Proteolysis of von Willebrand Factor in Therapeutic Plasma Concentrates

By Pier Mannuccio Mannucci, Antonella Lattuada, and Zaverio M. Ruggeri

Therapeutic plasma concentrates containing von Willebrand factor (vWF) lack the largest, most hemostatically active multimers. To evaluate whether this abnormality results from proteolysis during manufacturing, we have analyzed the subunit structure of vWF in several commercial products and found a marked reduction in the relative content of intact 225-kD subunit, paralleled by an increase in the proteolytic fragments normally present in plasma, particularly that of 176 kD. There was no heightened vWF fragmentation in blood-bank cryoprecipitate prepared from platelet-poor, single-donor plasma; in contrast, there was a marked degree of fragmentation in cryoprecipitate prepared from pooled plasmapheresis plasma representing the starting fraction for the production of commercial concentrates. In cryoprecipitate prepared experimentally from plasma containing varying numbers of platelets, the degradation of vWF was proportional to the platelet count, but was greatly diminished by adding protease inhibitors to the plasma. On the basis of these findings, we postulate that the loss of the largest vWF multimers in commercial products results from the use of poorly centrifuged plasmapheresis plasma containing an excessive number of residual platelets and leukocytes. These cells, lysing when plasma is frozen and thawed for the preparation of cryoprecipitate, may liberate proteolytic enzymes that cleave the vWF subunit and contribute to the degradation of the largest multimers. Our results should help devise new approaches for the preparation of more effective concentrates for the treatment of von Willebrand disease.

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PLASMA CONCENTRATES containing vWF are used in the therapy of patients with von Willebrand disease unresponsive to the nontransfusion agent desmopressin (DDAVP). Many clinicians prefer concentrates to cryoprecipitate (until recently, the blood product of choice) because the former are treated with virucidal methods, as yet not applicable to cryoprecipitate, that minimize the risk of transmission of blood-borne infectious agents. All concentrates can correct the defect of vWF, their ristocetin cofactor activity (an indirect measure of the ability of vWF to support platelet adhesion and aggregation) is usually low relative to the vWF antigen (vWF:Ag) levels. Moreover, all the products lack the largest multimeric forms of vWF, a structural abnormality that may be, at least in part, responsible for their decreased hemostatic efficacy. A reasonable hypothesis is that vWF multimers are degraded during the preparation of concentrates as a result of proteolytic cleavage. Indeed, vWF is susceptible to proteolysis even under physiologic conditions, as shown by the observation that a small but consistent proportion of the protein in normal plasma is fragments of the 225-kD subunit, with apparent molecular mass of 189 kD, 176 kD, and 140 kD, respectively. Although the proteolytic enzymes responsible for this physiologic cleavage of the vWF subunit have not been directly identified, calcium-dependent neutral proteases (calpains) from platelets and leukocytes are currently considered the most likely candidates.

With this as a background, we investigated whether or not the degree of vWF fragmentation was greater in concentrates than in plasma and different kinds of cryoprecipitate, and whether the vWF degradation varied in concentrates produced with different fractionation procedures and virus-inactivating methods. The results of our studies provide evidence for the occurrence of heightened vWF fragmentation in almost all the concentrates tested. They also support the view that the cause of vWF fragmentation is the use by manufacturers of plasmapheresis plasma contaminated by platelets, and possibly leukocytes, releasing proteolytic enzymes.

