A Technique for Specific Removal of Factor IX Alloantibodies From Human Plasma: Partial Characterization of the Alloantibodies

By Bo Theodorsson, Ulla Hedner, Inga Marie Nilsson, and Walter Kisiel

A method for specific removal of large amounts of factor IX:C alloantibodies by a resin to which highly purified factor IX was linked (factor IX CH-Sepharose) is described. Factor IX was isolated from human plasma by a three-step procedure, including barium citrate adsorption and elution, DEAE-Sepharose CL-6B chromatography, and dextran sulfate agarose chromatography. Approximately 100 mg factor IX was obtained from 60 liters of plasma. The preparation was about 95% pure as judged by SDS-PAA gel electrophoresis. Its specific coagulant activity was 160 U/mg (IX) and its factor IX clotting antigen (IX:Ag) 500–600 U/mg. Essentially quantitative coupling of the factor IX preparation to activated CH-Sepharose 4B was obtained (4 mg factor IX/ml gel: 2300–3000 U/IX:Ag/ml). This resin bound 1500–2000 U factor IX inhibitor/ml gel and could be re-used at least 5 times without any loss in binding capacity. The binding capacity was dependent on the flow rate. No signs of activation of the coagulation, fibrinolytic, or complement system were observed in vitro. Using this factor IX resin, factor IX alloantibodies were isolated and found to consist of two portions, one minor bound to the resin only in the presence of Ca\(^{2+}\) and another major portion Ca\(^{2+}\)-independent. The specific inhibitory activity/milligram IgG of the Ca\(^{2+}\)-dependent alloantibodies was about 5 times higher in the presence of Ca\(^{2+}\). It is concluded that 25 ml of the factor IX resin described can remove about 40,000 factor IX inhibitor units (comparable to 120,000 Bethesda U) in one run, provided the flow rate does not exceed 20 ml/hr. By using such a technique for removal of antibodies it seems feasible to convert hemophilia-B patients complicated with inhibitors against factor IX into ordinary hemophilia-B patients for treatment at an emergency or in association with major surgery.

ONE OF THE GREATEST challenges in the treatment of patients with hemophilia-B, an inherited deficiency of factor IX coagulant activity (IX:C), involves those patients who synthesize circulating IgG antibodies to the factor IX protein (IX:Ag). In most of the cases studied, these alloantibodies are polyclonal in nature, belonging to the IgG4 subclass containing both kappa and lambda light chains. Recently, a subpopulation of factor IX alloantibodies has been described whose activity is expressed and quantitated in the presence of divalent metal ions. Presumably, these alloantibodies are directed against that region of the factor IX molecule that contains the \(\gamma\)-carboxyglutamic acid residues.

The appearance of circulating alloantibodies to factor IX in hemophilia-B occurs most frequently in patients with severe hemophilia, and these cases continue to pose a substantial problem to the clinician in life-threatening bleeding episodes. While it is possible in most cases to neutralize the alloantibodies and restore hemostasis by conventional replacement therapy, some patients with high alloantibody titers necessitate lowering or removal of the alloantibody prior to factor IX administration. Adsorption of the alloantibody to protein-A-Sepharose CL-4B has been employed successfully in one patient, permitting replacement therapy prior to orthopedic surgery. Clearly, there is a need for a more potent, selective immunoadsorbent in reducing antibody levels, particularly in those patients subjected to extended periods of treatment with secondary response levels of alloantibodies. This article describes a procedure for the isolation and immobilization of human factor IX and emphasizes the clinical potential of this resin for removing high titer alloantibodies to factor IX extra-corporeally. In addition, information gathered from experiments regarding the nature of the alloantibodies derived from such resins is also presented.

