Circulating Anticoagulant in a Family With Prolonged Bleeding Time and Factor VIII Deficiency

By Maria Diez-Ewald, Eric Chun-Yet Lian, Roger Nunez, Daniel Deykin, and Donald R. Harkness

A circulating anticoagulant against factor VIII activity was demonstrated in the plasma of a boy from a family with both factor VIII deficiency and prolonged bleeding time. However, the factor VIII-related antigen, ristocetin-induced platelet aggregation activity, platelet retention in glass bead columns, platelet aggregation with adenosine 5'-diphosphate, collagen and epinephrine, and clot retraction among affected members were normal. The electrophoretic mobility of factor VIII-related antigen on crossed immunoelectrophoresis was normal. The inactivation of factor VIII activity by the inhibitor was time dependent and was nonlinear as the concentration of the inhibitor was increased. Immunotyping showed that the inhibitor was IgG with κ light chains.

Von Willebrand disease is an autosomally inherited hemorrhagic disorder generally diagnosed by the simultaneous presence of a prolonged bleeding time and a deficiency of factor VIII (antihemophilic factor, AHF) coagulant activity. With recent advances, it has been further demonstrated that the platelet retention in a glass bead column, factor VIII-related antigen, and ristocetin-induced platelet aggregation activity are decreased in patients with classical von Willebrand disease. Recently, Holmberg and Nilsson have described a group of patients with both prolonged bleeding times and low factor VIII activities who differed from those with classical von Willebrand disease in that the disease was sex-linked, factor VIII-related antigen was quantitatively normal, and there was normal or only slightly decreased platelet retention in glass bead columns. However, the existence of this as a separate disease entity has been questioned.

Circulating anticoagulants have been reported rarely in patients with hereditary prolonged bleeding time and low factor VIII activity. To our knowledge, factor VIII anticoagulant in such patients has never been characterized. Investigation of the properties of such an inhibitor may provide additional insight into the pathogenesis of factor VIII inhibitors.

In this communication we describe the heretofore unreported occurrence of a circulating anticoagulant in a family affected with a bleeding disorder similar to that reported by Holmberg and Nilsson. The kinetic behavior and immunologic properties of the inhibitor are described.
MATERIALS AND METHODS

Laboratory Procedures

Blood was withdrawn by using a double plastic syringe technique and was collected in plastic tubes containing 3.8% sodium citrate (9 parts of blood to 1 part of citrate). Platelet-poor plasma was obtained after centrifugation of blood at 2400 g for 20 min at 4°C. Platelet-rich plasma was prepared by centrifugation of blood at 183 g for 10 min at room temperature. Aliquots of platelet-poor plasma were stored at −80°C in plastic tubes for the study of factor VIII-related antigen, ristocetin-induced platelet aggregation activity, and inhibitor properties. Studies using platelet-rich plasma were carried out within 2 hr of blood collection.

The prothrombin time and activated partial thromboplastin time were determined in a fibrometer using rabbit brain thromboplastin (Hyland, Costa Mesa, Calif.) and Thrombofax (Ortho Diagnostics, Raritan, N.J.), respectively.7 Factor VIII, IX, XI, and XII coagulant activities were assayed by kaolin-activated partial thromboplastin time using congenitally deficient plasma.9

Bleeding times were performed by the modified Ivy techniques using a Mielke template.10 Platelet retention in glass bead columns was measured by methods described by Salzman2 and Bowie and Owen.11 Platelet counts were performed in a Model 2B1 Coulter counter according to the method of Bull et al.12 Clot retraction was performed by the method of Budtz-Olsen.13 Platelet aggregation was performed in a platelet aggregometer (Chrono-Log Corp., Broomall, Pa.) using a 609 nm red filter. After addition of each aggregating agent to 0.5 ml stirred platelet-rich plasma at 37°C, the change in optical density resulting from platelet aggregation was recorded. The final concentrations of the aggregating agents added to the platelet-rich plasma were as follows: epinephrine (Sigma Chemical Co., St. Louis, Mo.), 3 x 10⁻⁶ M; adenosine 5'-diphosphate (ADP, Sigma), 3 x 10⁻⁶ M; collagen (Sigma) approximately 6 g/ml; and ristocetin (Abbott Laboratories, North Chicago, Ill.), 1.2 mg/ml.

