Congenital Abnormality of the Prothrombin Molecule (Factor II) in Four Siblings: Prothrombin Barcelona

By F. Josso, J. Monasterio de Sanchez, J. M. Lavergne, D. Menache, and J. P. Soulier

A family is described in which four siblings suffer from an unusual hemorrhagic syndrome. In the patient’s plasma, the prothrombin level is only 5% of normal by standard clotting assay and 100% both by staphylococcal assay and immunochemical assay. Results of further investigations in these patients and their family are in favor of a genetic defect of the prothrombin molecule transmitted as a recessive autosomal trait. This abnormal prothrombin is slowly activated by prothrombinase and its electric charge differs from that of normal prothrombin.

Congenital Deficiency in a biologically active protein may arise either from the lack of synthesis of the molecule or from the synthesis of an abnormal molecule, devoid of activity. These mechanisms cannot be distinguished by a quantitative assay resting on biological activity alone.

Plasma prothrombin can now be tested by several methods based on different properties of the molecule: its conversion to thrombin by prothrombinase formed during the physiological mechanism of coagulation; its ability to form with staphylococcal protein a molecular complex endowed with thrombin-like activity; and its antigenic properties allowing an immunochemical assay using specific antibodies.

In a previously reported case of congenital hypoprothrombinemia (J.B.L.), the three types of assay gave identical results, consistent with the hypothesis of a quantitative deficiency.

The present report concerns a Spanish family, four members of which presented with a hemorrhagic syndrome thought to be congenital hypoprothrombinemia on the basis of routine clotting tests. The discrepancies between the results of the various types of prothrombin assay led to the discovery of an abnormal prothrombin molecule.

Case Report

One of us (J.M.S.) has observed in Barcelona a family of eight children (Fig. 1), four of whom from early childhood had a hemorrhagic syndrome clinically resembling mild
hemophilia: easy bruising, hematomas but not hemarthroses, hematuria, and prolonged bleeding after dental extractions. The affected children are two boys (P. and J.) and two girls (M.J. and M.A.). In the eldest sister, the first menses required several blood transfusions. The four other siblings exhibit no hemorrhagic tendency. The parents are clinically normal and unrelated. Routine investigation of the clotting system in the four patients showed an isolated prothrombin deficiency that was not corrected by vitamin K1 administration.

**MATERIALS AND METHODS**

Prothrombin was assayed by various methods, which are described in detail elsewhere. These include specific clotting assay, both by the one-stage Owren method and the Seegers two-stage method, slightly modified by the use of reagents of human origin instead of reagents prepared from animal material (that is, tissue extract, factor V, and fibrinogen); assay by staphylocoagulase; immunochemical assay using specific antihuman prothrombin antibodies, according to the method described by Laurell. This method cannot be applied to the assay of serum residual prothrombin because serum contains inactive prothrombin derivatives reacting with antiprothrombin antibodies.

Factors VII and X were assayed by the one-stage Owren method using congenitally deficient plasmas as substrate reagents. The factor IX assay was also performed by the one-stage method of Soulier and Larrieu in the presence of kaolin.

Adsorption of factors II, VII, IX, and X was performed by the addition of tricalcium phosphate to oxalated plasma at a concentration of 1%; the adsorbed factors were then eluted from the phosphate by 0.18 M sodium citrate.

The possibility of an inhibitory effect of the patients' plasma on the prothrombin assay was investigated as follows: normal plasma was diluted from 1:4 to 1:32 either in patient's plasma, or in Ca$_3$(PO$_4$)$_2$-treated normal plasma; each dilution was tested in the one-stage prothrombin assay system and the clotting time plotted against the reciprocal of the dilution (Fig. 4). As demonstrated by Hemker et al., the regression lines thus obtained intersect to the left of the Y-axis when an inhibitor is present.

Kinetics of the conversion of prothrombin into thrombin in the absence of antithrombin were studied using plasma euglobulin preparations. Euglobulins of normal and one patient's plasma (J.) were precipitated by diluting the plasma 1:20 in distilled water and adjusting the pH to 5.4 by addition of acetic acid. The precipitate was dissolved in 0.02 M citrated buffer. An equal volume of 0.025 M CaCl$_2$ was added to the euglobulin solutions. Thrombin formed in the mixtures was tested at intervals on fibrinogen and compared with the NIH human thrombin standard.