MATERIALS AND METHODS

Concentrates were chosen among the preparations containing factor VIII and vWF and used for the treatment of patients in the last 10 years, thus encompassing different fractionation methodologies, degrees of purification, and virucidal procedures (some nonvirally inactivated concentrates were also evaluated) (Table 1). Although these concentrates are licensed for the treatment of patients with hemophilia, many of them (with the exception of products obtained by immunoaffinity chromatography, numbers 18 to 20 in Table 1) are also used in patients with von Willebrand disease. Only one batch of each concentrate was tested, on the assumption that source plasma and the manufacturing process were uniform enough to make this representative of the particular product. We also analyzed three batches of a concentrate designed specifically for use in patients with von Willebrand disease containing mainly vWF with relatively little factor VIII (no. 21, Table 1; prepared by Centre
<table>
<thead>
<tr>
<th>Name and Manufacturer</th>
<th>Preparation Method</th>
<th>Virucidal Method</th>
<th>FVIII:C IU/mg</th>
<th>vWF:Ag U/mL</th>
<th>Ricof U/mL</th>
<th>Ricof/vWF:Ag</th>
<th>Multimers Missing</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Kryobulin (Immuno)</td>
<td>Cryoprecipitation plus precipitation/adsorption</td>
<td>None</td>
<td>0.4</td>
<td>188</td>
<td>37</td>
<td>0.20</td>
<td>Large and intermediate</td>
</tr>
<tr>
<td>2. Hemophil (Hyland)</td>
<td>As above</td>
<td>None</td>
<td>0.8</td>
<td>133</td>
<td>21</td>
<td>0.16</td>
<td>As above</td>
</tr>
<tr>
<td>3. Koate (Cutter)</td>
<td>As above</td>
<td>None</td>
<td>1.1</td>
<td>139</td>
<td>37</td>
<td>0.26</td>
<td>As above</td>
</tr>
<tr>
<td>4. UmanCry VIII (Biafini)</td>
<td>As above</td>
<td>None</td>
<td>0.5</td>
<td>200</td>
<td>28</td>
<td>0.14</td>
<td>As above</td>
</tr>
<tr>
<td>5. Hemophil T (Hyland)</td>
<td>As above</td>
<td>Dry heating (60°C, 72 h)</td>
<td>1.5</td>
<td>292</td>
<td>79</td>
<td>0.27</td>
<td>As above</td>
</tr>
<tr>
<td>6. Kryobulin TIM2 (Immuno)</td>
<td>As above</td>
<td>Steam heating (60°C, 1 h)</td>
<td>0.5</td>
<td>190</td>
<td>14</td>
<td>0.07</td>
<td>As above</td>
</tr>
<tr>
<td>7. Kryobulin TIM3 (Immuno)</td>
<td>As above</td>
<td>Steam heating (60°C, 10 h)</td>
<td>0.8</td>
<td>258</td>
<td>34</td>
<td>0.13</td>
<td>As above</td>
</tr>
<tr>
<td>8. Profilate HS (Alpha)</td>
<td>As above</td>
<td>Heptane suspension (60°C, 20 h)</td>
<td>2.1</td>
<td>176</td>
<td>23</td>
<td>0.13</td>
<td>As above</td>
</tr>
<tr>
<td>9. Koate HS (Cutter)</td>
<td>As above</td>
<td>Pasteurization (60°C, 10 h)</td>
<td>4.0</td>
<td>134</td>
<td>37</td>
<td>0.28</td>
<td>As above</td>
</tr>
<tr>
<td>10. BY (BPL)</td>
<td>As above</td>
<td>Dry heating (60°C, 72 h)</td>
<td>2.9</td>
<td>170</td>
<td>67</td>
<td>0.39</td>
<td>As above</td>
</tr>
<tr>
<td>11. Hameate P (Behring)</td>
<td>As above</td>
<td>Pasteurization (60°C, 10 h)</td>
<td>3.5</td>
<td>95</td>
<td>60</td>
<td>0.63</td>
<td>Large</td>
</tr>
<tr>
<td>12. Emoclot V.I. (Aima)</td>
<td>As above</td>
<td>Solvent/detergent</td>
<td>1.9</td>
<td>196</td>
<td>62</td>
<td>0.32</td>
<td>Large and intermediate</td>
</tr>
<tr>
<td>13. Beriate P (Behring)</td>
<td>As above, plus chromatography</td>
<td>Pasteurization (60°C, 10 h)</td>
<td>29.0 (2.5)*</td>
<td>12</td>
<td>2</td>
<td>0.17</td>
<td>As above</td>
</tr>
<tr>
<td>14. Profilate HP (Alpha)</td>
<td>As above</td>
<td>Solvent/detergent</td>
<td>31.0 (3.4)*</td>
<td>450</td>
<td>60</td>
<td>0.13</td>
<td>As above</td>
</tr>
<tr>
<td>15. Emoclot Octa V.I. (Aima)</td>
<td>As above</td>
<td>Solvent/detergent</td>
<td>85.0</td>
<td>22</td>
<td>2</td>
<td>0.09</td>
<td>As above</td>
</tr>
<tr>
<td>16. High-purity FVII</td>
<td>(Biotransfusion)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17. Koate HP (Cutter)</td>
<td>As above</td>
<td>Pasteurization (60°C, 10 h)</td>
<td>50.2 (2.5)*</td>
<td>227</td>
<td>72</td>
<td>0.32</td>
<td>As above</td>
</tr>
<tr>
<td>18. Hemophil M (Hyland)</td>
<td>As above</td>
<td>Immunoaffinity chromatography</td>
<td>&gt;2,400.0 (3.4)*</td>
<td>0.4</td>
<td>0.2</td>
<td>0.50</td>
<td>As above</td>
</tr>
<tr>
<td>19. Monoclate (Armour)</td>
<td>As above</td>
<td>Dry heating (60°C, 30 h)</td>
<td>&gt;3,640.0 (4.5)*</td>
<td>8</td>
<td>0.6</td>
<td>0.08</td>
<td>As above</td>
</tr>
<tr>
<td>20. Monoclate P (Armour)</td>
<td>As above</td>
<td>Pasteurization (60°C, 10 h)</td>
<td>&gt;3,640.0 (6.1)*</td>
<td>4</td>
<td>3</td>
<td>0.08</td>
<td>As above</td>
</tr>
<tr>
<td>21. High-purity vWF concentrate</td>
<td>Cryoprecipitation plus precipitation/adsorption</td>
<td>Solvent/detergent</td>
<td>8 IU/mL</td>
<td>95</td>
<td>91</td>
<td>0.95</td>
<td>Large</td>
</tr>
</tbody>
</table>

Abbreviation: Ricof, ristocetin cofactor activity.

