Studies of the Pathophysiology of Acquired von Willebrand’s Disease in Seven Patients With Lymphoproliferative Disorders or Benign Monoclonal Gammapathies

By P.M. Mannucci, R. Lombardi, R. Bader, M.H. Horellou, G. Finazzi, C. Besana, J. Conard, and M. Samama

In seven patients with acquired von Willebrand’s disease (AvWD) associated with lymphoproliferative disorders or benign monoclonal gammapathies, the platelet contents of von Willebrand factor antigen and ristocetin cofactor (vWF:Ag and vWF:RiCof, respectively) were normal. All the multimers of vWF:Ag could be seen in the 1.6% SDS-agarose gel electrophoresis patterns of plasma and platelet lysates. Infusion of 1-deamino-8-arginine vasopressin (DDAVP) augmented plasma levels of vWF:Ag and vWF:RiCof of all patients and corrected prolonged bleeding times (BT). However, compared with patients with congenital vWD type I and comparable degrees of baseline abnormality of the immune system, Three general mechanisms to explain the pathogenesis of AvWD have been proposed. The first implies that antibodies either inactivate the biologic activities of FVIII-vWF, bind to the complex without affecting the active sites, or induce rapid clearance of the complex from the circulation through the formation of immunocomplexes. The second proposes that AvWD is the result of the selective absorption of FVIII-vWF to abnormal lymphocytic clones or malignant cells, leading to low plasma levels. Finally, the third states that there is defective synthesis of FVIII-vWF in cellular compartments (megakaryocytes, endothelial cells) and/or there is defective release into plasma. We decided to apply three new approaches to investigating the problem of the pathogenesis of AvWD in seven patients with AvWD associated with lymphoproliferative disorders or benign monoclonal gammapathies. The first was the measurement of vWF (as vWF:Ag and vWF:RiCof) in platelets, which are easily accessible cells of megakaryocytic origin from which the vWF content of cellular compartments can be evaluated. The second was the assessment of the plasma release and clearance of FVIII-vWF by monitoring the changes of vWF:Ag, vWF:RiCof, and FVIII:C after infusion of 1-deamino-8-d-arginine vasopressin (DDAVP), a drug that probably releases FVIII-vWF from cellular compartments. The third was the electrophoretic analysis of the structure of FVIII-vWF, which consists of a multimeric series of oligomers of a protomer composed of a variable number of identical subunits, in plasma and platelets.

MATERIALS AND METHODS

Patients

The clinical details for the patients are listed in Table 1. The most typical features were the onset of mildly to moderately severe...
mucosal and postoperative bleeding in patients who had no previous history of excessive spontaneous or postoperative bleeding until their underlying diseases became manifest. None of the patients had any pertinent family history.

Five French patients were jointly examined by the two teams in Paris, and samples of plasma obtained before and after DDAVP and platelet lysates were transported in dry ice to Milan for further analysis. Plasma obtained before and after DDAVP and platelet lysates were obtained in Milan from two Italian patients.

**Methods**

The methods for collection of blood and plasma preparation have been published.28 Platelets were washed free of plasma constituents and then lysed according to previously published procedures.29

**Assay methods and standards.** FVIII:C was assayed by a one-stage clotting technique,28 and FVIII:CAg was assayed by a two-site immunoradiometric assay,27 using a homologous nonhemophilic antibody kindly provided by Dr. L. Holmberg (Malmö, Sweden). Concentrations of FVIII:C and FVIII:CAg were expressed in U/dL with reference to the First International Reference Preparation for Factor VIII-Related Activities in Plasma (National Institute for Biological Standards and Controls, London). VWF:Ag was measured by electroimmunoassay (EIA) and immunoradiometric assay (IRMA).26 VWF:RiCof activity was assayed with formalin-fixed platelets.26 These measurements were expressed in U/dL with reference to the International Reference Preparation.

**Inhibitor screening.** Inhibitors of FVIII:C were sought by the Bethesda method, after heating those plasmas that contained measurable FVIII:C at 56 °C for ten minutes. Inhibitors of VWF:Ag and VWF:RiCof were sought by incubating 2 vol of patient plasma with 1 vol of pooled normal plasma. Controls were mixtures of nine different normal plasmas (1 vol) with phosphate-buffered saline containing 3% albumin (2 vol). The first incubation was at 37 °C for one hour, then at 4 °C for an additional 12 hours. Patient and normal plasmas were also incubated, without mixing, under the same conditions. The samples were then centrifuged at 2,500 g for 20 minutes, and aliquots of the supernatants were assayed for VWF:Ag (IRMA) and VWF:RiCof against pooled normal plasma incubated under the same conditions (observed values). Expected values, assuming no inhibitor in the mixtures, were calculated from the values for patient plasma and normal plasma incubated and assayed separately and from their proportions in the mixture. Percentage differences between expected and observed values were also calculated, and the significance of these differences from zero was determined. Percentage differences between patients and controls were also compared.

