Increased Thrombin Generation in a Child With a Combined Factor IX and Protein C Deficiency

By C. Negrier, M. Berruyer, A. Durin, N. Philippe, and M. Dechavanne

We report a quantitative protein C deficiency combined with a factor IX deficiency in a one-year-old boy. The inheritance of the two deficiency states was independent, the factor IX defect coming from the mother and the protein C defect from the father. Both factor IX activity and antigen were below 1%, and protein C activity as well as antigen were close to 27% of normal values. This association raises a real therapeutic and prognostic question. Protein C deficiency is indeed associated with a significant thrombotic risk and some factor IX concentrates seem to carry a potential thrombogenicity, particularly following infusion of repeated doses. We evaluated in this patient the potential activation of the coagulation system by measuring the levels of prothrombin fragment F$_{1+2}$ at the basal state and after a single administration of 20 U/kg of a high purity factor IX concentrate. We found an unexpected basal activation of the hemostatic system before infusion (F$_{1+2}$ = 1.6 nmol/L), which further increased during 8 hours. Despite the clinical predominant expression of the hemophilic trait, our results seem to assess the biologic prevalence of the protein C deficiency. This emphasizes the need for a careful follow-up after infusions of repeated doses of factor IX, as used during a surgical procedure. Furthermore, this raises the question of the prognosis because the risk of thrombotic manifestations associated with a protein C deficiency increases with age. Finally, these results highlight a part of the in vivo activation process of prothrombin in case of failure of the intrinsic pathway of coagulation. The protein C defect seems to be responsible for an upregulation of the prothrombin activation through the extrinsic pathway.

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HUMAN PROTEIN C, which plays an important role in the regulation of the hemostatic system, is a vitamin K-dependent glycoprotein that circulates as an inactive zymogen.1 After activation by thrombin, activated protein C is able to inhibit the conversion of prothrombin to thrombin via factor Xa by inactivating factor Va and factor VIIIa.2,3 Furthermore, activated protein C has a stimulatory effect on fibrinolysis.3 Several reports have described the thrombotic manifestations associated with isolated heterozygous protein C defect.4-6 Moreover, Bauer et al7 recently noted that a significant increase of prothrombin fragment F$_{1+2}$ (F$_{1+2}$) levels could be detected in heterozygous protein C-deficient patients, reflecting an in vivo excessive plasma factor Xa activity. This biochemical abnormality was stable over a prolonged period of time in the nonanticoagulated patients, but its predictive value remains to be determined. Very few reports concerning the association of a protein C deficiency with another defect were published. A combined protein C and factor VII deficiency associated with peripheral pulmonary artery stenosis was reported by Takeuchi et al.8 An association of a protein C defect with either antithrombin III, plasminogen deficiency, or dysfibrinogenaemia was also identified.9-11 Conversely, factor IX deficiency is responsible for hemophilia B, which causes a tendency to severe bleeding, particularly when factor IX level is below 1%. We report on the case of a child with a combined deficiency of protein C and factor IX. To know which abnormality was predominant or whether one defect counterbalanced the other, we administered to this patient an infusion test of 210 U (20 U/kg) of a purified factor IX concentrate. We further evaluated the levels of plasma factor IX and F$_{1+2}$ before infusion and during the following 8 hours. Unexpectedly, we found an augmented pretreatment level of F$_{1+2}$, which further increased after infusion, reflecting an imbalance between the procoagulant and the anticoagulant pathways of hemostasis. These results suggest that, despite the bleeding tendency, the predominant in vivo biologic abnormality regarding the hemostasis balance was the protein C deficiency.

MATERIALS AND METHODS

Study design. This study was conducted in a young child presenting with a combined protein C and factor IX deficiency to assess the hemostasis modifications induced by the infusion of a purified factor IX concentrate. Factor IX concentrate was administered in the hospital, and plasma factor IX and F$_{1+2}$ were serially measured during the 8 hours following infusion.