* Values of specific activity were calculated after subtracting the protein content corresponding to added albumin; values obtained without subtracting the content of albumin are given in parentheses.
Regional de Transfusión Sanguínea, Lille, France; supplied by Dr D. Menache-Aronson, American Red Cross, Washington, DC). Specific activity of the concentrates is expressed in international units of factor VIII coagulant activity (FVIII:C) per milligram of total protein, because concentrates are licensed for use in hemophiliacs, and hence, their potency is expressed in relation to factor VIII (with the exception of the vWF factor concentrate; see Table 1). Intermediate-purity concentrates are arbitrarily defined as those with specific activity below 10 IU/mg; high-purity concentrates are those between 10 and 250 IU/mg; very high purity concentrates are those above 250 IU/mg and produced by immunoaffinity chromatography on monoclonal antibodies (MoAbs). Before lyophilization, human albumin was added to some concentrates (nos. 13, 14, 17 to 20, Table 1) to stabilize the factor VIII activity. Concentrates were reconstituted in sterile water as recommended by manufacturers.

Normal blood samples were drawn from members of the laboratory staff into polypropylene syringes and transferred immediately into polypropylene tubes containing one-tenth final volume of one of the following: 3.8% sodium citrate, 50 mmol/L EDTA and 60 mmol/L N-ethylmaleimide (NEM); or 50 mmol/L EDTA, 60 mmol/L NEM and 5,000 Kallikrein inhibitory units of aprotinin per mL (note that all final concentrations in blood were one-tenth of those stated). All volunteers who participated in this study gave their informed consent, according to the Declaration of Helsinki. Platelet-poor plasma was prepared by centrifugation of blood at 3,600 g for 20 minutes at 4°C. Platelet-rich plasma was prepared by centrifugal blood separators used to prepare pheresis, obtained frozen in plastic bags by courtesy of a commercial manufacturer of clotting factor concentrates (AIMA Plasmaderivati, Bolognana, Italy). Centrifugal blood separators were used to prepare this plasma, but the type of machine and other methodologic details were not known to the manufacturer.

Three different kinds of cryoprecipitate were tested. One was produced according to the method of Brown et al15 at the Blood Transfusion Center of the Ospedale Maggiore in Milan (courtesy of Dr Rebulla). Each unit was derived from a single bag of fresh-frozen plasma obtained from blood collected in citrate-phosphate-dextrose anticoagulant, immediately centrifuged at 4°C at high speed (5,000 g for 10 minutes) and frozen at −80°C within 2 hours of collection. Before freezing, these plasma preparations contained less than 10,000 platelets/μL. We also studied 28 industrial cryoprecipitate samples, obtained from six different manufacturers (Immuno, Vienna, Austria; AIMA Plasmaderivati; Alpha Therapeutic Corp, Los Angeles, CA; Hyland Laboratories, Glendale, CA; Novo Nordisk, Gentofte, Denmark; and Centre Regionale de Transfusion Sanguine), representing the initial fraction for the production of therapeutic factor VIII/vWF concentrates. Although no information was provided on the characteristics of the source plasma and on the methods for cryoprecipitate preparation, it is known that each batch of cryoprecipitate was made after pooling at least 10,000 single-plasma units obtained from plasmapheresis donors using centrifugal blood separators. Finally, cryoprecipitate was prepared on a small scale in our laboratory using the same method used by the blood bank (see above); starting material was frozen plasma collected in three different anticoagulants (citrate, EDTA plus NEM, or EDTA plus NEM and aprotinin) and containing varying amounts of platelets, prepared as described above. All cryoprecipitate samples were stored at −70°C for no longer than 1 month, thawed in a water bath at 37°C for 20 to 30 minutes and tested immediately after reconstitution.

