Immunodepletion of Human Plasma Factor VIII

By F. Ofosu, K. Cassidy, M.A. Blajchman, and J. Hirsh

Affinity chromatography of human cryosupernatants on anti-human factor VIII-Sepharose yielded a plasma devoid of detectable factor VIIIc, VIIIR:Ag, and VIIIR:WF activities. This plasma was indistinguishable from severe congenital hemophilic plasma when used as substrate in factor VIII coagulant assays.

PLASMA FROM INDIVIDUALS with severe hereditary deficiency of factor VIII are required to assay the levels of coagulant factor VIII in plasma, cryoprecipitates, and factor VIII concentrates. Such plasma is sometimes difficult to obtain in large quantities. We have therefore developed a method for artificially depleting human plasma of factor VIII by immunoabsorption with rabbit anti-human factor VIII insulobilized on Sepharose 4B for use in factor VIII assays.

Insolubilized antibodies have been used by several investigators to derive plasmas depleted of factors VIII, VII, and XII. Bouma et al.1 demonstrated that both Sepharose 4B and acrylamide are suitable immobilizing supports for the removal of plasma factor VIII by immunoabsorption. Their investigations and those of Holmberg and Ljung2 show that antibodies raised against each functional entity of factor VIII are capable of removing all aspects of factor VIII activity from plasma when the anti-factor-VIII antisera is insolubilized on Sepharose or acrylamide. Although Bouma et al.1 stated that recycled immunodepleted plasma could be used as a reagent for the determination of factor VIII activity, no further information about such use was presented. In addition, no information is presented on the effect of immunodepletion on the concentrations of other clotting factors. For example, Furlan et al.3 demonstrated that rabbit and human anti-factor-VIII immobilized on Sepharose CL-2B removed both factor VIII and fibrinogen from plasma. In this report, we compare the relative effectiveness of CL-Sepharose 4B, AH-Sepharose 4B, CH-Sepharose 4B, and Affigel-10 as immobilizing supports for the immunodepletion of plasma factor VIII and the suitability of the immunodepleted plasmas as substrate plasmas for one-stage factor VIII coagulant assays.

MATERIALS AND METHODS

Materials

Epsilon amino caproic acid (EACA) and soybean trypsin inhibitor (SBTI) were obtained from Sigma Chemicals, St. Louis, Mo.; heparin from Harris Laboratories, Brantford, Ontario; ethylenediaminetetraacetic acid—(EDTA); sodium diethylbarbiturate, cyanogen bromide, and polyethylene glycol (PEG 20,000) from Fisher Scientific, Toronto, Ontario. Factor-VIII-deficient plasma was obtained from George King Biomedical, Kansas City, Mo.; unflavored amphogel [Al(OH)₃] from Wyeth Ltd., Toronto, Ontario; CL-Sepharose 4B, AH-Sepharose, and CH-Sepharose from Pharmacia Fine Chemicals, Montreal, Quebec. Rabbit antiserum to human prothrombin was obtained from Behring Diagnostics, Montreal, Quebec; and antiserum to human alpha-2 macroglobulin, fibrinogen, and IgM from Meloy Laboratories, Inc., Springfield, Va. Complete Freund's adjuvant was obtained from Difco Laboratories, Detroit, Mich.; pertussis vaccine from Connaught Laboratories, Toronto, Ontario. Reagents for polyacrylamide gel electrophoresis, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, and Affigel-10 were purchased from BioRad Laboratories, Mississauga, Ontario.