Quantitative ristocetin-induced platelet aggregation activity (ristocetin cofactor activity, or so-called von Willebrand factor) assay in the plasma was modified from the methods described by Weiss et al.14 About 20-45 ml of normal platelet-rich plasma was placed in a 50-ml conical plastic tube. A volume of 0.8 ml of 30% bovine albumin (Dade Reagent, Miami, Fla.) was introduced at the bottom of the tube as a cushion. After centrifugation at 2000 g for 12 min at room temperature, a platelet pellet was formed between the albumin cushion and the plasma. The pellet was removed and resuspended in another conical plastic tube in 40 ml of Tris-saline buffer containing 1 part 0.15 M Tris Cl, pH 7.4, and 2 parts 0.85% NaCl and 1% disodium EDTA. Bovine albumin was again introduced at the bottom of the tube and the tube centrifuged as described above. The washing procedure was repeated four times using the Tris-saline EDTA buffer and twice with the same buffer but without EDTA. The washed platelets were adjusted to a final concentration of 187,000/cu mm with Tris-saline buffer. To a cuvette containing 0.4 ml of washed platelets and 0.1 ml of undiluted or diluted test plasmas, ristocetin was added to make a final concentration of 1.2 mg/ml, and the slope of platelet aggregation was recorded in a platelet aggregometer. The ristocetin-induced platelet aggregation activity of a test plasma was obtained by reading its slope against the normal curve constructed with the slopes from serially diluted normal pooled plasma samples on double-log paper.

Factor VIII-related antigen was measured directly in the plasma by Laurell’s immunoelectrophoretic technique,15 described elsewhere, using monospecific rabbit anti-human factor VIII antiserum prepared in our laboratory.16

Immunodiffusion was performed in 1% agarose using immunodiffusion cells (Cordis Laboratories, Miami, Fla.). In these cells, the thickness of agarose was 0.6 mm and each well allowed a sample volume of up to 50 μl; the distance between the centers of the wells was 2.5 mm. After the wells had been filled with plasma samples and rabbit antiserum, the cells were kept in humidified chambers for 4 days before they were photographed.

Two-dimensional crossed immunoelectrophoresis was performed as described by Laurell.17 The first electrophoresis was run in 1% agarose with 0.05 M barbital buffer, pH 8.6, at 1.5 mA/cm for 4–6 hr. The second electrophoresis was run at right angles with 1% agarose gel containing antiserum in the same buffer at 1.2 mA/cm for 18 hr.

Factor VIII inhibitor was assayed according to Kasper et al.18
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Antibody typing was performed by using rabbit antisera against human IgG, IgA, and IgM heavy chains, and and light chains (Pentese, Kankakee, Ill., and Meloy Laboratory, Springfield, Va.). Accelerated clotting intermediates which might be present in antisera were removed by adsorption with aluminum hydroxide and heating. A mixture of 1 ml antiserum and 0.1 ml half-diluted Amphojel (Wyeth) was incubated at 37°C for 30 min with mixing every 5 min. The mixture was centrifuged at 10,000 g for 20 min at 4°C and the supernatant was removed and incubated at 60°C for 1 hr. The precipitate formed after heating was discarded after centrifuging at 10,000 g for 20 min at 4°C. The plasma containing inhibitor was mixed with 31 volumes of the pretreated antiserum and incubated at 37°C for 1 hr and then at 4°C for 18 hr. The mixture was then centrifuged at 10,000 g for 20 min at 4°C and the residual titer of factor VIII inhibitor was assayed in the supernatant. At the same time, the supernatant had been shown to form a precipitant line in immunodiffusion against normal plasma, suggestive of excess rabbit antiserum in the mixture.

The patient's IgG fraction was purified from heated serum. After extensive dialysis against 0.01 M phosphate buffer, pH 8.0, the serum was chromatographed on DEAE-cellulose equilibrated with the same buffer. The flow-through fraction, which contained IgG only, had no factor VIII-related antigen or ristocetin cofactor activity.

Characterization of antibody was performed on the plasma sample drawn 3 yr after a single dose of cyclophosphamide. All the hemostatic studies were performed when the patients had been off all medication for at least 2 wk.

**Clinical Features**

The propositus, M.J., was an 8-yr-old white boy who had carried the diagnosis of "hemophilia" since birth when he experienced prolonged bleeding after circumcision. His factor VIII level was found to be less than 1% of normal. After 3 mo of age he developed multiple episodes of soft tissue bleeding following minor trauma, for which he was treated with cryoprecipitate. Factor VIII inhibitor was first discovered at 17 mo of age. Subsequently, he had several episodes of major bleeding into soft tissues and joints which resulted in muscle atrophy and joint deformities. He was treated with a single trial of concurrent administration of cyclophosphamide and factor VIII concentrate without success in controlling his circulating inhibitor.19

The father denied any history of bleeding. He was evaluated several times at Boston Children's Hospital Medical Center and was informed that all tests were normal. His mother bruised easily and had prolonged bleeding from the donor site of a skin graft.

One of his sisters bruised easily. She suffered from frequent epistaxes and several episodes of hemarthrosis in the knees and ankles following minor trauma. Each hemorrhagic episode was successfully controlled by factor VIII concentrate alone. Another sister also bruised easily. Thus far she had not had any surgical procedures.

