A Variant of von Willebrand’s Disease With Abnormal Expression of Factor VIII Procoagulant Activity

By Robert R. Montgomery, William E. Hathaway, Janet Johnson, Linda Jacobson, and Wolfgang Muntean

Reports on variants of von Willebrand’s disease are numerous, but many of these are based on tests that will show marked fluctuations with time and tests that might not be similar in affected family members. This report describes 8 patients with a new variant of von Willebrand’s disease in which there is a normal APTT, slightly reduced one-stage factor VIII:C assay (VIII:C-1), and a drastically reduced two-stage factor VIII:C assay (VIII:C-2). The VIII:C in this variant is more readily adsorbed to Al(OH)₃. This variability in VIII:C assays and excessive adsorption to Al(OH)₃ are corrected by the addition of either hemophilic plasma or hemophilic factor-VIII-related antigen. This variant is stable with restudy on multiple occasions and is inherited in a stable fashion in three generations of one family. The multimeric structure of the VIII:Ag appears normal, although the concentration is moderately reduced. The differences in functional activity, the adsorption to Al(OH)₃, and the differences between functional and antigenic (VIII:C Ag) assays of VIII:C suggest that this is a functional abnormality of type I von Willebrand’s disease.

MATERIALS AND METHODS

Materials

Reagents include aluminum hydroxide (Rehsorptar, Reheis Chemical Company, Phoenix, Ariz.), bovine thrombin (Parke Davis & Co., Detroit, Mich.), benzamidine (Sigma Chemicals, St. Louis, Mo.), Trasylol (FBA Pharmaceuticals, New York, N.Y.), celest (Johns Mansville, Denver, Colo.), cyanogen bromide (Sigma Chemicals), Sepharose 4B-CL (Pharmacia Fine Chemicals, Piscataway, N.J.), 4% agarose (Bio Gel A-15, Bio-Rad Laboratories, Richmond, Calif.), rehydratable agarose gels (Beckman Instruments, Fullerton, Calif.), and agarose (Sea Chem-ME, Marine Colloids, Rockland, Me.). All other reagents were of the best grade available.

Patient Population

All patients were studied in the Pediatric Coagulation Research Laboratory at the University of Colorado School of Medicine in conjunction with their evaluation by the Mountain States Regional Hemophilia Center. The study patients included 8 patients with the variant form of von Willebrand’s disease, 19 patients with von Willebrand’s disease, 11 patients with mild hemophilia (VIII:C, 10–20, U/dl), and 20 normal individuals. The variant von Willebrand’s patients were studied on 3 or more occasions.

Collection of Blood and Preparation of Plasma

Blood was obtained by clean venipuncture with a 2-syringe technique. Nine parts blood were drawn into 1 part anticoagulant (3 parts 0.1M sodium citrate and 2 parts 0.1M citric acid). The whole

Submitted December 28, 1981; accepted March 1, 1982

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R.R.M. is supported by NIH Young Investigator Award HL 21730, Basil O’Connor Research Award 5241 of the National Foundation/March of Dimes, and Grant 80-803 of the American Heart Association.

Blood, Vol. 60, No. 1 (July), 1982
blood was centrifuged immediately at 2500 g (4°C) for 20 min and the plasma removed. This plasma was then re-centrifuged at 34,000 g (4°C) for 30 min and frozen at –70°C or assayed fresh. In order to inhibit proteolysis in some experiments, inhibitors were added with a final concentration of 5 mM benzamidine, 10 U/ml Trasylol, and 10 U/ml heparin.

**Coagulation Assays**

Coagulation tests included a bleeding time (BT), activated partial thromboplastin time (APTT), prothrombin time, and thrombin time. A 1-stage assay for factor VIII procoagulant activity (VIII:C-1) and a 2-stage assay (VIII:C-2) were performed on each sample. VIII:vWF was performed as previously described.

**Immunologic Assays of VIIIIR:Ag, Vlll:C Ag, and vW AgIl**

VIIIIR:Ag was determined by quantitative immunoelectrophoresis. Assays for Vlll:C Ag were performed in Dr. Leon Hoyer’s laboratory at the University of Connecticut School of Medicine, Farmington, Conn. vW AgII was measured by quantitative immunoelectrophoresis as described elsewhere.

