Molecular Characterization of Commercial Porcine Factor VIII Concentrate

By Pete Lollar, Carlo G. Parker, and Russell P. Tracy

Commercial porcine factor VIII concentrate (Hyate:C) is effective in treatment of patients with hemophilia A who have circulating antibodies to factor VIII. The molecular forms of factor VIII in the concentrate were identified and evaluated in light of the known properties of porcine and human factor VIII. The factor VIII in the concentrate was isolated by tandem chromatography on gelatin-Sepharose and monochlonal anti-factor VIII-Sepharose. The factor VIII was 1% of the protein mass of the concentrate when calculated by either quantity of protein recovered or by radioimmunoassay. Both functional assay and Western blotting of the crude concentrate indicated that maximum coagulant function was achieved by proteolytic activation of procofactor forms of factor VIII. The factor VIII can be fractionated by cation-exchange high-performance liquid chromatography (HPLC) into two or three species of heterodimers depending on the lot. The specific activity of the purified porcine factor VIII was 550 U/mg using pooled porcine plasma at 1 U/mL as a standard. From this value, a factor VIII concentration in normal pig plasma of 2 μg/mL was calculated. This agreed well with a value of 3 μg/mL obtained by radioimmunoassay (RIA) of factor VIII in porcine plasma. In contrast, reported values for human factor VIII average 5800 U/mg, resulting in a calculated concentration in plasma of 0.2 μg/mL. The finding that porcine plasma contains a significantly higher circulating mass of factor VIII than human plasma appears to explain previous difficulties in comparing porcine and human factor VIII in standard assays.

FACTOR VIII can be defined as the factor that corrects the coagulation defect in the plasma of patients with hemophilia A. Acquired antibodies to factor VIII occur in ~5% to 10% of patients with severe hemophilia A. The resulting increase in dose requirement makes treatment in this setting difficult or impossible. Porcine plasma or plasma concentrates can give a better dose-response in patients with these inhibitors. Side effects due to contaminants prevented their use until an improved fractionation procedure was developed and tested clinically. Commercial porcine concentrate (Hyate:C) is now licensed in the United States and elsewhere for the treatment of bleeding in patients with hemophilia with low to intermediate levels of inhibitors.

Since the introduction of Hyate:C, the molecular properties of factor VIII have been determined in considerable detail as a result of successful procedures for its isolation from bovine, porcine, and human plasma and of the cloning of the factor VIII gene. Factor VIII is synthesized as a single polypeptide chain but probably undergoes proteolysis in vivo to several species of heterodimers. These inactive heterodimers can be further proteolytically modified to yield active factor VIII that participates as a cofactor in the activation of factor X by activated factor IX in the presence of a phospholipid surface and calcium. Both structural and functional studies indicate that porcine and human factor VIII are remarkably similar.

Dosage of Hyate:C is based on coagulant activity, which is complicated by the difficulties inherent in assaying a porcine concentrate against a human standard. For instance, our initial evaluation of Hyate:C indicated that its coagulant activity appeared lower than the manufacturer's value when 1 U factor VIII was defined as the amount of activity in 1 mL normal porcine plasma in a standard one-stage coagulation test. The method used by the manufacturer to define a unit has not been stated, but dose–response studies in humans indicate that it is based on a human standard. Thus, possible difficulties in comparing porcine and human factor VIII concentrates are that porcine plasma contains more factor VIII than human plasma or that Hyate:C contains molecular forms that differ from those previously described for highly purified porcine or human factor VIII. The latter possibility might occur from proteolytic alteration of factor VIII during the preparation of the concentrate resulting in partial activation of the factor VIII.

In this study, the factor VIII in Hyate:C was analyzed to determine (a) whether activated or nonactivated species are present, (b) whether it consists of molecular forms previously described in preparations of highly purified porcine factor VIII, and (c) the mass of factor VIII in the concentrate and in normal porcine plasma. Results indicate that porcine plasma contains a substantially greater mass of factor VIII than human plasma, which appears to explain satisfactorily the discrepancy between the two plasmas when used as standards. In addition, the procoagulant species correspond to previously described inactive forms that require proteolytic activation in vivo for clinical effectiveness.