**Table 1. Hemostatic Studies**

<table>
<thead>
<tr>
<th>Case</th>
<th>Bleeding Time (min)</th>
<th>F. VIII-related Properties</th>
<th>F. VIII Inhibitor (Bethesda units/ml)</th>
<th>Platelets (count/10⁹/liter)</th>
<th>Platelet Adhesiveness (%)</th>
<th>Platelet Aggregation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propositus</td>
<td>16</td>
<td>0 184 134</td>
<td>88</td>
<td>275</td>
<td>94 (B)</td>
<td>N</td>
</tr>
<tr>
<td>Mother</td>
<td>19</td>
<td>40 165 85</td>
<td>&lt;1</td>
<td>116</td>
<td>99 (B)</td>
<td>N</td>
</tr>
<tr>
<td>Sister 1</td>
<td>&gt;20</td>
<td>11 160 70</td>
<td>&lt;1</td>
<td>224</td>
<td>33.5 (S)</td>
<td>N</td>
</tr>
<tr>
<td>Sister 2</td>
<td>—</td>
<td>105 68</td>
<td>&lt;1</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>± 25D</td>
<td>1–8 50–200 50–200 50–200</td>
<td>&lt;1</td>
<td>150–400</td>
<td>70–95 (B)</td>
<td>N</td>
</tr>
</tbody>
</table>

*Ristocetin-induced platelet aggregation activity of the plasma (Ristocetin cofactor activity, "von Willebrand factor").

†B, Bowie method; S, Salzman method.

Platelet aggregation with epinephrine, collagen, ADP, and ristocetin; N, normal.
RESULTS

The results of the coagulation studies on the propositus were as follows: prothrombin time, 12.2 sec (control 12.3 sec); activated partial thromboplastin time, 85.7 sec (control 35 sec); factor VIII activity, < 1%; factor IX, 100%; factor XI, 50%; factor XII, 83%; factor VIII inhibitor, 88 Bethesda units/ml; platelet count, 275,000/cu mm; bleeding time, 16 min; platelet retention (method of Bowie and Owen), clot retraction, and platelet aggregation with epinephrine, collagen, and ADP, were normal. His factor VIII-related antigen was 184% and quantitative ristocetin-induced platelet aggregation activity was 134%.
As shown in Table 1, his mother as well as his sisters had both prolonged bleeding times and decreased factor VIII coagulant activities. However, their factor VIII-related antigen, platelet retention in glass bead columns, ristocetin-induced platelet aggregation activity, and other platelet function studies were entirely normal.

Immunodiffusion revealed that rabbit anti-human factor VIII antiserum formed similar precipitin lines with plasmas from normal and hemophilic subjects and the propositus. On crossed immunoelectrophoresis (Fig. 1), the migration of factor VIII-related antigen of the propositus and his relatives was the same as that of the normal control. There was no precipitin line formed between this inhibitor and normal plasma.

As shown in Fig. 2, the inactivation of factor VIII activity by the inhibitor was a time-dependent first-order reaction. When the amount of inhibitor was raised, factor VIII residual activity was inversely proportional to the antibody concentration in a curvilinear fashion (Fig. 3).

Immunoglobulin typing experiments demonstrated that the inhibitor was neutralized by anti-IgG and anti-κ antisera from either Pentese or Meloy Laboratories (Table 2).

Table 2. Antibody Typing of Factor VIII Inhibitor

<table>
<thead>
<tr>
<th>Incubation Mixture</th>
<th>Time (sec)</th>
<th>Residual Activity (%)</th>
<th>Inhibitor Titer in the Mixture (Bethesda units/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Normal human serum + buffer) + normal plasma</td>
<td>80.5</td>
<td>100</td>
<td>4.6</td>
</tr>
<tr>
<td>(Patient plasma + buffer) + normal plasma</td>
<td>127.8</td>
<td>4.3</td>
<td>4.2</td>
</tr>
<tr>
<td>(Patient plasma + anti IgA) + normal plasma</td>
<td>128.0</td>
<td>4.2</td>
<td>4.7</td>
</tr>
<tr>
<td>(Patient plasma + anti IgG) + normal plasma</td>
<td>83.3</td>
<td>85.4</td>
<td>0.2</td>
</tr>
<tr>
<td>(Patient plasma + anti IgM) + normal plasma</td>
<td>121.5</td>
<td>6.3</td>
<td>4.0</td>
</tr>
<tr>
<td>(Patient plasma + anti κ) + normal plasma</td>
<td>85.0</td>
<td>75.0</td>
<td>0.5</td>
</tr>
<tr>
<td>(Patient plasma + anti λ) + normal plasma</td>
<td>122.4</td>
<td>6.0</td>
<td>4.1</td>
</tr>
</tbody>
</table>

Mixture of 0.005 ml patient plasma and 0.155 ml heat-treated and aluminum hydroxide-adsorbed rabbit antiserum was incubated at 37°C for 1 hr and then at 4°C for 18 hr. Residual inhibitor titer of the mixture was assayed by incubating with equal amounts of normal pooled plasma at 37°C for 2 hr and measuring the factor VIII coagulant activity.
Normal Plasma + 0.00

Normal Plasma + IgG from M. J.