**SDS Agarose Gel Electrophoresis**

One-microliter samples of test plasma were electrophoresed through 0.65% agarose gels (Beckman rehydratable agarose gel kits) that were equilibrated with 0.025 M veronal buffer, pH 8.6, containing 0.1% SDS. Test samples were 2% SDS. Electrophoresis was carried out in a Gelman deluxe electrophoresis apparatus by applying 9 mA/gel for 120 min at room temperature. The gel was fixed for 45 min in a solution of 25% isopropanol and 10% acetic acid and then washed for 1 hr in 4 changes of distilled water. The gels were then soaked for 60 min in 1% nonimmune rabbit serum in veronal buffer, followed by soaking for 18 hr in 121I-labeled rabbit anti-VIIIIR:Ag which had been immunopurified on highly purified VIIIIR:Ag that was coupled to cyanogen-bromide-activated Sepharose CL-4B. The immunopurified antibody was labeled with 121I by the chloramine-T method. The gel was then washed in 0.5 M NaCl and 0.1% Trition X-100 for 8 hr, and 0.15 M NaCl with 0.1% Trition X-100 for an additional 19 hr, dried at 60°C, and analyzed further by autoradiography, as described by Ruggeri and Zimmerman. Using our techniques, 8–12 multimeric bands of VIIIIR:Ag were identified.

**Special Coagulation Experiments**

The time course of the activation of variant and von Willebrand’s disease plasmas was evaluated by activated PTT, nonactivated PTT, VIII:C-1, factor IX, and factor XI assays. Thrombin activation of VIII:C-1 was evaluated in normal, variant, and von Willebrand’s disease plasmas. The test plasma was incubated with bovine thrombin (0.1 U/ml) and VIII:C-1 assays performed at various times.

Since VIII:C-1 and VIII:C-2 assays differ in the fact that the VIII:C-2 assay uses an aluminum hydroxide [Al(OH)3] absorption, VIII:C-1 assays were done before and after this absorption to look at the direct effect of the absorption. Since Al(OH)3 only partially adsorbs factor XI, factor XI assays were performed before and after absorption to see if some nonspecific activator was Al(OH)3 adsorbed.

Mixing experiments of variant plasma with either severe hemophilia-A plasma or severe von Willebrand’s disease plasma were performed. VIII:C-1 and VIII:C-2 assays were performed to look at the change in the assays following the addition of normal VIIIIR:Ag (i.e., severe hemophilic plasma).

**RESULTS**

**Clinical and Laboratory Evaluation**

All eight patients had clinical and laboratory evidence of a mild to moderate coagulopathy with a history of mucosal bleeding (epistaxis, menorrhagia, dental bleeding, and bleeding after tonsillectomy and adenoidecotomy) and bruising. By history the affected individuals could be distinguished from their asymptomatic family members. The coagulation tests are summarized in Table 1. Of particular note, all of the individuals have APTT values that are well within the normal range, even though their VIII:C-2 assays are below 15 U/dl. All of these individuals have been studied on three or more occasions. The APTT results were always normal, and there was always a 2–4-fold difference between the VIII:C-1 and VIII:C-2 assays.

Figure 1 shows the factor VIIIIR:Ag multimers on SDS agarose electrophoresis of six of the individuals with variant von Willebrand’s disease. All have normal multimeric size distribution with apparent decreased staining due to their decreased VIIIIR:Ag antigen. Thus, they would be classified as type I variants.

Table 2 shows sequential studies over a 2-yr period on one of these patients. Again, all APTT values were normal and all VIII:C-1 and VIII:C-2 assays showed a marked discrepancy. At this level of VIII:C-1, the APTT is usually prolonged in our laboratory.

Table 3 summarizes the means of the APTT, VIII:C-1, VIII:C-2, and ratios in the 20 normals, 7
VARIANT vWd WITH ABNORMAL VIII:C

longed, but again there is agreement between the two VIII:C assays. Note the marked difference in ratios of the two assays in the variant plasmas. In normals, von Willebrand’s disease, and mild hemophilia-A, there is good agreement, but not in the variant von Willebrand’s disease plasma.

Patients A, B, C, D, E, and F all come from the same family and are depicted in Fig. 2. The maternal grandmother, although she has an elevated VIIIIR:Ag, has a threefold discrepancy between her two assays for VIII:C. Thus, this variant is present in three generations of one family.

