Molecular Characterization of Commercial Porcine Factor VIII Concentrate

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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 isolated 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 monoclonal 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.

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137
Superfine (Fast Desalting Column) and monoS (HR 5/5) resins, Pharmacia (Piscataway, NJ); tography resins, Pharmacia (Piscataway, C8 anti-human Factor VIII, (CNBr)-activated Sepharose 4B, Sepharose 4B, Sephadex G25 C8 anti-human Factor VIII was generously provided by Dr D. N. Fass, Mayo Clinic, Rochester, MN. W3-3-Sepharose 4B was prepared either as described, or by coupling the antibody at a concentration of 3 to 6 mg/mL to CNBr-activated Sepharose 4B according to the instructions supplied by the manufacturer.

Coagulation proteins. Porcine factors IX, IXa, and X, prothrombin, thrombin, and \(^{125}\)I-factor VIII were prepared as described previously.\(^3\) Porcine factor VIII was isolated either from whole blood essentially as described by Fass et al\(^3\) or from Hyate:C. In the latter method, 4 mL 0.1 mol/L NaCl, 0.01 mol/L histidine, 5 mmol/L CaCl\(_2\), pH 6.0 (buffer A), plus 0.25 mol/L CaCl\(_2\) were added to each of ten bottles of Hyate:C at room temperature. The resulting solution was seen and was applied at a flow rate of 20 mL/hour to a 10-mL column of gelatin-agarose connected in series to 4 mL W3-3-Sepharose. An insoluble calcium salt formed slowly in the solution as it passed through the column. The columns were then disconnected and the W3-3-Sepharose column was washed with 3 to 4 vol buffer A followed by buffer A plus 2 mol/L NaCl until the absorbance at 280 nm of the eluate was <0.005. The factor VIII activity falling through the column was <5% of the applied activity. Factor VIII was then eluted with buffer A plus 2 mol/L NaCl diluted 1:1 with ethylene glycol. Fractions containing factor VIII were stored at \(-20^\circ\)C. Factor VIII prepared in this way is referred to as immunoaffinity-purified factor VIII. The resin was washed with 0.5 mol/L EDTA disodium salt, pH 7.5 and reused. Alternatively, the precipitate can be avoided by using 0.1 mol/L MES (2[N-morpholino] ethanesulfonic acid), 0.1 mol/L NaCl, 0.25 mol/L MgCl\(_2\), pH 6.0, as the application buffer (D. N. Fass, personal communication).

Factor VIII was fractionated further by high-pressure liquid chromatography (HPLC) using a HR 5/5 MonoS column. A sample of the pooled factor VIII from the W3-3-Sepharose column was diluted 1:10 into buffer A of 0.01% Tween 80 and applied at a flow rate of 1 mL/minute. Factor VIII was eluted with a 20-mL gradient consisting of 0.1 to 0.6 mol/L NaCl in the same buffer.

Factor VIII and von Willebrand factor (vWF) assays. Factor VIII and thrombin-activated factor VIII were measured by one-stage and two-stage assays, respectively, in an activated partial thromboplastin time assay using human factor VIII-deficient plasma as a substrate.\(^4\) One unit of porcine factor VIII was defined as the amount of factor VIII in normal citrated porcine plasma. Porcine blood was obtained from eight normal adult animals by venipuncture or arteriopuncture, anticoagulated with 0.38% (wt/vol) NaHCO\(_3\), pH 9.5, for 3 hours at room temperature. The samples were then prepared for 1% SDS-PAGE without reduction of disulfide bonds as described above. Western blotting was done using a biotin-avidin horseradish-peroxidase detection system.\(^2\) The primary antibody used was the C8 murine monoclonal anti-human factor VIII described by Rotblat et al\(^2\) that is specific for the heavy chain-derived polypeptide containing the A2 domain of factor VIII (residues 375 through 719).\(^3\)

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 al\(^1\) (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 bovine serum albumin (BSA), 0.01% Tween 80. Plates then were washed three times with PBS and used immediately, or were stored in this buffer at \(-20^\circ\)C until use. For determination of the amount of factor VIII in Hyate:C, \(^125\)I-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 \(\beta\)-mercaptoethanol (\(\beta\)-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, \(\beta\)-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 \(\beta\)-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 \(\text{H}_2\text{O}\), 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 markers respectively.

Extinction coefficients. The extinction coefficients of immunoaffinity-purified factor VIII and Hyate:C at 280 nm were determined using a modification\(^1\) of the method of van Iersel et al.\(^1\) The following values (\(E_{\text{280}}^\text{nm}\)) 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.\(^1\) Samples containing 1% (wt/vol) SDS with or without 1% to 2% (vol/vol) \(\beta\)-ME were heated for 2 to 5 minutes in a heating block maintained at 100°C. Proteins were visualized by silver staining.\(^3\) Pretreated mol mass standards used were myosin (200 kd), phosphorylase b (97 kd), BSA (68 kd), ovalbumin (43 kd), chymotrypsinogen (26 kd), \(\beta\)-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 mg/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.\(^2\) The primary antibody used was the C8 murine monoclonal anti-human factor VIII described by Rotblat et al\(^2\) that is specific for the heavy chain-derived polypeptide containing the A2 domain of factor VIII (residues 375 through 719).\(^3\)

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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.²⁰ 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 previously have been identified by SDS-PAGE of chromatography fractions revealed that the light chain noncovalently associated with heavy chain poly-
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 $2.0 \mu g/mL$ can be calculated, since the concentration of factor VIII in porcine plasma (using porcine plasma as standard) is $1 \mu g/mL$ 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 by thrombin or factor Xa. Figure 5 (lane 1) shows that the antibody detects the 166-kd porcine factor VIII heavy chain. The 130- and 82-kd chains are also faintly detected. 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 (t½) of ~5 minutes. Activation in the presence of factor IXa and phospholipid increased the t½ to >30 minutes. These results are identical to those previously described for porcine factor VIII.13

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\(^{1,4}\); 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\(^{12}\) 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. Thus, 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.\(^{14}\) 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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Molecular characterization of commercial porcine factor VIII concentrate

P Lollar, CG Parker and RP Tracy