MATERIALS AND METHODS

DEAE-Sepharose CL-6B, Sepharose 4B, dextran sulfate, agarose B (low electroendosmosis), CNBr-activated Sepharose 4B, activated CH-Sepharose, protein-A-Sepharose CL-4B, and low molecular weight sodium dodecyl sulfate (SDS) electrophoresis standards were products of Pharmacia (Uppsala, Sweden). d-Phenylalanyl-l-pipe- coyl-t-arginine-p-nitroanilide (S-2238) and H-d-valyl-leucyl-l-lysine-p-nitroanilide (S-2251) were obtained from KabiVitrum (Stockholm, Sweden). Antisera against human \(\beta\)-lipoprotein, human IgG, and to human IgG\(k\)-light chains and IgG\(\lambda\)-light chains were obtained from Dakopatt (Copenhagen, Denmark). Bovine serum albumin, Coomassie Brilliant Blue G-250, factor-H-deficient bovine plasma, factor-X-deficient bovine plasma, Tris (Trizma) base, and benzamidine hydrochloride were purchased from Sigma (St. Louis, MO). Affi-Gel 10, acrylamide, N,N'-methylenbisacryl-
amide, N,N,N',N'-tetramethyl-ethylenediamine, and ammonium persulfate were obtained from Bio-Rad (Richmond, CA). Sodium dodecyl sulfate (SDS) was from British Drug House (Poole, England). A monospecific, precipitating antibody against human factor IX was prepared by immunization of a goat with human factor IX as previously described. The goat antibody against factor IX was affinity purified by chromatography of the antiserum on a human factor-IX-Sepharose column (described below) equilibrated with TBS (0.05 M Tris-HCl, pH 7.5/0.15 M NaCl)/Na2N. Anti-factor-IX was eluted from this column at 4°C with 0.1 M glycine-HCl (pH 2.5), and collected in 0.5 M Tris-HCl (pH 8.5). The eluted anti-factor-IX was precipitated by the addition of an equal volume of saturated ammonium sulfate solution, collected by centrifugation, and stored at -70°C in TBS/Na2N. Human IgG was isolated from normal plasma and from plasma containing factor IX alloantibody by adsorption to protein-A-Sepharose CL-4B columns followed by elution with 0.1 M glycine-HCl (pH 2.5). The IgG fraction was collected in tubes containing an aliquot of 0.5 M Tris-HCl (pH 8.5), sufficient to immediately neutralize the low pH glycine. Dextran sulfate agarose was prepared as described.

The total cholesterol and triglycerides were assayed principally according to Smith et al.

Plasma

Plasma was prepared from normal individuals and hemophilia-B patients with inhibitors to factor IX by withdrawing nine parts of whole blood from the antecubital vein into polyethylene tubes containing one part 3.8% sodium citrate, followed by centrifugation at 2400 g for 20 min at 4°C. Pooled, citrated plasma from 20 normal individuals was used as a reference base for the coagulation assays and was arbitrarily defined as containing 1 U clotting factor/ml plasma.

Coagulation Assays

Activated partial thromboplastin time (APTT, General Diagnostics Automated APTT) was assayed according to the manufacturer's specifications. Factor XII, factor XI, factor V, Owen's P&P (prothrombin plus factor VII plus factor X), fibrinogen, thrombin time, reptilase time, and fibrinogen/fibrin degradation products (FDP) were assayed as previously described. Factor VIII coagulant activity (VIII:C) and factor-VIII-related antigen (VIIIIR:Ag) were assayed according to Nilsson and Holmberg et al., respectively. Antithrombin-III and α2-antiplasmin were assayed by electroimmunoassay as well as by amidolytic assay employing S-2238 and S-2251, respectively. Factor X and prothrombin were assayed by one-stage assays according to Bachmann et al. and Hjort et al., respectively. Factor IX coagulant activity (IX:C) was assayed by a one-stage assay, and factor IX antigen (IX:Ag) was assayed by the immunoradiometric assay as described by Holmberg et al.

Quantitative Determination of Inhibitors of Factor IX

Inhibitors to factor IX in plasma samples were quantitated as described by Nilsson and Hedner. The inhibitory activity of the plasma was expressed as the number of units of IX:C inactivated by 1 ml of the plasma. The corresponding Bethesda units of inhibitor/milliliter is obtained by multiplying by a factor of 3. For assessing the inhibitory capacity of factor IX alloantibody isolated by protein-A-Sepharose or factor-IX-Sepharose column chromatography, a modification was used. In this assay, all anti-factor-IX IgG samples were dialyzed against TBS prior to assay. Into each of 3 plastic tubes, an 80-μl aliquot of an appropriate dilution of anti-factor-IX IgG was mixed with 15 μl Preconativ (KabiVitrum) containing 5 U factor IX/ml. To 1 tube, 5 μl of 200 mM CaCl2/TBS was added; to the second, 5 μl of 200 mM EDTA/TBS was added; to the third, 5 μl of TBS was added. Each system was incubated at 37°C for 15 min. Following the incubation, the samples were diluted 1/5 and 1/10 with TBS (or TBS/1 mM CaCl2 in the calcium chloride incubation mixture) and the remaining factor IX:C activity assayed as regards its ability to correct the recalcification time of platelet-rich hemo-phil-B plasma (<1% IX:C). All assays were performed in the presence of 1% bovine serum albumin (BSA). Factor IX:C activity remaining in each incubation mixture was quantitated from a standard curve generated from various dilutions of Preconativ incubated under identical conditions as the test samples, but in the presence of IgG (0.2 mg/ml) prepared from normal plasma by protein-A-Sepharose as described above. The specific activity of the anti-factor-IX IgG was expressed as the number of units of factor IX inactivated by 1 mg of the anti-factor-IX IgG preparation. The protein content of anti-factor-IX IgG preparations was determined by the method of Bradford using bovine serum albumin as the reference protein.