Patients and controls. The propositus was a 1-year-old boy with a combined factor IX and protein C deficiency. The hemophilic trait was discovered at the age of 7 months after a forehead hematoma that did not require factor IX substitution. Protein C level was also measured because his father and some members of the paternal branch of the pedigree had a protein C deficiency (Fig 1). There was no evidence of consanguinity. The father (II-3) had no history of thrombosis but the paternal grandfather (I-1) had presented recurrent episodes of venous thrombosis and was treated with long-term oral anticoagulant therapy. Patient II-1 had also inherited the same protein C deficiency, and she had experienced several episodes of superficial thromboses, particularly during and after her two pregnancies. The mother (II-4) was asymptomatic as were the other relatives. Informed consent from all these patients was obtained. The parents were informed that the factor IX infusion was performed with the goal of a better future management of the substitution schedules in the child. Twenty units per kilogram (total dose of 210 U of factor IX) were administered, and blood samples were collected before infusion and at different postinfusion time points (30, 60, 120, 240, and 480 minutes). In each relative, one citrated blood sample was collected in the absence of any anticoagulant treatment (except patient I-1). A control group of unrelated hemophilia B patients with factor IX <1% (n =
Hemophilia B carrier: 0.

deficiency: 0.0. Combined Protein C and factor IX deficiency:

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I

II

III

Fig 1. Family pedigree; arrow denotes the proband. Protein C deficiency: 33. 0. Combined Protein C and factor IX deficiency: 33. 0. Hemophilia B carrier: 0.

10, mean age = 17 years, range 2 to 44 years) was also collected in the absence of any treatment. Another control group of healthy volunteers without coumarin derivatives (n = 39, median age = 29 years, range 18 to 51 years) was collected to assess the normal range for the F1+2 assay in our laboratory using the same phlebotomy technique and identical processing steps. Factor IX and F1+2 levels were also measured in a 15-year-old severe hemophilia B patient before and during the 12 hours after administration of 50 U/kg (total dose of 2,500 U) of factor IX administered to better manage a planned abdominal surgery.

Concentrate. The coagulation factor IX concentrate (factor IX; H.P. Biotransfusion, Les Ulis, France) was provided in vials containing 25 U/mL factor IX coagulant activity after reconstitution in 10 mL distilled water. This high purity concentrate was virus-inactivated by a solvent-detergent method, and heparin (5 U/mL) was added to stabilize the product. The concentrate was administered as a 5-minute intravenous infusion.

Collection of plasma samples. Venipunctures were performed with 19-gauge butterfly infusion sets. Blood was drawn into plastic syringes and immediately mixed with the anticoagulant solution or directly collected into siliconized glass vacuum tubes (Vacutainer; Beckton Dickinson, Meylan, France) containing 0.1 volume 3.8% (wt/vol) sodium citrate. Plasma was prepared by centrifugation at 2,000g for 15 minutes at +4°C, and stored in aliquots at −80°C until tested.

Laboratory studies. The assays were performed using commercially available kits. Protein C activity was determined by an amidolytic assay according to the recommendations of the manufacturer (Stachrom protein C; Diagnostica-Stago, Asnières, France). Protein C antigen was assessed by an immunoenzymatic technique (Asserachrom protein C, Diagnostica-Stago). Factor IX coagulant activity was measured by a one-stage method using human factor IX-deficient plasma (Immuno, Vienna, Austria), and factor IX inhibitor assay was performed according to the Bethesda recommendations. Factor IX antigen was determined by enzyme-linked immunosorbent assay (ELISA) using a monospecific antiserum to human factor IX (Asserachrom factor IX, Diagnostica-Stago). The prothrombin fragment F1+2 was determined using a recently developed enzyme immunoassay according to the recommendations of the manufacturer (Behringwerke, Marburg, Germany). Following the sandwich principle, the ELISA was constructed using antibodies against F1+2 for capture and prothrombin, respectively. The capture antibody was a rabbit polyclonal antibody raised against a synthetic peptide containing 14 amino acids located within the carboxyterminal end of F1+2. After incubation, the bound plasma fragment 1+2 was revealed by anti-human prothrombin rabbit antibodies labeled with peroxidase. Calibration of the assay was accomplished with definite concentrations (0.04 to 10 nmol/L of purified F1+2 added to F1+2-depleted plasma). The lowest limit of sensitivity of the assay was 0.02 nmol/L. In addition to the normal range found in the healthy adult population from our laboratory, precision data were established with a normal plasma control provided by the manufacturer and a frozen plasma pool constructed by combining equal volumes of plasma from 25 patients who had undergone extracorporeal circulation for cardiac surgery. The intraassay coefficients of variation (n = 30) were 10.8% and 12.3%, respectively. The interassay coefficients of variation (n = 17) were 13.8% and 14.2%, respectively. All the determinations were performed in triplicate. The values for the F1+2 assay found in each group were: (1) 0.62 ± 0.085 nmol/L (mean ± SD) for the normal plasma control provided by the ELISA manufacturer; (2) 0.74 ± 0.32 nmol/L (mean ± SD, range 0.15-1.5 nmol/L) for the normal values of our laboratory (n = 39); (3) 1.62 ± 0.3 nmol/L (mean ± SD) for the extracorporeal circulation group (n = 25).