MATERIALS AND METHODS

The following materials were purchased from commercial suppliers: ovalbumin, lysozyme, avidin, radioimmunoassay (RIA) grade bovine serum albumin (BSA), porcine immunoglobulin G (IgG), gelatin-agarose, Tween 20, Tween 80, blue dextran, and NAD, Sigma Chemical Co. (St Louis); activated partial thromboplastin, General Diagnostics (Morris Plains, NJ); human factor VIII-deficient plasma and normal human citrated plasma, George King Biomedical (Overland Park, KA); ristocetin and fixed human platelets, Bio-Data (Hatboro, PA); porcine factor VIII concentrate
Superfine (Fast Desalting Column) and monoS (HR 5/5) chromatography resins, Pharmacia (Piscataway, NJ); murine monoclonal C8 anti-human Factor VIII, IGB Products (San Leandro, CA). The W3-3 hybridoma cell line producing mouse monoclonal antiporcine factor VIII was generously provided by Dr D. N. Fass, Mayo Clinic, Rochester, MN. W3-3-Sepharose 4B was prepared either as described,6 or by coupling the antibody at 1:10 of the pooled factor VIII from the W3-3-Sepharose column was eluted with a 20-mL gradient into buffer A 1% Tween 80. Plates then were washed three times with PBS and used immediately, or were stored in this buffer at -20°C until use. For determination of the amount of factor VIII in Hyate-C, 125I-factor VIII was then added to a final concentration of 30,000 cpm/well, followed immediately by the addition of dilutions of unlabeled factor VIII or Hyate-C. All samples were in buffer A, 0.01% Tween 80, 0.1% BSA, 0.035 mol/L β-mercaptoethanol (β-ME). After a 3-hour incubation, the wells were washed three times with PBS and the remaining radioactivity was measured. Determinations of factor VIII in plasma were done in the same way except that calcium was omitted from buffer A. Before plasma was used in the assay, β-ME was added to a final concentration of 0.035 mol/L, and the plasma was dialyzed for 2 hours against buffer A minus calcium containing 0.01% Tween 80 and 0.035 mol/L/β-ME in addition.

Gel permeation chromatography of Hyate-C. A column (1.5 x 25 cm) of Sepharose 4B was equilibrated in buffer A plus 0.01% Tween 80. A bottle of Hyate-C was reconstituted in 3 mL H2O, a 0.5-mL sample was applied at a flow rate of 30 mL/hour, and 1.2-mL fractions were collected. Samples were assayed for absorbance at 280 nm, factor VIII coagulant activity, and ristocetin cofactor activity. Blue dextran and NAD were used as void volume and internal volume markers respectively.

Extinction coefficients. The extinction coefficients of immunoaffinity-purified factor VIII and Hyate-C at 280 nm were determined using a modification15 of the method of van Lersel et al.14 The following values (ε280nm) were obtained: factor VIII, 1.2; Hyate-C, 1.4.

Electrophoresis. Discontinuous sodium dodecyl sulfate-7% polyacrylamide gel electrophoresis (SDS-PAGE) was done using the buffer system of Laemmli.13 Samples containing 1% (wt/vol) SDS with or without 1% to 2% (vol/vol) β-ME were heated for 2 to 5 minutes in a heating block maintained at 100°C. Proteins were visualized by silver staining.13 Prestained mol mass standards used were myosin (200 kD), phosphorylase b (97 kD), BSA (68 kD), ovalbumin (43 kD), chymotrypsinogen (26 kD), β-lactoglobulin (18 kD), and lysozyme (14 kD) (Bethesda Research Laboratories, Gaithersburg, MD).

Western blotting. Immunoaffinity-purified factor VIII or Hyate-C was dialyzed into 0.0625 mol/L Tris-Cl, pH 6.8. The final concentrations were 0.034 and 1.6 mg/mL, respectively. The samples were activated by addition of porcine thrombin to a final concentration of 0.3 μg/mL for 2 hours at room temperature. The samples were then prepared for 7% SDS-PAGE without reduction of disulfide bonds as described above. Western blotting was done using a biotin-avidin horseradish-peroxidase detection system.18 The primary antibody used was the C8 murine monoclonal anti-human factor VIII described by Roblat et al19 that is specific for the heavy chain-derived polypeptide containing the A2 domain of factor VIII (residues 375 through 719).20

