abnormal fibrinogen with defective release of fibrinopeptide A. *J. Clin. Invest.* **53:** 1308–1319.
29. Soulier, J. P., et O. Prou-Wartelle. 1966. Etude comparative des taux de confacteur de la staphylocoagulase (CRF) et des taux de facteur II (prothrombin) dans diverses conditions. *Nouv. Rev. Fr. Hematol.* **6:** 623–631.
30. Denson, K. W. E., R. Barrett, and R. Biggs. 1971. The specific array of prothrombin using the Taipan snake venom. *Br. J. Haematol.* **21:** 219–226.
31. Denson, K. W. E. 1961. The specific assay of Prower-Stuart factor and Factor VII. *Acta Haematol. (Basel).* **25:** 105–120.
32. Weeke, B. 1973. Crossed immunoelectrophoresis. *Scand. J. Immunol.* **2**(Suppl. 1): 47–56.
33. Blanchard, R. A., B. C. Furie, M. Jorgenson, S. F. Kruger, and B. Furie 1981. Acquired vitamin K-dependent carboxylation deficiency in liver disease. *N. Engl. J. Med.* **305:** 242.
34. Mann, K. G. 1976. Prothrombin. *Methods Enzymol.* **60:** 123.
35. Lowry, O. H., N. J. Rosenbough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. *J. Biol. Chem.* **193:** 265.
36. Weber, K., and M. Osborn. 1969. The reliability of molecular weight determination by dodecylsulfate-polyacrylamide gel electrophoresis. *J. Biol. Chem.* **244:** 4406.
37. Saito, H. 1977. Purification of high molecular weight kininogen and the role of this agent in blood coagulation. *J. Clin. Invest.* **60:** 584–594.