Immunochromat Assays

The rocket technique of Laurell was used for assaying human IgG employing a rabbit antiserum against purified IgG (Dakopatts). Human IgG samples obtained from protein-A-Sepharose or factor-IX-Sepharose columns were carbamylated prior to electrophoresis essentially by the procedure described by Laurell. Cross-immunoelectrophoresis of α2-antiplasmin was performed according to Ganrot. IgG λ-light chains and IgG κ-light chains were assayed according to Laurell using the rocket technique, as was protein C during the factor IX purification procedure. Activation of complement factor 3 was assessed by cross-immunoelectrophoresis as described.

Purification of Human Factor IX

Human plasma employed in the isolation of factor IX was obtained by therapeutic plasmapheresis from patients with acute glomerular nephritis. All patients were negative when checked for HbsAg and HbsAb. A Haemonetic Model A-30 Blood Processor was used to separate ACD anticoagulated blood into its cellular and plasma components. The plasma was stored at -20°C, and routinely, 20 liters were thawed overnight at room temperature, the cryoprecipitate removed by centrifugation (5000 g), and the cryosupernatant used for the isolation of factor IX. All steps in the purification procedure were performed at 4°C. Next, 1600 ml of 1 M BaCl2 was added to 20 liters of human citrated cryosupernatant and the mixture stirred for 60 min. The further purification procedure included ammonium sulfate precipitation, ion exchange chromatography on DEAE-Sepharose CL-6B, and dextran sulfate agarose chromatography principally as previously described. The dextran sulfate agarose chromatography step was performed essentially as described by Pepper and Prowse. Those fractions from the dextran sulfate agarose column containing factor IX were pooled, made 10 mM in benzamidine HCl, and 65% saturated in ammonium sulfate by the addition of the solid salt. This mixture was stirred for 60 min and centrifuged (16,000 g, 15 min). The pellet was redisolved in 50 ml of 0.1 M NaHCO3 (pH 8) containing 0.5 M NaCl and stored at -70°C until further use.

Preparation of Human Factor-IX-Sepharose

Human factor IX was coupled to activated CH-Sepharose essentially by the procedure recommended by the manufacturer. Approximately 350 mg of human factor IX obtained from dextran sulfate
agarose chromatography, dissolved in 75 ml of 0.1 M NaHCO₃ (pH 8)/0.5 M NaCl, was sterilized by filtration through a Millex HA-0.45 μm membrane (Millipore S.A., Molsheim, France) into a sterile plastic culture flask in a laminar airflow hood. Under aseptic conditions, 30 g of hydrated and washed activated CH-Sepharose containing (-Tris-HCl (pH 8)/0.5 M NaCl. After settling of the resin, the supernatant was removed and the resin was resuspended in 200 ml sterile 20 mM sodium phosphate (pH 6.5) containing 150 mM NaCl and 0.2% methyl p-hydroxybenzoate (Merck).

Two other factor-IX–Sepharose batches were prepared for analytical purposes in parallel with the large scale preparation described above. The same factor IX preparation was used, and the procedure was the same except that some procedures were not followed. These resins were stored in 0.1 M Tris-HCl (pH 8.0)/0.5 M NaCl/0.01 M benzamidine/0.02% NaN₃.

In Vitro Effect of Factor-IX–Sepharose on Various Coagulation System Parameters

Inasmuch as our long-range plan included use of the factor-IX–Sepharose column to remove factor IX alloantibody extracorporeally, it was important to test this resin in vitro as regards its potential to affect either the coagulation or complement system. Two different approaches were taken to evaluate this possibility. In the first approach, 0.5 ml of factor-IX–Sepharose was incubated for 3 hr with 12 ml of normal human plasma at room temperature in a plastic culture tube on a reciprocating shaker. In order to evaluate the effect of the Sepharose on the system, 0.5 ml of Sepharose 4B was incubated with 12 ml of normal human plasma at room temperature in a plastic culture tube on a reciprocating shaker. In order to evaluate the effect, if any, that successive sample applications and desorptions from the factor-IX–Sepharose had on subsequent plasma samples passed through the resin. In a 3-ml plastic syringe, 0.5 ml of factor IX Sepharose was packed and washed with TBS.

Two-hundred microliters of normal plasma were passed through the column, followed sequentially by 0.05 M Tris-HCl (pH 7.5)/0.5 M NaCl and TBS. The column was eluted next with 10 ml of 0.1 M glycine-HCl (pH 2.5), followed by regeneration with TBS. Following regeneration, a new 1-ml aliquot of plasma was passed through the column and then the wash (desorption/regeneration) processes repeated. Then, a third 1-ml aliquot of plasma was passed through the column. All three plasma samples passed through the column were then evaluated by the same battery of tests as in the first approach. These values were compared to three plasma samples passed through a control column of Sepharose 4B (0.5 ml) (Table 2).