RESULTS

Isolation of factor VIII from Hyate-C. After reconstituted Hyate-C was passed over gelatin-Sepharose, it was applied to W3-3 mouse monoclonal antiporcine factor VIII-Sepharose and eluted as described in the Materials and Methods section. A single peak with a trailing edge was observed, similar to that described by Fass et al6 (data not shown). The gelatin-Sepharose step was necessary to remove a high-mol-wt contaminant that has not been identified and fibronectin, which was identified by SDS-PAGE after the column was eluted with 4 mol/L urea. Adsorption to the immunoaffinity column was done in the presence of 0.255...
mol/L CaCl₂ or 0.255 mol/L MgCl₂, since factor VIII did not bind under a variety of other conditions. This is presumably due to the presence of vWF in Hyate:C, since high concentrations of divalent cations are known to dissociate it from factor VIII.²¹ Further evidence for this association resulted from gel permeation chromatography of Hyate:C on Sepharose 4B performed as outlined in the Materials and Methods section. Examination of two separate lots of Hyate:C compared with immunoaffinity-purified factor VIII revealed that 100% of the factor VIII activity in Hyate:C eluted ahead of immunoaffinity-purified factor VIII. Factor VIII in Hyate:C chromatographed as a single peak that eluted in the void volume along with ristocetin cofactor activity (data not shown).

The results of a typical purification of factor VIII from Hyate:C are given in Table 1. The specific activity is defined as the units of factor VIII per milligram. A unit is defined as the amount of factor VIII in 1 mL of normal citrated porcine plasma. The mass of factor VIII is calculated from its absorbance and extinction coefficient at 280 nm as described in the Materials and Methods section. In 24 preparations involving four batches of Hyate:C, the average specific activity was 550 U/mg. It is interesting to compare this specific activity with that obtained in our laboratory using the purification procedure described by Fass et al.²⁶ for porcine factor VIII and also with published reports for the specific activity of human factor VIII (Table 2). The lower specific activity of porcine factor VIII suggests that the circulating mass of porcine factor VIII is approximately tenfold greater than that of human factor VIII.

HPLC chromatography of immunoaffinity-purified factor VIII. Depending on the batch of Hyate:C used, the factor VIII that eluted from the immunoaffinity column consisted of three or four distinct chains when analyzed by SDS-PAGE. These chains have previously been identified by their SDS-PAGE apparent mol-masses of 166, 130, 82, and 76 kd.³⁻³ In one lot, the 130-kd band was consistently absent. Cation-exchange chromatography of the immunoaffinity-purified material under non-denaturing conditions resulted in partial resolution of these forms of factor VIII (Figs 1 and 2). Previous work with both human and porcine factor VIII has shown that factor VIII is heterogeneous when isolated from plasma and is derived from a single polypeptide precursor with a dominant sequence of A₁-A₂-B-A₃-C₁-C₂.¹¹ The heterogeneity is due to proteolysis within the B domain, which, in the case of porcine factor VIII, has led to the identification of heterodimers consisting of a common 76-kd light chain noncovalently associated with heavy chain polypeptides lacking variable amounts of the B domain.⁶ SDS-PAGE of chromatography fractions revealed that the light chain was associated with all the fractions, with the high-mol-mass heterodimers (166 + 76 kd) eluting first (Fig 2A). Thrombin activation of the same samples followed by SDS-PAGE resulted in cleavage of both heavy and light chain polypeptides (Fig 2B).

Factor VIII coagulant levels in porcine plasma. The measurement of factor VIII in porcine plasma in a one-stage clotting assay as described in the Materials and Methods section also suggests that the plasma concentration of porcine factor VIII is higher than that of human factor VIII. Pooled porcine plasma was compared with pooled human plasma in an assay in which 1 U factor VIII was defined as the amount in 1 mL of human plasma. The pooled porcine plasma contained 7 U/mL. Equivalently, undiluted porcine plasma resulted in a shortening of the clotting time of human factor VIII-deficient plasma to 35 seconds as compared with a clotting time of 50 seconds obtained with undiluted human plasma.

Radioimmunoassay of porcine factor VIII. A competition RIA was developed for factor VIII using solid-phase W3-3 antibody and immunoaffinity-purified factor VIII as competing ligand in the standard curve. The assay was used to measure the amount of factor VIII in Hyate:C and in porcine plasma. Hyate:C inhibits the binding of radioiodinated factor VIII, as shown in Fig 3. The inhibition curve appears parallel to the curve resulting from the inhibition of binding by immunoaffinity-purified factor VIII, indicating that the antigens appear to be equivalent in this assay. Calculation of the amount of factor VIII in Hyate:C was done by least-squares regression of the data over the linear region of the curves. Factor VIII constitutes 1.0% ± 0.17% of the protein mass of Hyate:C. This is consistent with the data in Table 1, in which an 88-fold purification was required to isolate factor VIII in Hyate:C. In contrast, when human factor VIII concentrate (e.g., Koate) was used as starting material for the purification of factor VIII, an 8,000-fold purification was required to achieve isolation,²² indicating that the porcine concentrate is substantially less contami-