**RESULTS**

**Patients' Study**

(1) Specific one-stage prothrombin clotting assay revealed a very low prothrombin activity (5%), identical in all four patients, whereas factors VII, IX, and X were normal (Table 1). By the two-stage clotting assay, the prothrom-
Table 1.—Family H.V.: Assay of Clotting Factors of Prothrombin Group in Four Clinically Affected Patients

<table>
<thead>
<tr>
<th>Factor II Assay</th>
<th>One-Stage Clotting Assay (Per Cent)</th>
<th>Two-Stage Clotting Assay (Per Cent)</th>
<th>Staphylocoagulase Assay (Per Cent)</th>
<th>Immunochemical Assay (Per Cent)</th>
<th>Factor VII (Per Cent)</th>
<th>Factor IX (Per Cent)</th>
<th>Factor X (Per Cent)</th>
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<tbody>
<tr>
<td>Barcelona patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>J.</td>
<td>5</td>
<td>12</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>P.</td>
<td>4,5</td>
<td>13</td>
<td>80</td>
<td>90</td>
<td>100</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M.A.</td>
<td>4,5</td>
<td>12,5</td>
<td>100</td>
<td>100</td>
<td>75</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>M.J.</td>
<td>4</td>
<td>12,5</td>
<td>65</td>
<td>75</td>
<td>125</td>
<td>100</td>
<td>75</td>
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Fig. 2.—Two-stage prothrombin assay. N, control; B, Barcelona patient (J.). The results were exactly similar in the three other patients.

Fig. 3.—Immunochemical prothrombin assay (Laurell's method). From right to left: increasing dilutions of control plasma (T); then the undiluted plasma of the four patients; finally the undiluted plasma of one patient with true hypoprothrombinemia (J.B.L.).
CLOTTING TIME - SECONDS -

Fig. 4.—One-stage prothrombin assay by diluting normal plasma either in Ca$_3$(PO$_4$)$_2$-treated normal plasma (1) or untreated patient plasma (2). The two regression lines intercept the Y-axis at the same point. This indicates the absence of inhibition. (The regression lines break between the dilutions $\frac{1}{4}$ and $\frac{1}{8}$, as the Lineweaver-Burk equation is not valid at high concentrations of the rate-limiting factor.)

In Ca$_3$(PO$_4$)$_2$-treated plasma, the presence of a prothrombin molecule could no longer be demonstrated. In the citrate eluate from the packed Ca$_3$(PO$_4$)$_2$, only traces of prothrombin activity were recovered, as tested by the one-stage clotting assay. However, the amount of prothrombin activity in this eluate, as measured by staphylocoagulase and immunochemical assays, was as high as in an eluate from normal plasma.

(3) Kinetic studies of the conversion of normal plasma prothrombin in the

Fig. 5.—Immunodiffusion of plasma and serum against antihuman prothrombin antibodies (central wells). p, plasma; s, serum; N, normal; J.B.L., patient with quantitative hypoprothrombinemia; P., J., M.J., and M.A., Barcelona patients. In serum M.A. a double line is seen, one presumably corresponding to serum prothrombin derivative, the other to residual prothrombin (incomplete prothrombin consumption).
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Fig. 6.—Plasma immunoelectrophoresis in agarose gel against antihuman prothrombin antibodies (Veronal 0.075 M, calcium lactate 0.025 M buffer, pH 9.5). J., patient (homozygote); M, mother (heterozygote); N, control.

presence of the patient's plasma at various concentrations showed that the latter had no inhibitory effect on the reaction (Fig. 4).

(4) Qualitative immunological studies (Ouchterlony) showed an identity reaction between the prothrombin of normal plasma and that of the Barcelona patients (Fig. 5). However, plasma immunoelectrophoresis against antihuman prothrombin antibodies showed that the prothrombin migration was clearly more anodic in the patient's plasma than in the control (Fig. 6).

(5) The patients' prothrombin was apparently unable to generate more than traces of thrombin activity but, nevertheless, was "consumed" during clotting. In the serum, factor II activity, assayed by staphylocoagulase, was much lower than in the original plasma; this activity completely disappeared if plasma was clotted by recalcification in the presence of tissue extract. The double diffusion method, using antihuman prothrombin antibodies, showed the same inactive derivative of prothrombin in the patients' serum as in normal serum (Fig. 5).