**Multimeric analysis.** The multimeric composition of FVIII-vWF was analyzed by SDS thin-layer agarose gel electrophoresis, using the discontinuous buffer system of Ruggeri and Zimmerman,25 and differed from their procedure in that electrophoresis was for 18 hours at a constant current of 6 mA/gel (instead of five to six hours and 10 to 12.5 mA/gel) and multimers were identified by exposing the gels to 121I-labeled affinity-purified rabbit (instead of emu) antibodies, prepared as described elsewhere.26 Agarose gel concentrations were 1.6%.

**DDAVP infusion.** 1-Deamino-8-D-arginine vasopressin (DDAVP) was infused IV into patients at a dose of 0.4 μg/kg, with blood samples and bleeding times (BT) (Simplate II, General Diagnostics, Milano, Italy) obtained before and after the infusion according to a published protocol.22 All subjects were aware of the experimental nature of the studies and gave their informed consent, and all experiments were performed in accord with the Declaration of Helsinki.

**Analysis of results.** To eliminate the large between-patient differences in baseline FVIII-vWF measurements, responses after DDAVP were expressed not only as absolute concentrations in U/dL, but also as relative changes over baseline values, taken as 1. Values were transformed logarithmically before testing the significance of differences between groups; they were then evaluated by means of two-way analysis of variance and the Student's t test for paired data. The rate of return of FVIII-vWF measurement to resting values after their maximal DDAVP-induced rise was determined by using the best curve fitted by the method of least squares on the posttreatment values, expressed as percentage of the peak value reached after DDAVP. Half-disappearance times and the corresponding slopes were then calculated for each FVIII-vWF measurement, assuming first-order kinetics.28 Good agreement with a one-compartment model of distribution of the FVIII-vWF mea-

### Table 1. Clinical Details of Seven Patients With Acquired von Willebrand's Disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age* (yr)</th>
<th>Sex</th>
<th>Underlying Disease</th>
<th>Family History</th>
<th>Previous Hemostatic Challenge</th>
<th>Nature of Acquired Bleeding Diathesis</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>45</td>
<td>F</td>
<td>Monoclonal gammopathy, IgG kappa</td>
<td>None</td>
<td>Tonsillectomy; multiple tooth extractions</td>
<td>Recurrent epistaxis requiring transfusion; posttraumatic hematomas</td>
</tr>
<tr>
<td>2</td>
<td>39</td>
<td>M</td>
<td>Monoclonal gammopathy, IgG lambda</td>
<td>None</td>
<td>Multiple tooth extractions</td>
<td>Posttraumatic hematomas and hemarthromas; massive gastrointestinal bleeding; bleeding after hernia repair</td>
</tr>
<tr>
<td>3</td>
<td>57</td>
<td>F</td>
<td>Monoclonal gammopathy, IgG lambda</td>
<td>None</td>
<td>Hysterectomy</td>
<td>Easy bruising; spontaneous and posttraumatic hematomas; epistaxis; menorrhagia</td>
</tr>
<tr>
<td>4</td>
<td>50</td>
<td>F</td>
<td>Myeloma, IgG kappa</td>
<td>None</td>
<td>None</td>
<td>None (fortuitous finding of abnormal hemostasis tests)</td>
</tr>
<tr>
<td>5</td>
<td>48</td>
<td>F</td>
<td>Myeloma, IgA kappa</td>
<td>None</td>
<td>Appendectomy</td>
<td>Easy bruising; gum bleeding; prolonged bleeding after tooth extraction</td>
</tr>
<tr>
<td>6</td>
<td>60</td>
<td>M</td>
<td>Primary macroglobulinemia</td>
<td>None</td>
<td>Orthopedic surgery</td>
<td>Easy bruising; excessive bleeding from superficial cuts</td>
</tr>
<tr>
<td>7</td>
<td>72</td>
<td>M</td>
<td>Chronic lymphocytic leukemia</td>
<td>None</td>
<td>Multiple tooth extractions</td>
<td>Epistaxis</td>
</tr>
</tbody>
</table>

*Age at presentation with bleeding.
measurements was observed. This was also checked by testing the regression curves for linearity. Half-disappearance times were compared by the Wilcoxon matched-pair signed ranks test.