FVIII:C, vWF:Ag, and ristocetin cofactor activity were measured as described previously,16 except that, for the latter assay, formalin-fixed platelets were used instead of washed platelets, and plasma from untreated patients with severe hemophilia A or von Willebrand disease was used instead of buffer to dilute the reconstituted concentrates to values of FVIII:C and vWF similar to those of normal plasma. The concentration of FVIII:C, vWF:Ag, and ristocetin cofactor were expressed in units per deciliter with reference to a laboratory standard calibrated against the 2nd International Standard for Factor VIII Related Activities (87/718, National Institute for Biological Standards and Controls, Potters Bar, UK). The multimeric structure of vWF was evaluated by sodium dodecyl sulfate (SDS)-agarose gel electrophoresis using a low-resolution gel system (that partially resolves large vWF multimers and allows the detection of abnormalities in their relative distribution).17 Concentrates and cryoprecipitates were adjusted before electrophoresis to the same concentration of vWF:Ag (approximately 0.20 U/mL) present in the normal plasma analyzed in the same gel for comparison.

vWF was immunopurified from plasma and cryoprecipitate with the anti-vWF MoAb AB5.5.72, coupled to cyanogen bromide-activated Sepharose CL 4B (Pharmacia, Uppsala, Sweden) at a density of 4 mg of IgG per mL of beads, as previously described.18 It was unnecessary to immunopurify vWF from concentrates, because paired samples obtained with and without immunopurification gave comparable results in terms of subunit composition. For all samples, vWF was reduced with 65 mmol/L dithiothreitol in the presence of 2% SDS for 15 minutes at 60°C.

Reduced vWF was analyzed by 5% polyacrylamide gel electrophoresis (PAGE) in the presence of SDS.19 Protein was transferred from the gels onto nitrocellulose membranes, incubated with specific antibodies, and visualized by incubation with appropriate 35S-labeled second antibody (rabbit-antiimmune IgG or goat-antirabbit IgG) and autoradiography.18 The specific primary antibody used was, in most instances, a previously described pool of murine anti-vWF MoAbs.19 Twelve selected concentrate samples (nos. 3, 4 to 7, 9, 11, 13, 16 to 19; Table 1) were also probed with polyclonal antibodies produced by immunizing rabbits with synthetic peptides reproducing sequences of the vWF subunit immediately before or after the bond Tyr542-Met543 that is cleaved physiologically in plasma vWF.20 One antibody reacts selectively with an epitope located between residues Met542 and Tyr542 at the carboxyl terminus of the normal 140-kD subunit,20 whereas the other reacts selectively with an epitope located between residues Met489 and Pro517 at the amino terminus of the normal 176-kD fragment.20 Because these antibodies react poorly with the intact subunit,21 the latter was visualized after an autoradiography was obtained to document the reactivity of the fragment-specific antibodies; for this, the same blots were probed with the pool of MoAbs followed by 35S-labeled rabbit-antiimmune IgG and a second autoradiography. The relative proportion of vWF fragments was determined by cutting out bands from nitrocellulose membranes (using the autoradiography as a guiding template), counting radioactivity in a γ scintillation counter and expressing the counts corresponding to each band as percentage of the total sum of counts in all bands. The reproducibility of this method has been established.20 Alternatively, the relative amounts of subunit and fragments were quantified by densitometric analysis of the autoradiographies using the computerized system Cliniscan 2 (Helena Laboratories, Milan, Italy), assuming that the intensity of the corresponding bands was representative of the amount of protein present. The results were expressed as ratios of the integrated areas corresponding to the 176-kD or 140-kD bands (visualized with the two antipeptide antibodies) relative to the areas corresponding to the 225-kD subunit (visualized with the pool of MoAbs).

Two dimensional, nonreduced/reduced agarose/PAGE was performed in selected samples of plasma and concentrates; this method has been described elsewhere.18 Intact vWF is resolved into multimers in the first dimension (SDS-agarose gel electrophoresis)
Five lanes contain samples obtained from commercial manufacturers labeled Industrial. From left to right they are: (P), a plasma sample obtained by plasmapheresis before cryoprecipitation; (CR), a cryoprecipitate prepared on industrial scale from plasmapheresis plasma; (CO 121), a commercial concentrate representative of those lacking large- and intermediate-size multimers (the number refers to the listing in Table 1); (CO 11), a commercial concentrate lacking large multimers only; (CO 21), the high-purity vWF concentrate. The last lane on the right contains single-donor blood-bank cryoprecipitate (CR).

Electrophoresis was performed in a low-resolution gel system (0.8% low-gelling temperature agarose).and the vWF subunit and fragments in each multimer are then resolved in the second dimension (SDS-PAGE) after reduction of disulfide bonds. Briefly, after discontinuous SDS-agarose gel electrophoresis using a low-resolution gel, each lane was excised, soaked in stacking gel buffer containing SDS and dithiothreitol to reduce vWF, applied as single strips to the top of the PAGE-stacking gel, and sealed with SDS-agarose. Polypeptides separated by SDS-PAGE in the second dimension were then transferred onto nitrocellulose membranes, probed with pooled anti-vWF MoAbs, and visualized by autoradiography with 125I-labeled rabbit-antimouse IgG. For each unreduced sample, a lane was processed after the discontinuous SDS-agarose gel electrophoresis to serve as a marker for the position of multimers; similarly, an aliquot of each reduced sample was applied directly to the SDS-PAGE to serve as a standard for the localization of subunit and fragments.