Special Coagulation Assays

Table 4 shows the effect that the addition of proteolytic inhibitors to the freshly drawn blood had on the VIII:C-1 assay in five of the individuals. The addition of proteolytic inhibitors did not affect the VIII:C-1 assay.

When the VIII:C-1 assays, APTT, nonactivated PTT, factor IX, and factor XI assays were done with 1–10-min incubation times, no relative differences between the variant, von Willebrand’s, or hemophilic plasmas were identified. Thus, nonspecific activation was not identified.

In order to evaluate the effect of thrombin on the VIII:C-1 assays in normal and variant von Willebrand’s disease plasmas, Fig. 3 shows the effect of 0.1 U/ml of thrombin on the VIII:C-1. Thus, the kinetics for thrombin activation of the VIII:C appears to be similar to normal.

**Effect of A1(OH)₃ on the VIII:C-1 Assay**

Since A1(OH)₃ absorption is one of the major differences between an VIII:C-1 and an VIII:C-2 assay, the VIII:C-1 assay was performed after absorption of the sample with various concentrates of A1(OH)₃ (see Fig. 4). At high concentrations of A1(OH)₃ (20%), almost all of the VIII:C was adsorbed in all of the samples. Figure 4 shows the effect of lower concentrations. The normal and von Willebrand samples were in very close agreement on the percent adsorbed to the A1(OH)₃, but the variant plasmas reached a plateau at 45% VIII:C-1 activity where decreasing A1(OH)₃ was not associated with a change in the VIII:C activity adsorbed to A1(OH)₃. Thus, in variant von Willebrand plasma, A1(OH)₃ adsorbed more VIII:C than normal, and since the concentration of A1(OH)₃ used in the VIII:C-2 assay is 3%, this accounts for the striking differences seen between the VIII:C-1 and VIII:C-2 assays. At those concentrations of A1(OH)₃, no adsorption of VIIIIR:Ag could be demonstrated.
Fig. 2. This figure demonstrates the family tree in a family with this variant of von Willebrand's disease. Although a child with severe von Willebrand's disease is due to a probable homozygous state with her father having type 1 vWd, the variant is present on the maternal side and is represented by the "cross-hatched" designation. Note that there are three generations with this defect.

Table 3. Summary of Coagulation Abnormalities in Similar Coagulopathies

<table>
<thead>
<tr>
<th></th>
<th>n</th>
<th>APTT (sec)</th>
<th>VIII:C-1 (U/dl)</th>
<th>VIII:C-2 (U/dl)</th>
<th>Ratio</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variant vWd</td>
<td>7</td>
<td>44.5</td>
<td>44</td>
<td>11</td>
<td>4.08±0.67</td>
<td>3.10–5.2</td>
</tr>
<tr>
<td>vWd</td>
<td>19</td>
<td>56.0</td>
<td>40</td>
<td>43</td>
<td>1.00±0.21</td>
<td>0.61–1.4</td>
</tr>
<tr>
<td>Hemophilia, mild</td>
<td>11</td>
<td>73.4</td>
<td>15</td>
<td>17</td>
<td>0.98±0.39</td>
<td>0.45–1.6</td>
</tr>
<tr>
<td>Normal</td>
<td>20</td>
<td>43.9</td>
<td>98</td>
<td>98</td>
<td>1.00±0.11</td>
<td>0.84–1.1</td>
</tr>
</tbody>
</table>

Effect of VIIIIR:Ag on the Adsorption of VIII:C to A1(OH)3

In order to test the effect of normal VIIIIR:Ag on the adsorption of VIII:C to A1(OH)3 and the differences between the VIII:C-1 and VIII:C-2 assays, the variant plasma was mixed with severe von Willebrand's disease plasma or severe hemophilic plasma. Table 5 demonstrates that severe von Willebrand's disease plasma (no VIIIIR:Ag) does not change the residual VIII:C after A1(OH)3 adsorption in the VIII:C-2 assay, but severe hemophilic plasma corrects the VIII:C-2 assay to the level expected with the VIII:C-1 assay. This same effect is seen when VIII:C-1 assays are done before and after adsorption with A1(OH)3.