### Table 1. The Effect of Factor-IX–Sepharose on Various Coagulation Parameters

<table>
<thead>
<tr>
<th>System</th>
<th>Coagulation Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor-IX–Sepharose</td>
<td>APTT (sec)</td>
</tr>
<tr>
<td>+ 12 ml plasma</td>
<td>46</td>
</tr>
<tr>
<td>Sepharose 4B</td>
<td>45</td>
</tr>
<tr>
<td>Control plasma (incubated at RT)</td>
<td>41</td>
</tr>
</tbody>
</table>

APT'T: VIII:C, IX:C, VII, X, V, fibrinogen, antithrombin III, P&P, XII, XI, thrombin time, reptilase time, and α₂-antiplasmin. The results from each test system (factor-IX–Sepharose and Sepharose 4B) were compared to those obtained from a plasma sample incubated in parallel with the two test systems for 3 hr in a plastic tube with no shaking (Table 1). The second approach was aimed at evaluating the effect, if any, that successive sample applications and desorptions from the factor-IX–Sepharose had on subsequent plasma samples passed through the resin. In a 3-ml plastic syringe, 0.5 ml of factor IX Sepharose was packed and washed with TBS. Twelve milliliters of normal plasma were passed through the column, followed sequentially by 0.05 M Tris-HCl (pH 7.5)/0.5 M NaCl and TBS. The column was eluted next with 10 ml of 0.1 M glycine-HCl (pH 2.5), followed by regeneration with TBS. Following regeneration, a new 1-ml aliquot of plasma was passed through the column and then the wash (desorption/regeneration) processes repeated. Then, a third 1-ml aliquot of plasma was passed through the column. All three plasma samples passed through the column were then evaluated by the same battery of tests as in the first approach. These values were compared to three plasma samples passed through a control column of Sepharose 4B (0.5 ml) (Table 2).

### Capacity Studies on the Factor-IX–Sepharose and the Effect of Repeated Alloantibody Adsorption and Desorption

The alloantibody binding capacity of the factor-IX–Sepharose was determined as follows. Two-hundred microliters of factor-IX–Sepharose was thoroughly equilibrated with TBS/Na₃ in a 10-ml plastic disposable column. To this resin was added 8 ml of high-titer factor IX alloantibody plasma. The column was sealed and placed on a reciprocating shaker at room temperature for 2 hr. The gel was allowed to settle for 1 hr, after which the plasma was eluted and assayed for factor IX:C inhibitor activity. This value was compared to the inhibitor activity of the original plasma sample, and the effect of repeated addition of plasma was repeated. Then, a third 1-ml aliquot of plasma was passed through the column. All three plasma samples passed through the column were then evaluated by the same battery of tests as in the first approach. These values were compared to three plasma samples passed through a control column of Sepharose 4B (0.5 ml) (Table 2).

### Table 2. The Effect of Regeneration of the Factor-IX–Sepharose Immunoaffinity Column on the Coagulation Parameters of Future Plasma Samples

<table>
<thead>
<tr>
<th>Resin</th>
<th>Coagulation Parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor-IX–Sepharose</td>
<td>APTT (sec)</td>
</tr>
<tr>
<td>(1)*</td>
<td>41</td>
</tr>
<tr>
<td>(2)</td>
<td>42</td>
</tr>
<tr>
<td>(3)</td>
<td>39</td>
</tr>
<tr>
<td>Sepharose 4B control</td>
<td>(1)</td>
</tr>
<tr>
<td>(2)</td>
<td>46</td>
</tr>
<tr>
<td>(3)</td>
<td>47</td>
</tr>
</tbody>
</table>

*Number in parentheses refers to the number of the 12-ml plasma sample applied in succession to the gel.

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the difference in inhibitor content before and after factor-IX-Sepharose treatment was assumed to represent the amount of alloantibody bound specifically to the resin.

Isolation of Factor IX Alloantibodies

The resins prepared for analytical reasons were tested for binding capacity by passing inhibitor plasma (2 ml; inhibitor level: 50 U/ml) through the column. After extensive wash (0.05 M Tris-HCl, pH 7.5/0.5 M NaCl and 0.05 M Tris-HCl, pH 7.5/0.15 M NaCl) the bound alloantibodies were eluted with 0.1 M glycine-HCl (pH 2.5). Recovery of the eluted antibody was assayed in the eluate immediately adjusted to pH 7.4 by addition of 0.5 M Tris-HCl (pH 8.5). The procedure was repeated after regeneration of the resin by 0.1 M Tris-HCl (pH 7.5)/0.5 M NaCl, followed by washing with 0.1 M Tris-HCl (pH 7.5)/0.15 M NaCl all together 5 times.