RESULTS

Characteristics and multimeric structure of the concentrates tested. Table 1 gives the names, methods of preparation, virucidal methods, factor VIII/vWF measurements, and multimeric patterns of the concentrates studied. In all but one of the concentrates, there was a relative decrease of ristocetin cofactor activity, regardless of vWF:Ag levels. Unlike other prepreparations, the high-purity vWF concentrate specifically manufactured for patients with von Willebrand disease (no. 21, Table 1) contained almost exclusively vWF with little FVIII:C and had an essentially normal ratio of ristocetin cofactor/vWF:Ag.

All concentrates contained vWF with abnormal multimeric structure, in most cases characterized by a deficiency of both large- and intermediate-size multimers (Table 1; see Fig 1 for a representative example). Only in one factor VIII/vWF concentrate (no. 11, Table 1) and in the high-purity vWF concentrate (no. 21, Table 1) was the multimeric structure less defective, with multimers of intermediate size present (Fig 1). Moreover, all cryoprecipitate preparations from commercial manufacturers used for the production of concentrates lacked large- and intermediate-size multimers, implying that the structural alteration of vWF occurred at the stage of cryoprecipitation and not in subsequent manufacturing steps (Fig 1). The vWF abnormality in industrial cryoprecipitate was at variance with the typical findings of cryoprecipitate prepared from single-donor plasma in a blood bank or in our laboratory, both of which contained the largest multimers present in normal plasma (Fig 1). Of relevance, eight samples of the starting material used for the preparation of commercial cryoprecipitate, ie, plasma collected by plasmapheresis of paid donors, had an intact multimeric structure similar to that of individual plasma samples collected in the presence of protease inhibitors (Fig 1).

Subunit structure of vWF in cryoprecipitate and concentrates. Electrophoretic analysis of reduced vWF in concentrates showed the same polypeptides seen in all normal plasma samples, with no evidence for novel proteolytic species. This was shown using peptide-specific antibodies that recognize sequences flanking the Tyr<sup>341</sup>-Met<sup>433</sup> bond in the mature vWF subunit, whose cleavage generates the 140-kD and 176-kD fragments present in all normal plasma samples<sup>59</sup>; these antibodies have previously<sup>12</sup> been shown to have only minimal reactivity with the intact vWF subunit (see Figs 2 and 3). The antibody raised against the peptide spanning from Met<sup>433</sup> through Pro<sup>457</sup> reacted with the amino terminus of the major 176-kD fragment both in normal plasma and in concentrates (Fig 2A). However, there was a significant difference in the concentration of fragment relative to intact subunit, the latter visualized by reactivity with a pool of MoAbs (Fig 2A). Indeed, the 176-kD fragment was apparently increased in the twelve concentrates used for these experiments (Fig 2B). Parallel evaluation performed with the antibody reacting with the carboxyl terminus of the 140-kD fragment, the sequence spanning from residues Leu<sup>328</sup> through Tyr<sup>432</sup>, showed the presence of the same polypeptide both in normal plasma and in concentrates (Fig 3A). The
Fig 2. Immunoblotting analysis of reduced vWF. (A), normal plasma (n) and two representative concentrates (c1 and c2) were probed with the antiserum reacting specifically with the mature vWF sequence from Met through Pro, representing the amino terminus of the 176-kD fragment (labeled Anti-peptide); this was followed by the MoAb pool (labeled mAb pool). Reduced vWF was analyzed in a 5% polyacrylamide gel containing 0.1% SDS. Polypeptides were transferred onto nitrocellulose by Western blotting and probed first with the antipeptide antibody followed by 125I-labeled goat-antirabbit IgG. After an autoradiography was obtained, the same blot was probed with the pool of MoAbs followed by 125I-labeled rabbit-antimouse IgG. The apparent molecular mass for each polypeptide is indicated on the left. (B), semiquantitative evaluation of the concentration of 176-kD fragment relative to intact subunit. Values are expressed as ratios between the intensity of the 176-kD band (visualized with the peptide-specific antibody) and that of the 225-kD band (visualized with the MoAb pool) measured by densitometric analysis (see Materials and Methods). (○), ratios obtained in normal plasma, (●), those obtained in the concentrates.