The VIII:C assay is not reduced much after Al(OH)3 adsorption if either severe hemophilic plasma or void volume VIIIIR:Ag from hemophilic plasma is mixed with the vWd variant. On the other hand, variant or severe vWd plasma do not prevent this decrease in VIII:C-1.

VIII:C Ag Assays

VIII:C Ag assays were performed in Dr. Leon Hoyer’s laboratory, and the results are expressed in Table 6. Note that in the 6 individuals tested, the VIII:C Ag levels were 2–4 times higher than the VIII:C-2 assays. In contrast, the VIII:C-1 assays were 24%–98% higher than the VIII:C Ag assays.

Table 4. Effect of Proteolytic Inhibitors on VIII:C-1 Assay

<table>
<thead>
<tr>
<th>Patient</th>
<th>Citrate (U/dl)</th>
<th>Special Anticoagulants* (U/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>B</td>
<td>28</td>
<td>25</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>38</td>
</tr>
<tr>
<td>E</td>
<td>32</td>
<td>25</td>
</tr>
<tr>
<td>F</td>
<td>54</td>
<td>58</td>
</tr>
<tr>
<td>Mean ±1 SD</td>
<td>36.4 ± 10</td>
<td>36.6 ± 13</td>
</tr>
</tbody>
</table>

*Citrate, heparin, benzamidine, and Trasylol.

DISCUSSION

Current understanding of the factor VIII molecular complex is that it is comprised of two separate proteins—one of which is under autosomal control (VIIIIR:Ag) and the other under X chromosome control (VIII:C). Thus, the abnormalities in VIII:C seen in von Willebrand's disease appear to be a result of the decreased or abnormal VIIIIR:Ag associated with that disorder.

Variants of von Willebrand’s disease have mainly been associated with discrepancies between the VIII:C, VIII:vWF, and VIIIIR:Ag assays, although other variations have been reported. These include carbohydrate abnormalities, association with low factor XII (von Willebrand—San Diego), and abnormal mobility on crossed immunoelectrophoresis. Although attempts at the subclassification of von Willebrand’s disease based on VIII:C, VIII:vWF, and VIIIIR:Ag have been made, the subclassifications on these variables alone appear not to be “true” variants. Thus, one individual will have variability in his laboratory testing so that he appears to have different variants when studied at different times, while another study showed siblings and offspring to have different but reproducible variants.

Since variants of a disease should be stable with reference to restudy in the same individual and inherited as a similar variant in family studies, these subclassifications appear to not define a “true” variant. In studies of patients with abnormal mobility of VIIIIR:Ag on crossed immunoelectrophoresis, there appears to be genetic separation of subclasses of
von Willebrand's disease where this abnormality is present on multiple occasions and is seen in several generations of one large family. Thus, we believe a variant should be so classified only if it is stable with respect to restudy on multiple occasions and if it is similarly present in family members. With the classification of von Willebrand's disease into type I and type II von Willebrand's disease used by various laboratories, the variants have been reclassified to the type II classification. Our study describes a variant of von Willebrand's disease with normal VIIIIR:Ag mobility on SDS agarose electrophoresis for VIIIIR:Ag subunits, but in which there appears to be an abnormal expression of the VIII:C activity with an increased susceptibility of the VIII:C activity to Al(OH)₃ adsorption. Since hemophilic VIIIIR:Ag can correct this abnormal adsorption, this is a functional abnormality of type I von Willebrand's disease. Since this abnormality is stable with restudy in time and in family members, it is a "true" variant of von Willebrand's disease.

Studies were undertaken to explore the nature of the abnormal coagulation findings. It appeared not to be caused by a testing artifact or a nonspecific activator of coagulation, since time course studies of the VIII:C, IX, XI, PTT, and APTT showed no kinetic differences between normal, variant, and von Willebrand's plasma. Although thrombin-activated VIII:C might have explained a difference in the two assays due to its adsorption to Al(OH)₃, the kinetics of thrombin activation of variant VIII:C in a one-stage assay were not different. The addition of proteolytic inhibitors that would prevent thrombin activation or other protease activation of factor VIII also failed to decrease the apparent VIII:C activity (see Table 4). Since the abnormality in VIII:C-1 and VIII:C-2 assays was limited to this variant when other mild coagulopathies were studied (Table 3), and since it was corrected with hemophilic VIIIIR:Ag, this variant appears to be due to an abnormal interaction of VIIIIR:Ag with VIII:C. This abnormal interaction appears to cause two significant laboratory abnormalities: (1) a normal APTT with low VIII:C activity, and (2) lack of correlation between the VIII:C-1 and VIII:C-2 assays. Since most laboratories only do a one-stage assay for factor VIII, this abnormality might not be recognized unless the