### Table 1. Isolation of Factor VIII From Hyate:C

<table>
<thead>
<tr>
<th>Source</th>
<th>Total Protein (mg)</th>
<th>Factor VIII Activity (U)</th>
<th>Yield (%)</th>
<th>Activation Quotient (U/mg)</th>
<th>Specific Activity (U/mg)</th>
<th>Fold Purification</th>
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<tbody>
<tr>
<td>Hyate:C</td>
<td>68</td>
<td>560</td>
<td>—</td>
<td>81</td>
<td>9.3</td>
<td>88</td>
</tr>
<tr>
<td>W3-3 eluate</td>
<td>0.73</td>
<td>530</td>
<td>95</td>
<td>45</td>
<td>730</td>
<td>88</td>
</tr>
</tbody>
</table>

*Based on 1 U/mL in porcine plasma.
†Reconstitution of nine bottles.
‡E_{W3}^{C} = 1.4.
§E_{W3}^{C} = 1.2.

### Table 2. Specific Activity of Porcine and Human Factor VIII

<table>
<thead>
<tr>
<th>Source</th>
<th>Factor VIII Specific Activity (U/mg)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>From plasma</td>
<td>600 (n = 2)</td>
<td>Current study</td>
</tr>
<tr>
<td>From Hyate:C</td>
<td>550 (n = 24)</td>
<td>Current study</td>
</tr>
<tr>
<td>Human‡</td>
<td>2.300</td>
<td>7</td>
</tr>
<tr>
<td>4.700</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>6.800</td>
<td>23</td>
<td></td>
</tr>
<tr>
<td>7.000</td>
<td>24</td>
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<td>8.000</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>7.500</td>
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<td></td>
</tr>
<tr>
<td>4.500</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>Average</td>
<td>5.800</td>
<td></td>
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</table>

*With normal porcine plasma as a standard.
†With normal human plasma as a standard.
Figure 1: MonoS HPLC of immunoaffinity-purified Factor VIII. Factor VIII (110 U) was chromatographed as described in Materials and Methods. Fractions (0.7 mL) were analyzed for absorbance (dots), factor VIII clotting activity (circles), and specific conductance (squares).

Porcine plasma also inhibited the binding of radioiodinated factor VIII in the assay (Fig 4). The standard curves in Figs 3 and 4 were constructed using different preparations of $^{125}$I-factor VIII and are slightly different. The plasma inhibition curve also is parallel to the one constructed using immunoaffinity-purified factor VIII. The concentration of factor VIII in porcine plasma was calculated to be $3.0 \pm 0.8 \mu g/mL$. This is consistent with the specific activity of immunoaffinity-purified factor VIII (Table 1) from which a value of 2 $\mu g/mL$ can be calculated, since the concentration of factor VIII in porcine plasma (using porcine plasma as standard) is 1 U/mL by definition. In contrast, using an average specific activity of 5,800 U/mg for human factor VIII (Table 2), a plasma concentration of 0.2 $\mu g/mL$ is calculated.

Western blotting of factor VIII in Hyate:C. Following SDS-PAGE, immunoaffinity-purified factor VIII and Hyate:C were transferred onto nitrocellulose and detected with a heavy chain-specific antibody. The antibody binds to the region of human factor VIII which contains the A2 domain. A 35-kd chain of porcine factor VIII (Fig 2B) that contains the A2 domain is generated by cleavage of either the 166-, 130-, or 82-kd heavy chains. After cleavage of factor VIII by thrombin, these three bands disappear and the 35-kd chain is visualized (lane 2). The 35-kd band is visualized in Hyate:C only after pretreatment with thrombin (lanes 3 and 4). A thrombin-sensitive 130-kd band is also evident. The 166-kd band could not be identified because of the presence of porcine IgG in Hyate:C. Control experiments with purified porcine IgG showed that it reacted strongly with the antimurine IgG used in this detection system (data not shown). These results show that Hyate:C does not contain significant amounts of factor VIII that has been activated by thrombin or factor Xa.

