Immonoaffinity Purification of Factor IX From Commercial Concentrates and Infusion Studies in Animals

By Kenneth J. Smith

Thrombosis and transmission of viral diseases are the principal adverse effects of current replacement therapy for factor IX deficiency when using heat-treated concentrates of vitamin K–dependent coagulation factors. More highly purified factor IX preparations could decrease the risk of disease transmission, reduce patient exposure to allogeneic proteins, and reduce the risk of thrombosis. In this study, two immunoaffinity-purified factor IX preparations from commercial vitamin K–dependent coagulation factor concentrates had specific activities of 134 and 155 U/mg. Crude concentrates and purified factor IX preparations were tested for thrombogenicity in rabbits. One of two crude concentrates tested in the stasis-thrombosis assay caused large thrombi at doses of 50 U/kg. Purified factor IX from this concentrate was not thrombogenic at 106 to 234 U/kg. A heparin-treated concentrate that was not active in the stasis model at 100 U/kg caused significant (P < .05) delayed consumption of rabbit fibrinogen, platelets, antithrombin III antigen, and factor VIII activity at the same dose. Factor IX prepared from this concentrate caused no consumption of coagulation factors at 214 to 243 U/kg despite the presence of trace amounts of activated factor IX. These results indicate that more highly purified preparations could reduce the risk of thrombosis in replacement therapy for hemophilia B. Also, at least for the preparations tested, factor IX and factor IXa were not the thrombogenic components of the crude concentrates.

Highly purified factor IX can be produced by monoclonal antibody immunoaffinity techniques using currently available therapeutic materials as the factor IX source. Potential advantages of highly purified factor IX for therapeutic use are decreased disease transmission, less exposure to extraneous allogeneic protein antigens, and reduced risk of thrombosis.

Physical separation of factor IX from detectable hepatitis B surface antigen and human immunodeficiency virus (HIV) antigen can be accomplished with immunoaffinity purification using calcium- or magnesium-dependent specific rabbit antibodies to factor IX. Purification can be as high as 1,000-fold when HIV antigen is added to a plasma source of factor IX and could aid in reducing the risk of virus transmission when combined with procedures for virus inactivation of the product for transfusion.

Current therapeutic materials have a specific factor IX activity of 1 to 2 U/mg. A recently described therapeutic factor IX concentrate prepared by using diethyl aminoethyl (DEAE)-Sephadex batch adsorption and sulfated dextran chromatography had a specific activity of approximately 9 U/mg. This preparation had markedly decreased thrombogenicity in rabbits when compared with the material processed by DEAE-Sephadex alone.

In the current report, monoclonal antibody immunoaffinity purification of factor IX was tested as a means of reducing the thrombotic risk of replacement therapy since this method can be readily adapted to produce factor IX in large amounts. High–specific activity factor IX preparations (greater than 100 U/mg) and the crude concentrates from which they were purified were tested for activity in the stasis thrombosis assay and for delayed effects on the rabbit coagulation system.

MATERIALS AND METHODS

Materials. Affigel 10 and Affiprep 10 were from Bio-Rad, Richmond, CA. Staphylococcal protein A–Sepharose CL-6B was from Pharmacia CBK Biotechnology Laboratories, Piscataway, NJ. Peroxidase was from Boehringer Mannheim, Indianapolis, IN. Biotin- N-hydroxysuccinimide ester was from Biosearch, San Rafael, CA. Peroxidase-conjugated streptavidin was from Bethesda Research Laboratories (BRL), Gaithersburg, MD. Heparin was from Wyeth Laboratories, Philadelphia. o-Phenylene-diamine, rabbit brain cephalin, Tween 20, and o-ovalbumin (grades II and III) were from Sigma Chemical, St Louis. Benzamidine-HCl was from Aldrich Chemical Co, Milwaukee. All other chemicals were of the highest available purity from either Fisher Scientific, Pittsburgh, or Sigma. Outdated commercial concentrates of factor IX were from Armour Pharmaceutical, Inc, Kankakee, IL, or from Cutter Biological, Berkeley, CA. The concentrate from Armour contained added heparin. Neither concentrate was heat treated.