Preparation of Purified Factor VIII

Factor VIII was purified by gel filtration of human cryoprecipitate.4-5 Cryoprecipitate was obtained from fresh platelet-poor human plasma anticoagulated with CPD. When so anticoagulated, the plasma contained 0.002 M citric acid, 0.012 M sodium citrate, 0.001 M Na₃HPO₄, and 0.018 M glucose. The platelet-poor plasma also contained 1% w/v of PEG (average mol wt 20,000) and the following inhibitors; EACA, 0.5%; SBTI, 0.01 mg/ml; heparin, 2 U/ml; and EDTA, 0.1 mM. The plasma, in 200-ml aliquots, was frozen at −60°C for 1 hr, thawed at 4°C in a circulating water bath, and the cryoprecipitate recovered by centrifugation at 4°C at 7000 g. Ten to 20 ml of 0.03 M diethylbarbiturate buffer, pH 7.35, containing 0.15 M NaCl and 0.5% EACA, was added to the cryoprecipitate from 1 liter of plasma and incubated at 37°C. The cryoprecipitate thus dissolved was absorbed with 10% v/v Al(OH)₃ for 10 min at 20°C and centrifuged at 18,000 g for 20 min at 20°C to remove adsorbed proteins. The resulting supernatant from 1 liter of plasma was subjected to gel filtration on 2 liters of CL-Sepharose 4B packed in a 5 cm × 100 cm column at a flow rate of 80 ml/hr. The eluting buffer was 0.03 sodium diethylbarbiturate, pH 7.35, containing 0.15 M NaCl, 0.5% EACA, and 0.02% NaN₃. Factor VIII in the eluates, located by a one-stage assay with congenital factor-VIII-deficient plasma as substrate,6 was concentrated against 60% PEG at 4°C, dialyzed against the elution buffer and stored at −60°C. Purity of the factor VIII was determined by electrophoresis on 4% polyacrylamide gels containing sodium dodecyl sulfate (SDS)7 and by quantitative rocket immunoelectrophoresis8 with monospecific antifibrinogen, anti-IgM, and anti-alpha-2-macroglobulin. Factor VIII eluted as a sharp peak in the void volume on Sepharose 4B and on concentration to 0.4–1 mg/ml.
reacted only with rabbit anti-human factor VIII on quantitative immunoelectrophoresis.

Preparation and Immobilization of Anti-Factor-VIII

New Zealand white male rabbits (approximately 2 kg weight) were immunized by intradermal injections of 40 μg of purified human factor VIII emulsified in complete Freund’s adjuvant. Each rabbit was also injected intradermally with 0.5 ml of pertussis vaccine at the time of the initial immunization. Rabbits were boosted with 40 μg of factor VIII 4 wk after the initial injection and weekly thereafter for 4–6 mo.

Rabbit anti-human factor VIII was obtained from the immunized rabbits and rendered monospecific to human factor VIII by absorption with the 3%–8% ethanol precipitate of cryosupernatant. The antiserum was absorbed with 10% v/v of Al(OH)₃, and the IgG was isolated by chromatography on DEAE-cellulose. It was then coupled to CL-Sepharose 4B by the method of Cuatrecasas at a ratio of 10–15 mg IgG/g of the gel. The gel was then washed under suction with 1–2 liters of CPD, pH 7.4, containing 0.15 M NaCl. When the gel was suspended in 150 ml of 1 M glycine-HCl, pH 3.5, in a 250 ml Buchner funnel for 10–15 min. The gel was then washed under suction with 1–2 liters of CPD, pH 7.4, containing 0.15 M NaCl.

Immunodepletion of Plasma Factor VIII

The plasma to be subjected to immunodepletion was anticoagulated with CPD. Fifty to 75 ml of platelet-poor plasmas from 15 to 20 normal donors were pooled and centrifuged at 17,000 g for 10 min to remove cellular debris, pooled, and stored frozen in 50-ml aliquots at −70°C. Cryosupernatant was obtained from each 50-ml aliquot and subjected to affinity chromatography on a 10-ml column of anti-factor-VIII coupled to CL-Sepharose 4B and previously equilibrated with CPD, pH 7.4, containing 0.15 M NaCl. Two-milliliter fractions were collected at a flow rate of 8 ml/hr using CPD/NaCl as the eluant. The resulting plasma was then subjected to clotting factor assays to determine the levels of factors II, V, VII, VIII, IX, X, XI, XII, and fibrinogen relative to the starting cryosupernatant. The levels of factor VIII antigen and von Willebrand cofactor were also determined. In addition, cryosupernatants, 50-ml aliquots of the plasma were also subjected to immunodepletion.

After its use in immunodepletion, the anti-factor-VIII–CL-Sepharose 4B was regenerated as follows. The column was developed with CPD, pH 7.4, containing 0.15 M NaCl. When the OD₂₈₀ of the eluate was less than 0.1, the gel was suspended in 150 ml of 1 M glycine-HCl, pH 3.5, in a 250 ml Buchner funnel for 10–15 min. The gel was then washed under suction with 1–2 liters of CPD, pH 7.4, containing 0.15 M NaCl.