RESULTS

Plasma FVIII-vWF Measurements

Baseline values for these measurements and the BT are given in Table 2. Patients 1, 2, and 3 were the most severely affected patients, with plasma vWF:Ag (EIA method) and vWF:RiCof below the lower limits of detection of the methods (6 U/dL). Very low concentrations of vWF:Ag, however, could be measured by the IRMA method, which gave dose–response curves parallel to those of normal plasma and maximal antibody-binding capacity like that of normal plasma. The BT was prolonged, but less than would have been expected from the severity of the plasma FVIII-vWF deficits. In the remaining four patients (No. 4 to 7), all the plasma FVIII-vWF measurements were less low than in the first three patients. The BT of patients 4 to 6 were normal.

Inhibitors of FVIII-vWF

In AvWD plasma heated to destroy FVIII:C, there was no evidence of inhibitory activity against FVIII:C (data not shown). After prolonged incubation of normal and AvWD plasmas, observed values of residual vWF:Ag and vWF:RiCof did not significantly differ from the values expected from the values of plasmas incubated separately (nor from the values of the control plasmas), indicating that no significant inactivation of vWF:Ag and vWF:RiCof was induced in normal plasma by incubation with AvWD plasma (Table 3).

Platelet FVIII-vWF Measurements

Platelet vWF:Ag and vWF:RiCof concentrations were normal in all patients except No. 5, who had low borderline values (Fig 1). This patient has myeloma and was anemic, and hence, it was difficult to avoid contamination with red cells in washed platelets and lysates. Such contamination is likely to have led to a higher protein content and thus to underestimation of platelet vWF:Ag and vWF:RiCof.

Table 2. Baseline Laboratory Findings of Seven Patients With Acquired von Willebrand's Disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Plasma Factor VIII-von Willebrand Factor-Related Measurements (U/dL)</th>
<th>Bleeding Time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>FVIII:C</td>
<td>FVIII:C Ag</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>4</td>
<td>31</td>
<td>32</td>
</tr>
<tr>
<td>5</td>
<td>19</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>15</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>13</td>
<td>—</td>
</tr>
<tr>
<td>Normal</td>
<td>50-165</td>
<td>47-151</td>
</tr>
</tbody>
</table>

Table 3. Inhibitory Activity Against vWF:Ag and vWF:RiCof in Plasmas of Seven Patients With Acquired von Willebrand's Disease

<table>
<thead>
<tr>
<th>Mixtures of 1 part + 2 parts of</th>
<th>vWF:Ag</th>
<th>vWF:RiCof</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expected</td>
<td>Observed</td>
</tr>
<tr>
<td>Normal + buffer (controls)</td>
<td>33</td>
<td>34*</td>
</tr>
<tr>
<td></td>
<td>(30-37)</td>
<td>(-8-+10)</td>
</tr>
<tr>
<td>Normal + patient 1 plasma</td>
<td>35</td>
<td>32</td>
</tr>
<tr>
<td>Normal + patient 2 plasma</td>
<td>35</td>
<td>30</td>
</tr>
<tr>
<td>Normal + patient 3 plasma</td>
<td>34</td>
<td>30</td>
</tr>
<tr>
<td>Normal + patient 4 plasma</td>
<td>55</td>
<td>57</td>
</tr>
<tr>
<td>Normal + patient 5 plasma</td>
<td>43</td>
<td>51</td>
</tr>
<tr>
<td>Normal + patient 6 plasma</td>
<td>42</td>
<td>38</td>
</tr>
<tr>
<td>Normal + patient 7 plasma</td>
<td>41</td>
<td>45</td>
</tr>
<tr>
<td></td>
<td>39†</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>(35-45)</td>
<td>(32-48)</td>
</tr>
</tbody>
</table>

Observed values are expressed as percentages of pooled normal plasma incubated under the same conditions as the mixtures, set arbitrarily at 100% (medians of three different experiments). Expected values were calculated from the values of normal and patient plasmas incubated and assayed separately. Percentage differences between observed and expected values were also calculated.

*Means and 95% confidence limits (between parentheses) for nine normal subjects. Percentage differences were not significantly different from those for patients.