(6) When the prothrombin was separated from the plasma antithrombins by precipitation at low ionic strength (euglobulins), it appeared that the patient's prothrombin could, in fact, be converted into thrombin with the same final yield as normal prothrombin. But the rate of this conversion was slow. Whereas normal prothrombin is entirely converted into thrombin after

Fig. 7.—Thrombin generation in euglobulin solution after recalcification. N, control; B, Barcelona patient (J.). This experiment was performed in duplicate in patient J. with near identical results. Other patients could not be investigated because adequate amounts of plasma were lacking.
Table 2.—Family H.V.: Assay of Clotting Factors of Prothrombin Group
in Heterozygote Subjects

<table>
<thead>
<tr>
<th>Patients</th>
<th>Factor II Assay (Per Cent)</th>
<th>Factor VII (Per Cent)</th>
<th>Factor IX (Per Cent)</th>
<th>Factor X (Per Cent)</th>
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<tbody>
<tr>
<td>Father</td>
<td>59</td>
<td>100</td>
<td>108</td>
<td>104</td>
</tr>
<tr>
<td>Mother</td>
<td>58</td>
<td>100</td>
<td>117</td>
<td>180</td>
</tr>
<tr>
<td>E.</td>
<td>57</td>
<td>80</td>
<td>98</td>
<td>100</td>
</tr>
</tbody>
</table>

15 min, conversion of the patient's prothrombin took 6 hr under the same conditions (Fig. 7).

Family Study

We were able to study plasma samples from the patients' parents, the paternal grandfather and three apparently normal siblings (E., A., and M.). The patients' relatives can be divided in two groups. In one group (grandfather, A., and M.) the prothrombin level was normal by all assay methods. In the other group, comprising the parents and one son (E.), the prothrombin level was approximately 60% by the one-stage and two-stage clotting assays and normal by both staphylocoagulase and immunochemical assays (Table 2). We were unable to obtain the picture of a prothrombin heterogeneity by immunoelectrophoresis in these patients. The electrophoretic mobility of prothrombin was intermediate between that of homozygotes and that of normal subjects (Fig. 6).

Discussion

The plasma of four siblings of a Barcelona family contained a protein similar to prothrombin in two respects: antigenicity and ability to generate a thrombin-like activity in the presence of staphylocoagulase. The patients' plasma, however, was unable to generate thrombin in more than minute amounts when standard methods were used. This finding was not due to the presence of an inhibitor of prothrombin activation by prothrombinase. The protein resembling prothrombin was consumed during the clotting process, a fact that indicates that it was a substrate for prothrombinase. Moreover, after clotting, the patients' serum contained a prothrombin derivative indistinguishable by immunodiffusion from that described in normal subjects by Shapiro and Ganrot and Nilén.

Although the molecule seemed to be completely transformed during the clotting process, the yield of thrombin was very low as tested by the prothrombin clotting assays. This observation could be explained by a low rate of the conversion of prothrombin into thrombin by prothrombinase. In this case, thrombin formation would not be detected by the one-stage clotting method, which measures a reaction rate. This does not apply to the two-stage method and, in fact, more prothrombin activity was found in our cases, using this type of assay. However, plasma antithrombins would influence the final results if the thrombin formation were slow enough. To test this possibility,
we have eliminated the interference of plasma antithrombins by measuring the thrombin formation in plasma euglobulins. In this situation, the final thrombin yield was the same in the patient's euglobulins as in the normal but the rate of its formation was 20 times lower. This finding explains the discrepancies between the results of the various types of prothrombin assay.

The patients' prothrombin gave an identity reaction with normal prothrombin by the double immunodiffusion method using antihuman prothrombin antibodies. Nevertheless, plasma immunoelectrophoresis against the same antibodies showed that the electrophoretic migration of prothrombin in the patients' plasma was more anodic than that of normal prothrombin.

The hypothesis that best fits with these data postulates the existence in these patients of an abnormal prothrombin molecule: prothrombin Barcelona. The molecular defect would be responsible for both the low affinity of prothrombin for prothrombinase and its abnormal electrophoretic migration.

The synthesis of an abnormal prothrombin molecule may be induced in normal subjects by vitamin K deficiency, as predicted by Hemker and later demonstrated by Josso et al. and Canrot and Niléhn by immunochemical methods. This abnormal molecule, also called "preprothrombin" by Hemker, differs from normal prothrombin by a slower activation by prothrombinase and a more anodic electrophoretic migration. This latter feature is only observed in the presence of calcium ions, which suggests that the abnormal prothrombin of vitamin K deficiency is unable to bind calcium ion. This is not the case with prothrombin Barcelona, as its electrophoretic mobility is not affected by the presence of calcium ions.

In the Barcelona family, the inheritance of the defect is autosomal recessive. Heterozygotes are clinically normal but the plasma prothrombin level was close to 60% by clotting assays and normal by immunochemical assay. Such findings have been recently observed by Shapiro, Martinez, and Holborn in several members of a single family whom they considered heterozygotes for a congenital dysprothrombinemia: prothrombin Cardeza. These authors found a normal electrophoretic mobility of prothrombin in their patients. In the Barcelona family prothrombin migration is abnormal in heterozygotes. These findings suggest that the molecular defect in the family described by Shapiro et al. differs from that of the Barcelona patients. Further studies will be needed to clarify this point.

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