The influence of flow rate on the factor IX alloantibody binding capacity was studied on one of the batches of factor-IX-Sepharose resins prepared for analytical purposes. Three different columns were made (1 ml, 1.6 x 0.5 cm each). Through each of these columns, 150 ml of plasma containing factor IX alloantibodies (6 U/ml plasma) was passed at different flow rates. The first column was run at a flow rate of 15 ml/hr (7.5 ml/sq cm/hr), the second 56 ml/hr (28 ml/sq cm/hr), and the third one at a flow rate of 196 ml/hr (98 ml/sq cm/hr). The amount of factor IX alloantibodies in the plasma was assayed after passage through the column, and the amount of alloantibodies removed from 100 ml of plasma was given.

Factor IX alloantibody was isolated by immunoaffinity chromatography from 100 ml of plasma obtained from a patient with high-titer alloantibodies. In order to quantitate the relative amount and activity of the putative alloantibody directed against the γ-carboxyglutamic acid region of the factor IX molecule, the total IgG from the patient's plasma was removed by adsorption and elution from protein-A-Sepharose as described above. The IgG/alloantibody fraction was dialyzed at 4°C against 2 liters of TBS/1 mM CaCl₂. The dialyzed sample was applied to a factor-IX–Sepharose column (1.6 x 1.3 cm) previously equilibrated at room temperature with TBS/1 mM CaCl₂. Following sample application, the column was washed with 50 mM Tris-HCl (pH 7.5) containing 0.5 M NaCl and 1 mM CaCl₂ to remove IgG nonspecifically bound to the resin. The column next was eluted with TBS/10 mM EDTA to elute protein bound to the resin via calcium ions. Following regeneration of the column with TBS, the remaining IgG was eluted from the column with a linear decreasing pH gradient generated from 40 ml of 0.2 M sodium phosphate (pH 7.2) and 40 ml of 0.2 M citric acid (pH 2). Flow rate was maintained at 0.5 ml/min by a peristaltic pump.

RESULTS

Preparation of Human Factor IX

Factor IX was isolated from human plasmapheresis plasma by a three-step procedure that included barium citrate adsorption and elution, DEAE–Sepharose CL-6B chromatography, and dextran sulfate agarose chromatography. Approximately 100 mg of purified factor IX was routinely obtained from 60 liters of plasma.

In the isolation procedure, factor IX was eluted from the barium citrate by ammonium sulfate essentially as described by Miletich et al. The procedure was adopted in preference to fractional ammonium sulfate precipitation in order to avoid difficulties in the sedimentation of the 30%-65% ammonium sulfate pellet. The majority of our attempts to produce a 30%-65% ammonium sulfate pellet from a barium citrate eluant resulted in a floating, lipid-rich protein aggregate following centrifugation at 5000 g for 30 min. Increasing the gravitational force to 15,000 g for 30 min failed to pellet the material completely. Whether this phenomenon relates specifically to the plasma used in these isolation attempts is uncertain.

Human factor IX was partially resolved from protein C and protein S by DEAE–Sepharose chromatography, but coeluted with prothrombin, factor X, and C-reactive protein (an acute phase protein present in the starting plasma). Factor IX was completely resolved from these proteins by dextran sulfate agarose column chromatography (Fig. 1). Factor IX was bound tightly to this resin at pH 7.4 and eluted in the gradient at approximately 0.8 M NaCl. Reduced and unreduced SDS-polyacrylamide gel electrophoresis of the purified factor IX (Fig. 1, inset) revealed that the preparation was about 95% pure. Factor IX exhibited a molecular weight of 65,000 in the presence or absence of mercaptoethanol. Contaminants present in the factor IX preparation invariably were high molecular weight proteins (Mol wt 110,000–130,000 in the presence or absence of reducing agent). SDS-gel electrophoresis of 50 μg of reduced factor IX failed to
reveal the presence of activated factor IX or activation intermediates of factor IX (factor IXα, factor IXαα, or factor IXαβ). The specific coagulant activity of the purified factor IX was about 160 U/mg, whereas the factor IX clotting antigen (IX:Ag), as measured by a solid-phase immunoradiometric method,13 was approximately 500–600 U/mg. The reason for this discrepancy is not fully understood, but in all likelihood reflects denaturation of the protein during purification, which adversely affects its biologic activity to a greater degree than the association of the protein determinants with the alloantibody used in the immunoradiometric assay.