Functional assay of Hyate:C. In coagulation tests, samples containing factor VIII typically yield higher values in a two-stage assay when compared with a one-stage assay. This presumably reflects incomplete activation of factor VIII during the course of the development of a fibrin clot in the one-stage assay. In the two-stage assay, thrombin is added exogenously to activate the factor VIII rapidly. In Table 1, the activation quotients (AQs), defined as the factor VIII level in the two-stage assay divided by that in the one-stage assay (described in the Materials and Methods section), is listed for Hyate:C and immunoaffinity-purified factor VIII. An unexplained 1.5-fold to 2-fold decrease in the AQ occurred routinely during the isolation procedure. The final AQ is experimentally indistinguishable from the value of 40 previously reported for porcine factor VIII.
The functional identity of factor VIII in Hyate:C to previously characterized porcine factor VIII is also supported by a plasma-free assay. In this assay, the rate of factor Xa formation is measured in a system that contains phospholipid vesicles, factor IXa, factor X, calcium, and limiting amounts of factor VIII as test substance. Porcine factor VIII has <1% of the activity of thrombin-activated factor VIII in this assay. Both Hyate:C and immunoaffinity-purified factor VIII contained <1% of the activity of the same samples after activation by thrombin. The immunoaffinity-purified factor VIII was unstable after activation by thrombin and had a half-life of ~5 minutes. Activation in the presence of factor IXa and phospholipid increased the t1/2 to >30 minutes. These results are identical to those previously described for porcine factor VIII.

DISCUSSION

Recent advances in the understanding of the structure and function of factor VIII have provided a more rational approach to replacement therapy of hemophilia A. The use of porcine factor VIII concentrate in patients with inhibitors is complicated by problems in the determination of dosage. The possibility of quantitative or qualitative species differences makes assignment of units difficult. Currently, a unit of Hyate:C is based on in vivo recovery of activity in humans in an assay in which normal human plasma is used as a standard, although the exact details of the assay have not been published.

Based on the results of this study, which includes the use of techniques not previously applied in the biochemical analysis of factor VIII, it is possible to make a detailed characterization of the procoagulant properties of Hyate:C. An accurate extinction coefficient for immunoaffinity-purified factor VIII has been determined by far ultraviolet absorption spectroscopy. This allows the use of a well-defined standard to develop an immunoassay to measure the mass of factor VIII in Hyate:C and porcine plasma. Approximately 1% of the protein mass of Hyate:C is due to factor VIII itself. The contaminants, which include vWF and fibronectin, can be removed by gelatin-Sepharose and immunoaffinity chromatography. Gel filtration studies of Hyate:C show that all of the factor VIII is associated with vWF. Both Hyate:C and the purified factor VIII require proteolytic activation to act as cofactors in the activation of factor X by factor IXa as shown by the following experiments: (a) The activation coefficients of Hyate:C and purified factor VIII are very similar to that described by Fass et al in their original report of the properties of isolated porcine factor VIII (this mate-
rial has been shown to require proteolytic activation by thrombin or factor Xa; and (b) Western blotting of Hyate:C with a monoclonal antibody specific for a heavy chain-derived polypeptide generated by either thrombin or factor Xa detects only the polypeptide after proteolytic activation of Hyate:C.

The purified factor VIII can be fractionated by HPLC into three heterodimers with apparent mol masses of 166 + 76, 130 + 76, and 82 + 76 kd. They can be activated by thrombin to activate cofactor molecules. The fractionation of various heterodimers of human factor VIII derived from concentrates has been reported by Fay et al. and emphasizes the similarity of human and porcine factor VIII as they exist in therapeutic concentrates.

The specific activity of porcine factor VIII is lower than published values for human factor VIII (Table 2). One explanation for this observation is that porcine plasma contains a greater mass of factor VIII than human plasma. This is supported by the observations that porcine plasma is more potent than human plasma in correcting the prolonged clotting time of human factor VIII-deficient plasma and by direct immunoassay of the factor VIII content in porcine plasma, which results in a value (3 μg/mL) that is approximately tenfold higher than that calculated for human plasma based on the specific activity of human factor VIII. Thus, our work strongly suggests that porcine and human factor VIII are functionally equivalent on a per mass basis, and that porcine plasma differs from human plasma in its antihemophilic potential by virtue of its greater mass of factor VIII.

The events leading to hemostasis following administration of Hyate:C are probably identical to those resulting from use of human concentrates and do not involve infusion of preactivated factor VIII or other activated complexes.

The response to infusions of Hyate:C has been measured by factor VIII coagulation assay using a human standard. Because our study indicates that the concentrate contains no undefined procoagulants, this appears to be a valid predictor of clinical response. In addition, because a unit of Hyate:C is not well defined, assay of the concentrate using the same standard also appears to be worthwhile to evaluate the response.

REFERENCES

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