Table 5. Mixture Experiments

<table>
<thead>
<tr>
<th>1:1 Mixture</th>
<th>VIII:C-1 (U/dl)</th>
<th>VIII:C-2 (U/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variant + variant</td>
<td>36</td>
<td>8</td>
</tr>
<tr>
<td>Variant + severe vWd</td>
<td>18</td>
<td>3</td>
</tr>
<tr>
<td>Variant + severe hemophilia</td>
<td>17</td>
<td>18</td>
</tr>
</tbody>
</table>

Table 6. Comparison of VIII:C and VIII:C:Ag Assays in Variant Plasmas

<table>
<thead>
<tr>
<th>Patient</th>
<th>VIII:C (IU/dl)</th>
<th>VIII:C:Ag (IU/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>vWd</td>
<td>5</td>
<td>3.5</td>
</tr>
<tr>
<td>50% Normal plasma</td>
<td>50</td>
<td>59</td>
</tr>
<tr>
<td>A</td>
<td>35</td>
<td>28</td>
</tr>
<tr>
<td>B</td>
<td>28</td>
<td>22</td>
</tr>
<tr>
<td>C</td>
<td>33</td>
<td>29</td>
</tr>
<tr>
<td>D</td>
<td>28</td>
<td>21</td>
</tr>
<tr>
<td>E</td>
<td>32</td>
<td>20</td>
</tr>
<tr>
<td>G</td>
<td>26</td>
<td>13</td>
</tr>
</tbody>
</table>

A-G       | 30 ± 3.5*     | 7 ± 1.4†       | 22 ± 5.8‡     |

*Mean ± 1 SD.
†Compared to VIII:C-1, p < 0.001.
‡Compared to VIII:C-1, p < 0.01; compared to VIII:C-2, p < 0.001.
VIII:C-1 and APTT assays did not agree. An alternative way to diagnose this variant is with VIII:C-1 assays before and after adsorption with A1(OH)3 (see Fig. 4).

The comparison of the VIII:C assays and the VIII:C Ag assays permit speculation on the defect in this disorder. Since the VIII:C-1 activity is more than the VIII:C Ag activity, this may indicate that the VIII:C that is present in this disorder has more activity per unit antigen, possibly because the VIII:C is less tightly associated and therefore more readily available for activation. This accessibility, however, makes it more susceptible to adsorption with A1(OH)3, thereby accounting for the significant decrease in VIII:C activity with the two-stage assay or the one-stage assay after A1(OH)3 adsorption. This wouldn’t explain the very normal APTT with a slightly low VIII:C. Experiments done in our laboratory suggest that the VIIIIR:Ag may inhibit some of the expression of VIII:C activity.27 Thus, when we assay the variant VIII:C, the tenfold excess of hemophilic VIIIIR:Ag is free to bind with the VIII:C, thereby decreasing its activity from what is apparent in the APTT, yet at the same time protecting the VIII:C adsorption by A1(OH)3 (Table 5). Further studies will be necessary to confirm this hypothesis.

The identification of this variant adds another complexity to the evaluation of patients with von Willebrand’s disease. The finding that VIII:C-1 assays before and after adsorption with A1(OH)3 can substitute for a two-stage assay will permit most laboratories to diagnose this variant without establishing the two-stage tests themselves. Since the VIIIIR:Ag has a normal multimeric distribution, this appears to be a functional abnormality of type I von Willebrand’s disease. Unlike many other forms of von Willebrand’s disease, this variant appears to be relatively stable with restudy at subsequent evaluation, thereby making its diagnosis easier.

ACKNOWLEDGMENT

We wish to thank Susan Clarke for her technical assistance and Wendy Stinebaugh for preparation of the manuscript. We would also like to acknowledge Dr. Leon Hoyer of the University of Connecticut for performing the VIIIIC:Ag determination.

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VARIANT vWD WITH ABNORMAL VIII:C


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