**Preparation of Human Factor IX-Sepharose**

In preliminary analytical scale experiments, human factor IX was reacted at room temperature with three commercially available coupling gels (activated CH-Sepharose 4B, CNBr-activated Sepharose 4B, and Affi-Gel 10) according to the manufacturer's coupling recommendations. In these experiments, the coupling of factor IX to the resin was monitored by electroimmunoassay12 of the postcoupling supernatant and subsequent washes. Essentially quantitative coupling of factor IX was observed in all lots (3) of activated CH-Sepharose 4B and CNBr-activated Sepharose 4B (2 lots) tested. Poor coupling (~20%–30% factor IX bound) was observed with the 2 lots of Affi-Gel 10 that were tested, and this resin was not given further consideration as a preparative matrix. While the factor-IX-Sepharose derived from the reaction of factor IX with either activated CH-Sepharose or CNBr-activated Sepharose possessed roughly equal inhibitor binding capacity (1500–2000 U/ml gel corresponding to 4500–6000 Bethesda inhibitor U/ml gel), the activated CH-Sepharose 4B was chosen over the CNBr-activated Sepharose 4B for a preparative scale column on the basis of the theoretical bond stability between ligand and matrix at neutral to slightly alkaline pH values (amide linkage versus isourea linkage, respectively). Using this technique, two factor-IX-Sepharose batches (4 mg factor IX/ml gel) were prepared for analytical purposes. While the factor-IX-Sepharose was stored at pH 6.5 in the presence of a bacteriostatic agent (methyl p-hydroxybenzoate).

**In Vitro Effect of Factor-IX-Sepharose on Various Coagulation and Complement System Parameters**

When factor-IX-Sepharose or Sepharose 4B was incubated at room temperature with normal plasma for 3 hr, no significant effect on various coagulation parameters was noted relative to a control sample of plasma (Table 1). Furthermore, no activation of complement C3 was noted in the control or test samples. When three different aliquots of plasma were passed through the factor-IX-Sepharose column following recycling of the gel, no significant changes in several coagulation parameters were observed between the three plasma samples relative to three plasma samples passed through Sepharose 4B under the same elution conditions (Table 2). From these observations, it does not appear that the factor-IX-Sepharose, initially or following regeneration procedures, affects the coagulation system or activates the complement system.

**Capacity Studies of the Factor IX Resins**

All these resins were found to bind 1500–2000 U factor IX inhibitor/ml gel (Table 3). Recovery of alloantibodies after elution from the factor IX resin by glycine-HCl was found to be 90%–100% in repeated experiments. Repeated adsorption and desorption of factor IX alloantibodies on one of the resins prepared for analytical purposes showed no loss of binding capacity after 5 consecutive runs. After the fifth run, the recovery of alloantibodies was still 90%.

The binding capacity of factor IX alloantibodies at different flow rates decreased somewhat with increasing flow rate. The amount of alloantibodies bound from 100 ml of patient plasma (6 U/ml factor IX alloantibodies) thus decreased from 570 U at a flow rate of 7.5 ml/sq cm/hr to 480 U at a flow rate of 28

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**Table 3. Capacity of the Factor-IX-Sepharose (1 ml) to Bind Factor IX Alloantibodies From a Patient Plasma**

<table>
<thead>
<tr>
<th>Resin no.</th>
<th>Alloantibodies Applied to 1-ml IX-Sepharose (U)</th>
<th>Alloantibodies Not Bound to 1-ml IX-Sepharose (U)</th>
<th>Alloantibodies Eluted From 1-ml IX-Sepharose (U)</th>
<th>Alloantibodies Bound/ml IX-Sepharose (U)</th>
</tr>
</thead>
<tbody>
<tr>
<td>no. 1</td>
<td>1,690</td>
<td>185</td>
<td>1,600</td>
<td>1,500</td>
</tr>
<tr>
<td>no. 2</td>
<td>2,140</td>
<td>560</td>
<td>1,580</td>
<td>1,500</td>
</tr>
<tr>
<td>no. 3</td>
<td>2,135</td>
<td>250</td>
<td>1,850</td>
<td>1,700</td>
</tr>
</tbody>
</table>
ml/sq cm/hr and to 430 U at a flow rate of 98 ml/sq cm/hr.

Isolation and Partial Characterization of Factor IX Alloantibodies

A human IgG fraction, derived from 100 ml of plasma from a patient with high-titer factor IX alloantibody, was equilibrated with calcium chloride and applied to a column of factor-IX-Sepharose (2.6 ml) previously equilibrated with TBS/CaCl2. Following removal of IgG nonspecifically bound to the resin, the column was treated with TBS/10 mM EDTA to elute the fraction of factor IX alloantibody that bound to the resin in the presence of Ca2⁺ (Fig. 2, fractions 2–7). The remaining factor IX alloantibodies were eluted from the column with a linear decreasing pH gradient. Analysis of the pH of these fractions (data not shown) indicated that the midpoint of this peak coincided with a pH value of 3.5. Fractions 2–7 (alloantibodies only bound to the resin in the presence of Ca2⁺) and fractions 45–60 (alloantibodies also bound to the resin without Ca2⁺ present) were pooled separately, dialyzed against TBS at 4°C, and further characterized as regards protein content, specific inhibitor activity, and antibody light chain composition.