†Means and 95% confidence limits (between parentheses) for seven AvWD patients. Percentage differences were not significantly different from zero nor from the control values.
ACQUIRED VON WILLEBRAND'S DISEASE

Fig 1. Platelet von Willebrand factor antigen (vWF:Ag) measured with IRMA (squares) and ristocetin cofactor (vWF:RiCof) (triangles) in nine healthy subjects (normals), seven patients with congenital von Willebrand's disease (CvWD, closed symbols), and seven patients with acquired von Willebrand's disease (AvWD, open symbols). No significant difference between groups for each measurement was found with a one-way analysis of variance.

vWF:Ag IRMA (■), vWF:RiCof (△).

FVIII-vWF Multimeric Pattern

All the multimers present in normal plasma could be seen in all patients (Fig 2), with the pattern similar to that for normal plasma and plasma from patients with the “classical” form of congenital vWD (type I). The relative intensities of the bands were decreased in rough proportion to the plasma concentrations of FVIII-vWF measurements. Larger multimers than were present in plasma were seen in the platelet lysates of all the patients tested. A representative example (patient 3) is shown in Fig 2.

DDAVP Studies

In order to evaluate the magnitude and time course of FVIII:C, vWF:Ag, and vWF:RiCof changes in AvWD patients after DDAVP, we have chosen to use as a control group seven patients with congenital vWD (CvWD) type I treated with DDAVP in the same manner (Table 4). The rationale for this choice was that these patients had the following features in common with the AvWD patients: low plasma levels of FVIII:C, vWF:Ag, and vWF:RiCof (Table 4); normal platelet vWF:Ag and vWF:RiCof (Fig 1); and intact multimeric structure in plasma and platelets. Bleeding times, however, were prolonged in all patients. Figure 3 compares the extent and time course of plasma changes in these measurements after DDAVP in the two groups. In both, there was a marked increase of FVIII:C, vWF:Ag, and vWF:RiCof over baseline values. The mean increase of vWF:Ag and vWF:RiCof for AvWD patients, however, was slightly less (P < .05) than for CvWD immediately after infusion. In AvWD, there was also a more rapid return of vWF:Ag and vWF:RiCof to the baseline after the end

Fig 2. Autoradiograph pattern of factor VIII-von Willebrand factor (FVIII-vWF) electrophoresed in 1.6% agarose in the presence of sodium dodecyl sulfate and detected by reaction with 125I-labeled affinity-purified antibody. The arrow indicates the origin of the running gel, and the anode is at the bottom of the gel. (A) From left to right: normal plasma; plasmas from AvWD patients 4, 5, 6, 2, and 3; platelet lysates from a normal subject and AvWD patient 3. (B) From left to right: plasmas from patient 1, normal control, and patient 7.
of the DDAVP infusion, as shown by their mean half-disappearance times reported in Table 5.

After DDAVP, electrophoretic analysis of vWF:Ag multimers showed the same patterns in patients with AvWD and CvWD. There was an increase in plasma concentration of all multimers and an early postinfusion appearance of larger multimers than were present in plasma before DDAVP (Fig 4). However, the staining intensity of all the multimers became weaker earlier in AvWD than in CvWD patients (Fig 4). Bleeding time became much shorter or returned to normal 30 minutes after DDAVP infusion in the CvWD and AvWD patients in whom it had been prolonged before DDAVP (Table 6). However, in AvWD, the BT tended to be longer again four hours after DDAVP, unlike that in patients with CvWD, despite their longer baseline BT.

DISCUSSION

Nine patients with AvWD were previously shown to have qualitative abnormalities of plasma FVIII-vWF, which did not show the more slowly moving forms on crossed immunoelectrophoresis (CIE). Only two of the 11 AvWD patients studied by this technique had shown a normal pattern (both had monoclonal gammopathies). Three of the nine patients with abnormal CIE had lymphoproliferative disorders, and the remaining three had carcinoma of the stomach, chronic myeloid leukemia, and secondary amyloidosis. In one patient, abnormalities were also detected by IRMA, indicating that in this assay, dysfunctional FVIII-vWF does not react with the same antigen–antibody reaction kinetics as normal plasma. The observed abnormalities have usually been attributed to the deficiency of larger FVIII-vWF multimers, selectively removed from plasma by increased catabolism or specific absorption to abnormal cell lines. We have reassessed this problem in seven patients with AvWD associated with lymphoproliferative disorders or benign monoclonal gammapathies by using IRMA and SDS agarose gel electrophoresis, which illustrate more directly than CIE the multimeric structure of FVIII-vWF. In AvWD plasma, the multimeric structure was intact. Moreover, the IRMA of vWF:Ag gave dose–response curves parallel with those of normal plasma and maximal antibody-binding capacities similar to those of normal plasma.