RESULTS

Immunodepletion of Plasma Factor VIII

The passage of 50 ml of pooled cryosupernatant plasma through a 10-ml column of anti-factor-VIII–CL-Sepharose 4B completely depleted the cryosupernatant of factor VIIIC, factor VIIIR:Ag, and factor VIIIR:WF from the first 25–30 ml of the plasma. Only this 25–30-ml fraction of depleted cryosupernatant plasma was suitable for substrate use. When 50 ml of whole plasma was similarly processed, only the first 10–15 ml was totally depleted of factor VIII. The immunodepletion process was not associated with significant loss of other clotting factors (Table 1). The lower values obtained after immunodepletion represents an approximately 25% dilution of the plasma that occurred during the immunodepletion procedure. After regeneration, the anti-factor-VIII-Sepharose columns could be reused up to 6 times, before a significant loss of the factor-VIII-binding capacity became apparent.

The use of anti-factor-VIII coupled to AH-Sepharose 4B resulted in the loss of factors II, VII, IX, and X in addition to factor VIII. The levels of factors II, VII, IX, and X were reduced to less than 40% of normal plasma in each experiment. When plasma was subjected to immunodepletion on anti-factor-VIII coupled to either CH-Sepharose 4B or Affi-Gel-10, the factor-VIII-depleted plasma contained less than 10% factor XI.

Comparison of Severe Factor-VIII-Deficient Hemophilic and Immunodepleted Plasma as Substrates for Factor VIII Assays

Two severe hemophilic plasmas (factor VIIIC < 1%) and 6 cryosupernatant plasmas immunodepleted of factor VIII were compared as substrates for the quantitation of VIIIC in various patient plasmas, cryoprecipitates, or fresh-frozen plasmas. The factor VIIIC levels in mixtures of hemophilic and pooled normal plasmas as well as mixtures of pooled normal plasma and immunodepleted plasma were also determined.

In Fig. 1 are shown the results obtained when factor VIIIC levels were determined with either severe hemophilic plasma or immunodepleted cryosupernatant plasmas as substrate. There is an excellent correlation

<table>
<thead>
<tr>
<th>Table 1. Coagulation Factor Assays of Cryosupernatant and Cryosupernatant Plasma Immunodepleted of Factor VIII on Anti-Factor-VIII–CL-Sepharose 4B</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Factor</strong></td>
</tr>
<tr>
<td><strong>Factor</strong></td>
</tr>
<tr>
<td>XII</td>
</tr>
<tr>
<td>XI</td>
</tr>
<tr>
<td>X</td>
</tr>
<tr>
<td>IX</td>
</tr>
<tr>
<td>VIIIC</td>
</tr>
<tr>
<td>VIIIR:Ag</td>
</tr>
<tr>
<td>VIIIR:WF</td>
</tr>
<tr>
<td>VII + X</td>
</tr>
<tr>
<td>V</td>
</tr>
<tr>
<td>II</td>
</tr>
<tr>
<td>Fibrinogen</td>
</tr>
</tbody>
</table>

* The low values obtained relative to the starting cryosupernatant are due to an approximate 25% dilution resulting from the immunodepletion procedure. The mean values were obtained from 9 to 12 batches of immunodepleted plasma.
† Lower limit of detection of the assay.
‡ Not determined.
FACTOR VIIIC (UNITS/mL) WITH V VIII - DEPLETED PLASMA

FACTOR VIIIC (UNITS/mL) WITH HEMOPHILIC PLASMA

Fig. 1. A comparison of the results obtained in factor VIIIC assays when either factor-VIII-immunodepleted cryosupernatant plasma or severe hemophilic plasma was the substrate plasma.

There were no statistically significant differences between values obtained with the two types of plasmas at the 0.05 level using the Wilcoxon test. Similar results were obtained when whole plasma completely immunodepleted of factor VIII was used as substrate plasma. However, the use of whole plasma required a lower flow rate (3 ml/hr) and gave substantially reduced volumes of immunodepleted plasma. When whole plasma was subjected to immunodepletion at a flow rate of 8 ml/hr, only 10–15 ml of plasma was completely depleted of factor VIII. Whole plasma processed at 8 ml/hr yielded plasma only partially depleted of factor VIII. This plasma was thus unsuitable for one-stage factor VIII assays. A second passage of the partially depleted plasma on anti-factor-VIII-Sepharose was required to achieve complete removal of factor VIII. This recycling of partially depleted plasma then resulted in a further dilution of the plasma.