Protein analysis of each antibody pool revealed that about 5% of the total IgG eluted from the column was found in the EDTA peak. The protein concentration of each alloantibody fraction as determined spectrophotometrically³⁷ (fractions 2–7 varied between 0.010 and 0.012 mg/ml; fractions 45–60 between 0.10 and 0.15 mg/ml) agreed reasonably well with the IgG content as determined by electroimmunoassay following carboxymethylation of the sample (fractions 2–7 varied between 0.010 and 0.020 mg/ml; fractions 45–60 between 0.12 and 0.19 mg/ml), indicating that the protein peaks reflect the IgG elution from the resin. Electroimmunoassay of each pool for kappa and lambda light chains revealed the presence of both kappa and lambda light chains in both the EDTA fraction and in the calcium-independent alloantibody fraction. Analysis of each pool for the presence of α-lipoprotein or β-lipoprotein proved negative. Each pool, however, contained significant levels of triglycerides. While cholesterol was detected in the IgG fraction applied to the factor-IX-Sepharose, no cholesterol was detected in either factor IX alloantibody fraction. Presumably, trace amounts of triglyceride-rich lipoprotein adsorb to the factor-IX-Sepharose column that elute in concentrations below the sensitivity of the electroimmunoassay, but sufficient for the more sensitive colorimetric assays for triglycerides and cholesterol.

Each factor IX alloantibody pool was tested for its inhibitory capacity in the assay described in Materials and Methods. Under these conditions, the inhibitory activity in the calcium-dependent alloantibody fraction was about 5 times higher when tested in the presence of calcium than in the presence of EDTA or TBS (Table 4). In the presence of calcium, this factor IX alloantibody fraction exhibited a specific inhibitor activity of about 50 inhibitory U/mg protein assayed as the factor IX:C inhibitory activity in a clotting system as described above.


discussion

Several procedures to lower the antibody in those hemophiliacs having a titer too high to be neutralized by the administration of factor IX concentrates have been considered. Initial studies demonstrated that extensive plasma removal of plasmapheresis resulted in a lowered antibody titer.⁴⁻²⁵ However, the use of plasma exchange alone, or in combination with administration of factor IX concentrates, has inherent limita-

| Table 4. The IX:C Inhibitory Capacity of the Different Pools of Alloantibody (U/mg IgG) |
|-------------------------------|---------|---------|---------|
|                               | in the Presence of Ca²⁺ | of EDTA  | of TBS  |
| Ca²⁺-dependent alloantibody   | 47      | 13      | 15      |
| Ca²⁺-independent alloantibody | 19      | 17      | 15      |
tions. Thus, it has been pointed out that the use of plasmapheresis as a method of lowering the antibody level is useful when the patient's inhibitor level is at its basic level, or approaching it after an earlier boost, but not in the patients in whom the antibody is rising. In such patients, the lowering effect achieved by plasmapheresis is likely to be compensated by the intensive antibody synthesis occurring in these cases. Furthermore, in most cases, serial plasmapheresis treatment has to be performed to achieve the necessary reduction of the antibody titer. Accordingly, such a treatment can rarely if ever be used in emergency situations and certainly not during a period of anamnestic antibody production. Furthermore, a prolonged serial plasmapheresis treatment is virtually impossible to carry out in small children. In these situations, a more effective way of reducing the antibody titer is obviously required. By means of extracorporeal immunoabsorption, the antibodies may be removed from the patient's plasma, and the plasma subsequently can be returned to the patient. Such an arrangement will allow much larger volumes of plasma to be cleared of their antibody content in a shorter period of time. Extracorporeal adsorption of antibodies against factor IX on protein-A-Sepharose was used in a hemophilia-B patient prior to orthopedic surgery. However, this kind of immunoabsorption removes not only the alloantibodies against factor IX, but also all gammaglobulins with the exception of those belonging to the subclass IgG3. Conceivably, under these conditions, the patient may become increasingly susceptible to infections. Therefore, a specific immunoabsorption on purified factor-IX-Sepharose should be the procedure of choice. Using such a technique, an extensive specific removal of the antibodies against factor IX can be achieved without the potentially deleterious side effects described above. The capacity of the factor-IX-Sepharose presented in this study will permit the removal of roughly the total plasma content of alloantibodies against factor IX in a patient (60 kg body weight) having an inhibitor level of about 10 U/ml plasma (corresponding to about 30 Bethesda U/ml plasma) by a single passage through 25 ml of factor-IX-Sepharose gel. In fact, 25 ml of the resin can remove about 40,000 inhibitor units (120,000 Bethesda U) in one run, provided the flow rate does not exceed 20 ml/hr. However, in order to be able to perform the whole treatment within a reasonable period of time (2 hr.), a flow rate in the order of 50 ml plasma/min is required. At this flow rate, a lower capacity must be expected. However, in most cases, it will be more than satisfactory to be able to remove substantially less than 40,000 U in one run. Furthermore, the gel was easily regenerated by glycine-HCl, making it strategically possible to reuse the resin within a short period of time if necessary. It should be stressed that two runs may be necessary, or at least advisable, in order to eliminate the alloantibodies distributed extravascularly.