RESULTS

Sixteen subjects from the same family were available for the study (Fig 1). Six of them exhibited a protein C deficiency but were asymptomatic at the time of evaluation, and none had sustained any thrombotic episode in the previous 12 months. There was no clinical or laboratory sign of liver or kidney disease. Hemostatic tests, including platelet count, and clotting factors were within the normal range (except for factor IX for III-4 and III-5). Antithrombin III, protein S (except in I-1 who was under coumarin therapy), plasminogen, and heparin cofactor II levels and aminotransferases also proved normal. The results of the protein C assays showed that antigen concentration as well as activity were close to 40% of normal values in the six protein C-deficient patients (Table 1), corresponding to a “classical” or type I deficiency state according to Bertina et al. The pedigree was concordant with a heterozygous state caused by the dominant transmission of the defective allele inherited from I-1.

Both activity and antigen factor IX were below 1% in the propusitus (III-5), reflecting severe hemophilia B (Table 1) without evidence of factor IX inhibitor. His asymptomatic 6-year-old sister (III-4) was probably a hemophilia B carrier, as evidenced by her factor IX activity and her antigen levels being close to 30%. She never experienced potential bleeding challenges (eg, surgeries, injuries, extractions, etc) that would be useful to indicate whether she may be a symptomatic carrier. Thus, the mother (II-4) must be considered as an obligatory carrier despite her normal factor IX values. There was no history of bleeding tendency in her family, and her parents (I-3, I-4), her sister (II-5), and her two first cousins (III-6, III-7) had normal factor IX levels (Table 1). Molecular biology analysis did not detect the defective allele, because the daughter (III-4), the mother (II-4), the aunt (II-5), and the maternal grandmother (I-4) were homozygous for the intragenic and extragenic polymorphisms usually described in hemophilia B (data not shown).

The daughter (III-4) and the proband (III-5) both exhibited the protein C deficiency and the factor IX defect, leading to a combined deficiency. Because of the protein C deficiency, factor IX and F1+2 levels were measured in the proband, be-
fore and after a factor IX bolus dose. Figure 2 shows the plasma levels of factor IX and F₁+₂ after the infusion. As expected, infusion of factor IX concentrate was followed by the attainment of a measurable plasma level of factor IX coagulant activity. Less expected was the finding that the basal F₁+₂ level was slightly higher (1.60 nmol/L) than the normal values established in the healthy adult population (0.74 ± 0.32 nmol/L, mean value ± SD) and much higher than the values found in a hemophilia B population (0.53 ± 0.17 nmol/L, mean value ± SD), as shown in Table 2. Furthermore, at subsequent time points (30, 60, 120, 240, and 480 minutes), F₁+₂ levels clearly increased after the factor IX infusion, showing an abnormal activation of the coagulation system. No clinical adverse reaction occurred during the factor IX administration or in the following 8 hours. On the contrary, as shown in Fig 3, an infusion of 50 U/kg of the same high purity factor IX concentrate to a 15-year-old hemophilia B patient with normal protein C level does not increase the F₁+₂ levels during the subsequent 12 hours.

The untreated relatives of the proband who exhibited the protein C deficiency (ie, his sister III-4 and his father II-3) also had increased F₁+₂ levels (ie, 1.7 nmol/L and 1.8 nmol/L, respectively), showing an abnormal basal activation of the coagulation system caused by the protein C defect. The grandfather (I-1) who presented the same protein C deficiency but was under long-term coumarin therapy had a normal F₁+₂ level (Table 1). F₁+₂ levels were also measured in the other two family members with a protein C deficiency (I-4 and II-3). These values were in the normal range for our laboratory as were those for the members with normal protein C level (I-2, II-2, III-1, and III-3).