Cryosupernatant plasma immunodepleted on anti-factor-VIII immobilized on AH-Sepharose 4B, CH-Sepharose 4B, and Afi-Gel-10 were unsuitable as substrate plasma for factor VIII assays because of the loss of additional clotting factors during immunodepletion.

von Willebrand Factor Assays

The method of McFarlane and Zucker was employed in determining the levels of ristocetin cofactor in the immunodepleted plasmas. Plasma immunodepleted of factor VIII did not support the aggregation of fixed human platelets in the presence of ristocetin. Factor-VIII-depleted plasma was also used as a diluent of pooled normal plasma instead of buffer in deriving standard curves for the levels of ristocetin cofactor. The predicted ristocetin cofactor levels were achieved in all cases, indicating that no detectable anti-factor-VIII was eluted from the anti-factor-VIII column. When factor-VIII-depleted plasma was used as a diluent for pooled normal plasma adjusted to contain 20% VIIIIR:WF, the mean factor VIIIIR:WF was found to be 17.4 ± 2.3 (1 standard deviation) in 17 separate determinations performed over a 10-wk period.

DISCUSSION

Rabbit anti-human factor VIII was coupled to CL-Sepharose 4B, AH-Sepharose, CH-Sepharose and Afi-Gel-10. Of the four types of immobilized anti-factor-VIII used, only anti-factor-VIII coupled to CL-Sepharose 4B resulted in the specific immunodepletion of plasma factor VIII. The use of AH-Sepharose 4B resulted in the removal of the vitamin-K-dependent clotting factors in addition to the factor VIII. This is most likely due to the presence of residual amino groups on this type of Sepharose. When CH-Sepharose 4B and Afi-Gel-10 were used to immobilize anti-factor-VIII, the loss of factor XI activity probably resulted from the presence of residual carboxylate groups.

Anti-factor-VIII was not detected in the immunodepleted plasma, as no detectable lowering of the factor VIIIC or VIIIIR:WF values was evident in comparative assays. Immobilization of anti-factor-VIII on CL-Sepharose 4B thus permits the removal of factor VIII in its various functional forms without the introduction of antibodies in the plasma, as is the case when antibody is simply added to the plasma to derive substrate plasma for factor VIII assays. In addition to its use as a substrate for factor VIIIC assays and as a diluent for ristocetin cofactor assays, the plasma immunodepleted on Sepharose 4B has also been used as an immunoabsorbent in the preparation of monospecific antisera to human factor VIII.

Solid-phase immunoabsorption has been used to derive factors VII and XII deficient plasmas for use as substrate plasmas for factors VII and XII assays, respectively. This procedure has also been used to derive factor-VIII-deficient plasma using anti-factor-VIII coupled to CL-Sepharose 4B. The use of CL-Sepharose 2B, however, resulted in the removal of fibrinogen in addition to factor VIII. Other investigators have used anti-factor-VIII-Sepharose 4B to immunodeplete plasma of factor VIII. However, these
investigators explored the use of antisera raised against specific functional entities of factor VIII to determine structure–function relationships. While these investigators suggest that antibody coupled to Sepharose 4B could provide factor-VIII-immunodepleted plasma for reagent use, no data are presented.

The use of cryosupernatant plasma where the initial levels of factor VIII was reduced before immunodepletion made it possible to increase the flow rate to 8 ml/hr from the 3 ml/hr required with whole plasma. The use of cryosupernatant plasma also enabled immunodepletion of factor VIII in a single filtration step, thus eliminating the need for recycling, as suggested by Bouma et al. The use of cryosupernatants as the starting plasmas for immunodepletion thus yielded larger volumes of plasma suitable as substrate plasma for one-stage factor VIII assays. Thus, the process of immunodepletion may prove to be of general applicability for the production of various deficient plasmas where the plasma constituent in question occurs in the microgram per milliliter range. These results show that the immobilization of anti-factor-VIII antibodies on CL-Sepharose 4B is a useful step for the preparation of factor-VIII-free plasma for reagent use.

ACKNOWLEDGMENT

The help of J. Russett and M. Johnston in the performance of the von Willebrand's factor and coagulation factor assays is gratefully acknowledged.

REFERENCES

Immunodepletion of human plasma factor VIII

F Ofosu, K Cassidy, MA Blajchman and J Hirsh