The specific immunoabsorption prepared makes it feasible to treat hemophilia-B patients with a high titer of factor IX alloantibodies efficiently even under emergency conditions. By this technique, such patients temporarily are converted into ordinary hemophilia-B patients or at least to patients with a low inhibitor titer. Excessive doses of commercial factor IX concentrates do initiate an activation of the coagulation system with, the appearance of circulating fibrin monomers. By lowering the antibody titer by specific immunoabsorption, it may be possible to reduce the doses of factor IX concentrate necessary for treatment of patients with high antibody titer.

Plasma separation techniques per se have been shown to induce major complement activation under certain conditions. However, these investigators also showed that addition of citrate to the plasma prevented such an activation. Our in vitro experiments, exposing the factor IX resin to citrated normal plasma, failed to show any signs of complement activation. Accordingly, it should be emphasized that any treatment with specific extracorporeal adsorption of alloantibodies on a resin should be performed in the presence of citrate ions. The presence of citrate may also prevent any activation of the coagulation and/or fibrinolytic systems, since no indications of such an activation were found on repeated exposure of normal citrated plasma to the factor IX resin. Such an activation was found on intensive plasma exchange on the cell separator by Chirnside et al. In their system, however, heparin was used as an anticoagulant.

A small subpopulation of alloantibodies seems to be directed against neoantigens in the factor IX molecule, revealed as the result of Ca\(^{2+}\) binding. Our studies of the alloantibodies adsorbed specifically on the factor IX resin confirmed the existence of a minor fraction bound to factor IX in the presence of calcium ions. The specific activities of those alloantibodies were 2–3 times higher in the presence of Ca\(^{2+}\) than in the presence of EDTA or TBS. On the contrary, the major fraction of alloantibodies was not influenced by the presence of Ca\(^{2+}\). In this study, total fraction of IgG bound to the resin only in the presence of Ca\(^{2+}\) did not exceed 5% of the total IgG. Also, if this IgG fraction had the highest specific activity as measured as inhibitory capacity against IX:C/mg IgG (Table 4), the total amount of this alloantibody population in the patient is lower than that of the alloantibodies also bound without Ca\(^{2+}\) present.
Both fractions of alloantibodies purified in our system by specific adsorption on a factor IX resin had both types of light chains. These findings are at variance with earlier results reported by Pike et al., who found only one heavy chain subclass and one single light chain type in human factor IX alloantibodies. However, using a cross-immunoelectrophoretic technique and enzyme-conjugated antisera, Ørstavik also found five different alloantibodies against factor IX to be polyclonal. In fact, one of the patients examined by her was the one described in the present report. Furthermore, it was pointed out that the factor IX alloantibodies produced following an anamnestic response may become increasingly heterogeneous. 2

In summary, this article described a method for specific removal of high amounts of factor IX:C alloantibodies by a resin on which highly purified factor IX was linked (factor-IX-CH-Sepharose). The capacity of this resin was high enough to remove about 40,000 inhibitor units (assayed with the method described by Nilsson and Hedner corresponding to about 120,000 Bethesda U.) in one run through 25 ml of factor IX resin, making it possible to convert a patient with an extremely high inhibitor level (≥ 10 U/ml plasma; ≥ 30 Bethesda U/ml plasma) into an ordinary hemophilia-B patient within hours of time. In fact, a resin prepared essentially in the same way was recently successfully used in a patient having an inhibitor titer against IX:C of 60 U/ml (~ 180 Bethesda U/ml plasma). These results will be published separately. Provided the procedure is performed in the presence of citrate, neither activation of the complement system nor of the coagulation/fibrinolytic systems seemed to occur. Furthermore, a small proportion (~ 5%) of the alloantibodies were found to bind to factor IX only in the presence of Ca2+, confirming earlier suggestions of an antigenic site in the γcarboxyglutamic acid region of the factor IX molecule.

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A technique for specific removal of factor IX alloantibodies from human plasma: partial characterization of the alloantibodies

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