Fig 3. Immunoblotting analysis of reduced vWF. (A), the experiment was essentially identical to that described in Fig 2, except for the use of the peptide-specific antibody reacting with the mature vWF sequence from Leu through Tyr (representing the carboxyl terminus of the 140-kD fragment). (B), semiquantitative evaluation of the intensity of the 140-kD fragment (visualized with the peptide-specific antibody) relative to that of the intact subunit (visualized with the MoAb pool). The methodology and symbols are identical to those described in the legend to Fig 2.

relative proportion of 140-kD fragment, like that of the 176-kD fragment, was apparently increased in most of the concentrates tested (Fig 3B). However, in these experiments, the relative concentration of each species was derived from the corresponding intensity of bands on autoradiography; thus, the results are only semiquantitative because they may be affected by nonlinear saturation of the x-ray film. Nevertheless, these studies suggest that the proteolytic cleavage of vWF in concentrates results from the activity of the same enzymes responsible for the small degree of subunit fragmentation seen also in normal plasma.

A more direct quantitative evaluation of the relative proportion of intact 225-kD subunit and its proteolytic fragments was performed in commercial concentrates and in different types of cryoprecipitate. Only the pool of MoAbs, not the peptide-specific antibodies described above, were used in these studies because the identity of individual polypeptides had already been established. In most concentrates
containing factor VIII and vWF (nos. 1 to 20, Table 1), there was a decrease of the 225-kD subunit and a corresponding increase of the proteolytic fragments (Fig 4), confirming the results shown in Fig 3. As a whole, the difference between concentrates (n = 20) and normal plasma samples (n = 20) was highly significant (P < .001) for a decrease of the intact subunit and an increase of the 176-kD fragment, but not significant for a change of the 189-kD and 140-kD fragments, although a trend towards increase of the latter was apparent. Heightened vWF fragmentation occurred in high-purity concentrates produced using complex fractionation procedures, but also, and to a similar degree, in less pure concentrates that had undergone fewer manipulations during manufacturing (Fig 4). There was no obvious relationship between the occurrence and degree of fragmentation and the use and type of virucidal methods (Fig 4). The only factor VIII/vWF concentrate with intermediate-size vWF multimers (no. 11, Table 1) had normal amounts of the 189-kD and 140-kD fragments, but increased amount of 176-kD fragment and decreased concentration of intact 225-kD subunit [225-kD subunit: 63% (normal: 73% to 89%); 189-kD fragment: 4% (normal: 0.9% to 5.9%); 176-kD fragment: 29% (normal: 5.1% to 15.5%); 140-kD fragment: 4% (normal: 2.2% to 8.0%)]. Hence, in this product vWF was not overly less proteolyzed than in the remaining concentrates lacking large- and intermediate-size multimers. Analysis of three lots of the high-purity vWF concentrate containing little FVIII:C showed that the intact 225-kD subunit was between 56% and 63%, the 189-kD fragment between 1.6% and 3.4%, the 176-kD fragment between 26% and 35%, and the 140-kD fragment between 13.4% and 16% (range of observed values). Hence, vWF fragmentation appears to have occurred in this concentrate at similar levels as in all the other factor VIII/vWF concentrates tested.

Like concentrates, all commercial preparations of cryoprecipitate tested exhibited a marked decrease of the intact subunit and a corresponding increase of all its proteolytic fragments (Fig 4). In this case, the difference between industrial cryoprecipitate (n = 28; from six different manufacturers) and normal plasma (n = 20) was highly significant (P < .001) for a decrease of the intact subunit and an increase of all the three major fragments of 189 kD, 176 kD, and 140 kD. In contrast, the relative proportion of intact subunit and fragments was within the normal range in blood-bank cryoprecipitate (n = 15; Fig 4), as well as in eight plasma samples collected by plasmapheresis and supplied by one manufacturer (not shown).

Subunit structure of individual vWF multimers in plasma and concentrates. To assess how subunit and fragments are distributed in multimers of different size, we used a two-dimensional electrophoretic technique that first separates multimers in SDS-agarose gels and then assesses the distribution and relative proportions of subunit and fragments in individual multimers. As previously shown,19 the relative proportions of subunit and fragments varied among the multimers. In plasma samples with a full set of multimers, the larger ones contained mostly intact subunit and very little fragments (Fig 5A). The relative intensity of the bands corresponding to the fragments of 140 kD and 176 kD (the minor 189-kD fragment was not well resolved in these gels) increased as multimer size decreased, whereas the band of 225 kD decreased (Fig 5A). In samples of concentrates lacking larger multimers there was an increase in the relative amounts of the 140-kD and 176-kD fragments, with a decrease of the 225-kD intact subunit (Fig 5B). Of note, this was apparent also in intermediate-size multimers that, in normal plasma, contain mainly intact subunit (compare Fig 5, A and B).