In AvWD platelets, vWF:Ag and vWF:RiCof were normal, as in CvWD platelets, and the multimeric structure was intact, indicating that the synthesis of FVIII-vWF in megakaryocytes is quantitatively and qualitatively normal. However, our studies certainly do not rule out the possibilities that FVIII-vWF abnormalities might occur in some patients with AvWD, because our patients constitute only a small group of those with the syndrome.

Two other possible pathogenetic mechanisms of...
AvWD—defective release of FVIII-vWF from cellular compartments or increased plasma clearance—were evaluated by comparing the effects of DDAVP in AvWD and CvWD patients, on the assumption that this agent should amplify the physiologic mechanisms for releasing FVIII-vWF from cellular compartments and clearing it from plasma. In AvWD, FVIII:C, vWF:Ag, and vWF:RiCof markedly increased soon after the infusion, and larger multimers, possibly released from cellular compartments, appeared transiently in the circulation. This behavior was similar to that of patients with CvWD type I, but differed in that the increase was of lesser magnitude and more short-lived, at least for vWF:Ag and vWF:RiCof. A more rapid plasma clearance of FVIII-vWF could probably explain both the lower peak values and the shorter half-disappearance times of vWF:Ag and vWF:RiCof, even though an additional role of defective release from cells cannot be excluded by our data. Rapid plasma clearance might be due to at least three causes: (1) specific autoantibodies that inactivate FVIII-vWF; (2) specific autoantibodies that bind FVIII-vWF but are not directed against the so-called FVIII-vWF active sites; and (3) nonspecific antibodies that form circulating immunocomplexes with FVIII-vWF and favor its clearance by Fc-bearing cells of the reticuloendothelial system.12,13 Unlike some previous studies,2–15,30 but in agreement with many others,12–19,29,31–33 our patients’ plasmas did not inactivate FVIII:C, vWF:Ag, and vWF:RiCof in normal plasma in vitro.

Table 6. Bleeding Times Before and After DDAVP in Patients With Congenital and Acquired von Willebrand’s Disease

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Diagnosis</th>
<th>Baseline</th>
<th>30 min</th>
<th>Postinfusion</th>
<th>4 h Postinfusion</th>
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<tbody>
<tr>
<td>1</td>
<td>AvWD</td>
<td>10</td>
<td>4</td>
<td>11</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>AvWD</td>
<td>11</td>
<td>6</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>AvWD</td>
<td>14</td>
<td>7</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>AvWD</td>
<td>5</td>
<td>ND*</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>AvWD</td>
<td>5</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>AvWD</td>
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<td>ND</td>
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<td>7</td>
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<td>8</td>
<td>ND</td>
<td>ND</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>CvWD</td>
<td>19</td>
<td>10</td>
<td>8</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>CvWD</td>
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<td>6</td>
<td>7</td>
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<td>CvWD</td>
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<td>11</td>
<td>CvWD</td>
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<td>14</td>
<td>CvWD</td>
<td>14</td>
<td>8</td>
<td>5</td>
<td></td>
</tr>
</tbody>
</table>

*ND, not done because baseline bleeding times were normal or borderline.
ruling out the presence of inactivating antibodies. Although we have not directly tested the other two possibilities, our findings would be compatible with either of them.

The results of this investigation might also have therapeutic implications. DDAVP transiently normalized the bleeding time in all AvWD patients and increased plasma concentrations of FVIII:C, vWF:Ag, and vWF:RIfCof. Even though the correction did not last as long as in patients with CvWD, DDAVP might be useful for treating bleeding episodes or securing hemostasis for surgical procedures without resorting to cryoprecipitate, with its inherent risk of hepatitis.

NOTE ADDED IN PROOF

Schneider et al. have previously reported a case of AvWD associated with a malignant lymphoma and characterized by the absence of the more slowly moving forms of plasma FVIII-vWF on CIE. We have now studied the FVIII-vWF multimeric structure in the plasma of this patient and found a defect of large and intermediate-sized multimers reverting to normal after clinical remission of the lymphoma.

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REFERENCES


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