Two months later, this hemophilia B child was admitted with a tongue bleeding. He received 30 U/kg of the same high purity factor IX concentrate, which stopped the bleeding. Factor IX and the F₁+₂ levels were measured again, before and during the subsequent 8 hours after factor IX infusion. Exactly the same activation profile of the coagulation system based on the levels of F₁+₂ was found (data not shown).

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### Table 1. Laboratory Values in the Family Members

<table>
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<th>Case No.</th>
<th>Factor IX Activity (%)</th>
<th>Factor IX Antigen (%)</th>
<th>Protein C Activity (%)</th>
<th>Protein C Antigen (%)</th>
<th>F₁+₂ Level (nmol/L)</th>
<th>Coumarin Therapy</th>
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Normal range: 60-140 (nmol/L)

Abbreviation: NM, not measured.
We report, to our knowledge, the first case of a combined protein C and factor IX deficiency in a French family. The propositus, a 1-year-old boy, presented with a bleeding tendency caused by severe hemophilia B (factor IX < 1%). The protein C deficiency allele was inherited from his father and the factor IX defect allele from one of the maternal chromosomes. His sister had exactly the same pathologic association. The thrombotic diathesis in families with inherited protein C deficiency has previously been reported. Bauer et al showed a significant elevation of prothrombin F1+2 levels in nonanticoagulated patients with an isolated protein C deficiency as compared with normal controls because of an excessive production of factor Xa enzymatic activity. These investigators also showed that F1+2 levels were markedly suppressed in patients with protein C deficiency chronically anticoagulated with coumarin derivatives. Our observation in a severe hemophilia B patient confirms these data. Moreover, Nesheim et al showed that factor Xa can protect factor Va from inactivation by activated protein C. We suggest that the basal increased level of F1+2 could be explained by a spontaneous generation of factor Xa via the extrinsic pathway of the coagulation cascade, which further back-activates factor VII and activates factor V. Small amounts of thrombin could be generated, leading to an increase of factor V and factor VIII activation rate because of the protein C defect. Although the samples were not drawn into the anticoagulant mixture proposed by Bauer et al, the fact that the F1+2 level was also found to be elevated in the sister and father argues for the validity of the test. Moreover, in the absence of any treatment, the basal F1+2 level measured after the same phlebotomy technique and identical processing steps was clearly normal in 10 unrelated severe hemophilia B patients (0.53 ± 0.17 nmol/L, mean value ± SD, range 0.15 to 0.97). However, the F1+2 values observed in the other two protein C–deficient patients were within the normal range, suggesting a certain degree of variability in the protein C–deficient patients from the same family. Bauer et al have previously reported the same biochemical profiles in heterozygous protein C–deficient individuals with similar plasma levels of the zymogen.

Many reports have described the thrombotic complications related to some factor IX concentrate administration, particularly when large and repeated doses were used. These complications included superficial and deep venous thromboses, myocardial infarction, and disseminated intravascular coagulation. The mechanisms responsible for the thrombogenicity of these concentrates are still not completely understood. Some hypotheses have been proposed, including high levels of unactivated factors II and X, the presence of activated factors VIIa, IXa, and Xa, or the presence of a coagulant-active phospholipid.

Purer concentrates, such as the one we used, are being developed and are thought to carry a lower thrombogenic risk. These purified factor IX concentrates have been shown to be less thrombogenic in animal models. Recently, Mannucci et al showed that, in hemophilic patients, the prothrombin fragment F1+2 was significantly increased in plasma after infusion of prothrombin complex concentrates but not after high purity factor IX concentrate. These investigators clearly concluded the absence of any activation of the hemostatic system after a single infusion of purified factor IX. In our patient, despite the use of a very high purity factor

![Graph](https://example.com/graph.png)