Effect of plasma contamination with platelets on the vWF
subunit structure of cryoprecipitate. To evaluate whether heightened vWF fragmentation in commercial concentrates and cryoprecipitate was caused by the action of platelet proteases contaminating the starting plasma, cryoprecipitate was prepared from plasma containing a varying number of platelets obtained by mixing appropriate volumes of homologous platelet-poor and platelet-rich plasma, collected in citrate anticoagulant with no added protease inhibitors. As shown in Fig 6, reduction of the 225-kD subunit and parallel increase of its major fragments were highly correlated with the residual platelet count in the starting plasma; the correlation between concentration of each polypeptide and number of platelets, varying between 5,000 and 160,000/µL, was inverse for the intact subunit (r = −0.82; P < .01) and direct for the fragments (r = 0.87 for the 176-kD fragment and 0.78 for the 140-kD fragment; P < .001). The 189-kD fragment did not show relevant changes in the various samples and its concentration was not significantly correlated with the platelet number (r = −0.14, P > .1). Of note, all vWF multimers were present in normal relative proportion in these preparations of cryoprecipitate, despite the observed increase in subunit fragmentation (not shown); therefore, with regard to multimer distribution, these preparations were different from industrial cryoprecipitate derived from pooled plasma. Moreover, protease inhibitors had a marked effect on the degree of vWF fragmentation in cryoprecipitate prepared from plasma containing a varying number of platelets. The degree of fragmentation was substantially reduced by EDTA and NEM, and was further reduced by the addition of aprotinin, a broad spectrum protease inhibitor (Fig 6).

**DISCUSSION**

A number of studies have previously suggested that vWF may be proteolyzed in therapeutic FVIII/vWF concentrates. Using two-dimensional immunoelectrophoresis, Montgomer and Johnson23 showed the occurrence of novel peaks not present in plasma and interpreted them as products of proteolytic fragmentation. Multimeric analysis of concentrates by intermediate resolution SDS-agarose gel electrophoresis showed that the two satellite bands in the “triplet” of smaller multimers were broader and more intense than the corresponding bands in plasma,24 a pattern thought to be caused by increased proteolytic cleavage of vWF.18 The low ratio of ristocetin cofactor activity and vWF:Ag found in all concentrates6,7 was also assumed to reflect heightened proteolysis because this pattern is obtained when vWF is degraded by proteolytic enzymes in vitro.22 Now, our results directly show that the absence of large vWF multimers in concentrates is accompanied by heightened proteolytic fragmentation of the constitutive subunit and that this is a magnification of a phenomenon already occurring under physiologic conditions because all fragments identified in concentrates are also present in normal plasma.

We found no obvious relationship between the degree of proteolytic degradation of vWF and the use of various virucidal methods during manufacturing. Moreover, there was no correlation between the degree of proteolysis and the nature and complexity of the industrial fractionation process because intermediate-purity concentrates were not less proteolyzed than high-purity ones. Rather, our findings indicate that the structural damage to vWF is caused by events occurring at earlier stages of the manufacturing process. In fact, plasma used as the starting material for the preparation of concentrates exhibited normal multimeric structure and normal degree of vWF fragmentation; in contrast, the first plasma fraction prepared during the industrial process, cryoprecipitate, lacked large multimers and exhibited increased fragmentation of the subunit. Thus, proteolysis of vWF seems to occur in connection with the procedure of cryoprecipitation, and the structural damage is not augmented during the subsequent steps of purification or viral inactivation used to arrive at the final product.

It has been established that plasma vWF multimers have heterogeneous subunit composition.19 The largest normal
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Fig 6. Relationship between the degree of vWF fragmentation in cryoprecipitate and the residual platelet count in the starting plasma. The relative proportion of intact subunit and fragments was calculated as percentage of the total radioactivity associated with bands identified by reactivity with the MoAb pool (see Fig 4 and Materials and Methods). Note that different scales (vertical axis) are used for subunits and fragments. The platelet counts reported on the horizontal axis are per μL (values divided by 1.000). (ο), values observed in three different samples of cryoprecipitate prepared from citrated plasma containing varying numbers of platelets; (●), corresponding values in the starting plasma (in this case, platelets were removed by centrifugation before analysis); (△), values in three samples of cryoprecipitate prepared from plasma collected in EDTA and NEM; (◊), values in three samples of cryoprecipitate prepared from plasma collected in EDTA, NEM, and aprotinin; (-----), limits of the normal values (mean ± 2 SD) for subunits and fragments, obtained in 20 normal plasma samples.