Butter on Ristocetin

0.52

<

B

A

TIME MINUTES

Fig. 4. (A) Absence of inhibition of the patient's antibody versus ristocetin-induced platelet aggregation. (B) In contrast, the rabbit anti-human factor VIII antiserum inhibits ristocetin-induced platelet aggregation. Mixtures of equal volumes of antiserum (or buffer) and normal plasma (or buffer) were preincubated at 37°C for 30 min before being added to washed platelets for testing.

The purified IgG fraction from the propositus possessed only anticoagulant activity and had no inhibitory activity against ristocetin-induced platelet aggregation (Fig. 4).

DISCUSSION

We have reported a family affected with a bleeding disorder similar to that reported by Holmberg and Nilsson.5 The affected members displayed a dual defect with both prolonged bleeding time and factor VIII deficiency, suggesting the presence of von Willebrand disease. However, their factor VIII-related antigen, platelet retention in glass bead columns, and quantitative ristocetin-induced platelet aggregation activity were normal, as they are in hemophilia. Holmberg and Nilsson demonstrated that there was no delayed rise of factor VIII activity after transfusion with factor VIII concentrate in their patients with this disease.

A severe deficiency of factor VIII activity in the affected male (referring to the level prior to development of inhibitor) and a mild deficiency of factor VIII in the affected females are findings compatible with sex-linked inheritance. Thus as pointed out by Holmberg and Nilsson, the disease cannot be classified either as von Willebrand disease or as hemophilia. It is very likely that the disease constitutes a distinct separate disease entity.

Our patients were different than those reported by Chesney et al.20 and Peake et al.21 Their patients had prolonged bleeding times, reduced factor VIII activities, and normal amounts of factor VIII-related antigen. However, the patients reported by Chesney et al. had combined features of factor VIII deficiency and thrombocytopenia, whereas the patients reported by Peake et al. had abnormal ristocetin-induced platelet aggregation activity and fast migra-
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tion of factor VIII-related antigen on two-dimensional crossed immunoelectrophoresis.

Our patient developed a factor VIII inhibitor early in life after he had been treated with cryoprecipitate for more than 1 yr. Similar to most other reports, the inactivation of factor VIII activity was time dependent, and the inhibitor in this case was also an IgG with \( \kappa \) light chains\(^{2,23} \) and did not form a precipitin line with normal plasma.

Recently a circulating inhibitor has been reported in patients with hereditary\(^{24} \) or acquired\(^{25} \) von Willebrand disease. However, that inhibitor was directed toward the ristocetin-induced platelet aggregating activity without anticoagulant effect. In two patients with von Willebrand disease in Silwer's series,\(^{26} \) a circulating anticoagulant was suspected but not studied in detail. Apparently the inhibitor activity of the antibody in our patient was limited to the coagulant activity of factor VIII since the quantitative ristocetin-induced platelet aggregation activity of the patient was normal and the purified IgG fraction from the propositus did not inhibit platelet aggregation induced by ristocetin.

The cause of the prolongation of bleeding time in these patients is not clear. It has been demonstrated by Holmberg and Nilsson\(^5 \) that the bleeding time can be shortened in these patients by infusion of factor VIII concentrate. Apparently platelets are not responsible for this abnormality, since clot retraction, platelet retention, and aggregations with ADP, epinephrine, collagen, and ristocetin are normal and platelet transfusion is not required for cessation of bleeding in these patients. It has been observed that factor VIII-related properties in von Willebrand disease can be raised to normal levels in plasma while the bleeding time remains prolonged.\(^{27,29} \) Transfusion studies have demonstrated that the immunofluorescence for factor VIII-related antigen in the vascular intima remains absent and that the bleeding time is still prolonged even after the factor VIII-related properties have been raised to normal in plasma by exogenous antigen.\(^30 \) It has been suggested\(^{29} \) that factor VIII exerts its effect on primary hemostasis locally in the vessel wall and that lack of exchange between plasma and endothelial factor VIII would explain why the bleeding time remains abnormal after factor VIII properties are normalized in the plasma following infusion of cryoprecipitate. However, the immunofluorescence reaction in the vascular intima of this disease has been shown to be normal.\(^30 \) One can speculate that the factor VIII-related antigen present in the vessel wall of these patients is functionally abnormal in primary hemostasis despite the fact that interaction between factor VIII and platelets as determined by platelet retention in glass beads and ristocetin tests are normal.

REFERENCES

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