**Fig 3.** Evolution of plasma factor IX activity (□) and F1+2 (●) levels after a single infusion of 50 U/kg high purity factor IX concentrate in a severe hemophilia B patient.
IX concentrate, we observed a sustained increase in prothrombin fragment F₁⁺² concentrations. This result was in accordance with a prethrombotic state in biochemical terms, particularly 8 hours after infusion. Two hypotheses could account for these results. First, the coagulation cascade could be enhanced by nonfactor IX proteins contained in the factor IX concentrate. The recent report of Mannucci et al. disagrees with this suggestion. Indeed these investigators found no postinfusion increase of factor II, factor VII, factor X, fibrinopeptide A, F₁⁺², or factor X activation peptide after infusion of single large doses, i.e., 50 and 100 U/kg, of the same high purity factor IX concentrate provided by the same manufacturer. Furthermore, F₁⁺² levels were not significantly higher than baseline value at all postinfusion intervals in a severe hemophilia B patient infused at our institution with 50 U/kg of the same purified factor IX concentrate at our institution. Second, factor IX infusion could locally enhance the indirect activation pathway of factor X by the action of tissue factor-factor VIIa complex in conjunction with the direct pathway. This excessive generation of plasma factor Xa activity should be able to increase the cleavage of the prothrombin molecule, accounting for the relatively high levels of F₁⁺² measured after factor IX infusion. The results also point out the delayed elevation in F₁⁺² levels after the administration of factor IX. The mechanisms responsible are not known, but these relatively late modifications were previously reported by Hedner et al. and by Mannucci et al. Two other possible explanations for the postinfusion elevation of F₁⁺² must be discussed. First, trace amounts of factor Xa in the concentrate, coupled with the low protein C level, might account for the further elevations postinfusion, and this would explain why his sister or father, with intermediate or normal factor IX levels, do not have greater F₁⁺² elevations. If this point remains debatable, Burnouf et al. found no measurable factor Xa in the product. Second, regarding the question of the anticoagulant mixture, it remains to be determined whether the observed elevations in F₁⁺² levels result from an in vitro cleavage artifact or reflect a true increase in prothrombin activation in vivo. Bauer et al. indeed observed a slight increase in F₁⁺² levels of samples collected into sodium citrate as compared with those collected with other anticoagulants. They also showed that, in patients with antithrombin III deficiency (but not in those with protein C deficiency), F₁⁺² is artifically high in blood collected in their heparin-containing anticoagulants (i.e., acid-citrate-dextrose, EDTA, adenosine, and heparin). On the other hand, Mannucci et al. using the same commercial ELISA method, recently confirmed the existence of a procoagulant imbalance in some protein C deficiency patients. Furthermore, F₁⁺² levels, measured with the same technique before and within the 12 hours after an infusion of 50 U/kg of the same factor IX concentrate, were not increased in a 15-year-old hemophilia B patient with normal protein C level. Finally, we observed 2 months later in the propositus, using the same measurement method, identical procoagulant imbalance after infusion of 30 U/kg of the same high purity factor IX concentrate.

In summary, elevation of the prothrombin F₁⁺² levels was observed before and after infusion of a single dose of 20 U/kg of a high purity factor IX concentrate to a 1-year-old boy presenting with a combined factor IX and protein C deficiency with a predominant clinical expression of the hemophilic trait. This raises the question of the cause of the bleeding diathesis in such a patient, particularly because of the enhanced basal prothrombin cleavage. It is reasonable to assume that both the intrinsic and the extrinsic pathways of the coagulation cascade are required for a suitable hemostasis during a local bleeding. Furthermore, Osterud et al. and Bauer et al. have shown that at low tissue factor concentrations following vascular injury, the activation of factor IX rather than factor X by the factor VIIa-tissue factor complex is likely to play a significant role in the initiation of the extrinsic pathway of coagulation. In the case of factor IX deficiency, even in the presence of excessive basal factor Xa enzymatic activity, the initiation of the coagulation cascade could not efficiently occur locally.

Though the predictive value of the biochemical modifications is uncertain, these results emphasize the active role of the protein C system in the regulation of the hemostatic balance. Furthermore, because the factor IX doses necessary to prevent postoperative bleeding usually vary from 30 to 50 U/kg and are repeated for several days, our data raise the question of the substitution schedule that will have to be used during future surgical procedures.

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