Species are composed predominantly of intact 225-kD subunit, but smaller multimers contain subunit fragments, suggesting that they may be proteolytic derivatives of the larger ones. Our results show that this heterogeneity is enhanced in concentrates, where subunit fragments could be detected in intermediate vWF multimers that are composed predominantly of intact subunit in normal plasma. This finding, similar to that observed in certain variant forms of von Willebrand disease, notably type II A,12 implies that the loss of larger multimers and the changes of subunit composition are associated events resulting from proteolysis. A clue to the cause of the observed structural modifications stems from the observation that commercial cryoprecipitate contains altered vWF, whereas the pooled plasma from which it is prepared contains normal vWF. This finding suggests that the step of cryoprecipitation is crucial in causing the degradation of vWF multimers; however, the nature of the starting plasma may also be relevant. In fact, blood-bank cryoprecipitate, prepared from single-blood donations centrifuged at high speed immediately after collection to make plasma as cell-free as possible, contains essentially intact vWF with normal subunit composition. Industrial cryoprecipitate, in contrast, is produced from pools of more than 10,000 plasma units obtained by plasmapheresis of paid donors. In this case, the procedure involves the use of blood separators that may be less efficient than regular centrifuges in removing leukocytes and platelets.23 Thus, commercial plasma is likely to contain relative large numbers of cells; these, lysing when plasma is frozen and thawed during fractionation, may liberate excessive amounts of proteolytic enzymes that degrade vWF.

This hypothesis is supported by the experimental demonstration that cryoprecipitate prepared from plasma collected in citrate anticoagulant and contaminated in vitro with varying amounts of platelets exhibited a degree of vWF fragmentation proportional to the platelet number; and that as little as 20,000 to 40,000 platelets/μL, a number that may contaminate plasma prepared with some cell separators,23 could cause degradation of the 225-kD vWF subunit. Further support to this hypothesis comes from the finding that vWF fragmentation was substantially less when plasma was col-
lected in EDTA and NEM, which are inhibitors of calcium-dependent proteases, and was further reduced by a broad-spectrum protease inhibitor such as aprotein. At present, it remains unexplained why this experimental cryoprecipitate, unlike industrial fractions, contained intact vWF multimers despite the appearance of subunit fragments. Perhaps cryoprecipitation per se contributes to the release of proteases but the industrial process, occurring in large tanks containing a mixture of 10,000 to 15,000 individual bags of plasma, may change the way these enzymes act on the substrate. In fact, as previously discussed with regard to the position of interchain and intrachain disulfide bonds in the polymeric vWF molecule,10 if subunits at the two ends of a multimer are cleaved at the 842-843 peptide bond, fragments are generated that may be as much as half the size of the original multimer. The industrial process may cause some denaturation of vWF molecules with exposure of subunits otherwise protected from the action of proteases. The presence of subunit fragments in multimers of intermediate size that contain mainly intact subunit in normal plasma (Fig 5) is in agreement with the concept that the subunit fragments derive from proteolysis of larger molecules. Also of note is the fact that all commercial cryoprecipitates showed a parallel increase of the two major vWF subunit fragments of 176 kDa and 140 kDa, whereas the latter was relatively less prominent in concentrates. This suggests that during some of the procedures involved in the preparation of the final product, species containing the 140-kDa fragment may be more readily removed than those containing the 176-kDa fragment.

The present findings of increased vWF subunit fragmentation in concentrates, and evidence obtained on the manufacturing steps responsible for its occurrence, may be of clinical relevance. It is reasonable to surmise that the lack of the largest, most hemostatically active multimers is responsible, at least in part, for poor concentrate efficacy in shortening the bleeding time in patients with von Willebrand disease. Perhaps an additional factor is the inhibitory effect that vWF fragments contained in relatively large amounts in concentrates may exert on platelet-vWF interactions.24 Admittedly, there must be causes other than the vWF multimeric defect and subunit fragmentation contributing to the poor efficacy of concentrates, because also blood-bank cryoprecipitate (the epitomy of products with an intact vWF multimeric structure and a normal degree of subunit fragmentation) sometimes fails to normalize a prolonged bleeding time in von Willebrand disease.25,26 In this regard, the most efficacious therapeutic intervention is the concomitant infusion of both cryoprecipitate and platelet concentrates, the latter providing a source of vWF apparently necessary to support primary hemostasis to a full extent.27 On the other hand, because both cryoprecipitate and platelet concentrates carry a small but definite risk of transmitting blood-borne infections, it is desirable to attempt to obtain more hemostatically effective and virally inactivated concentrates with as little proteolytic degradation of vWF as possible. Perhaps manufacturers should attempt to produce such concentrates from plasma containing less residual blood cells, using more robust centrifugation of blood before freezing the plasma. In theory, another technical approach would be to collect blood in anticoagulant containing inhibitors of proteases, but the presently available substances may be unsuitable for the optimal use of blood and its components. The methods and results presented here should help in the search and evaluation of new approaches to address these problems.

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