Immobilized A-7 antibody. A metal ion–dependent mouse monoclonal antibody that reacts with an epitope on the light chain of factor IXa and does not react with heat-decarboxylated factor IX was prepared from ascites fluid. All purification steps were at 4°C. After centrifugation at 40,000 g for 30 minutes, ascites fluid was filtered through a 0.45-μm filter (Nalgé, Rochester, NY) and applied to a 10-mL column of protein A–Sepharose in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2) (TBS). The column was washed with TBS until the absorbance at 280 nm was less than 0.01. Bound antibody was eluted with 0.1 mol/L glycine-HCl (pH 2.6) into collection tubes containing 0.1 mL 0.5 mol/L K2HPO4. Fractions containing antibody were dialyzed overnight in 0.1 mol/L HEPES (pH 7.0). Antibody was coupled to Affigel 10 (25 mL) by following the instructions provided by the manufacturer. Five milligrams of antibody bound per milliliter of gel as determined by absorbance at 280 nm with an extinction coefficient of 1.4 for a 1-mg/mL solution. A 60-mL column (2.5 cm × 12 cm) was used for these experiments.

Immunoaffinity purification. Factor IX concentrates were suspended in sterile water for injection and then diluted with an equal volume of sterile saline to achieve the desired concentration. The monoclonal antibody-immunoaffinity-purified factor IX concentrate was then added to each test tube, and the diluted concentrate was centrifuged to remove the antibody–factor IX complex.

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jugular vein segments were clamped 30 seconds after contralateral injection in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2). Peak fractions were dialyzed against 0.15 mol/L NaCl/0.01 mol/L sodium citrate (pH 7.2) before injection in animals. Immunoaffinity purification of factor IX after viral inactivation was tested in separate experiments by using a 3 x 50-mm high-performance liquid chromatography (HPLC) column (Rainin Instrument Co, Woburn, MA) containing approximately 2.5 mg of Affigel 10-linked A-7 antibody. Factor IX (2.0 mg/mL) was heated with 1% Triton X-100 and 1% tributyl phosphate for 15 minutes at 37°C in 0.15 mol/L NaCl. The reaction mixture was diluted eighthfold in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2)/0.005 mol/L CaCl2/0.001 mol/L MgCl2, Diluted factor IX (200 μL) was loaded on the column by using a Waters (Milford, MA) 6000A solvent delivery system at 0.5 mL/min. The absorbance was read on a Waters 480 model spectrophotometer and recorded on a Hewlett Packard 3390A integrator (Palo Alto, CA).

Residual Triton X-100 was estimated by using scanning UV spectroscopy. Residual tributyl phosphate was determined on a dried chloroform extract of factor IX by using a Hewlett Packard 5890A gas chromatograph with an Ultra 2 capillary column and a Hewlett Packard 597C Mass Selective Detector.

Animal studies. Stasis-thrombosis assays were performed on rabbits anesthetized with atropine, 0.1 mg (Elkins-Sinn, Inc Cherry Hill, NJ), ketamine, 40 mg/kg (Bristol Laboratories, Syracuse, NY), and xylazine, 8 mg/kg (Bayvet, Shawnee, KS). External jugular vein segments were clamped 30 seconds after contralateral marginal ear vein injection. Segments were inspected for clots after 15 minutes of stasis. Thrombus scores of 1 and 2 refer to fibrin strands and small clots, respectively. A score of 3 is a large clot, while a score of 4 indicated that the vessel was completely occluded by a thrombus.

Infusion studies were in rabbits similarly anesthetized. The femoral artery was cannulated for sampling before infusion with a 0.965-mm-outer-diameter polyethylene tube (Clay Adams, Parsippany, NJ) and maintained patent with 0.9% normal saline (Fenwal Laboratories, Deerfield, IL) to which 47% sodium citrate–citrac acid (Haemonetics, Braintree, MA) had been added to a final concentration of 0.016 mol/L.

Ion-exchange chromatography. Immunoaffinity-purified factor IX (0.2 mL of a 1.65-mg/mL solution) was injected on a 75 x 7.5-mm TSK-DEAE-3-SW column in 0.02 mol/L 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.0) at a flow rate of 0.5 mL/min. The column was washed for ten minutes and factor IX eluted with a linear gradient of 0.25 to 1.5 mol/L NaCl in 0.02 mol/L MES (pH 6.0).

Coagulation factor assays. Factor IX clotting activity of purified factor IX was determined by a single-stage partial thromboplastin time (PPT) using hemophilia B plasma and actin FS (Dade Diagnostics, Inc, Aguada, PR) and compared with a normal plasma pool from 17 individuals. One unit per milliliter of coagulation factor activity or antigen refers to that amount found in 1 mL of pooled normal plasma. Factors II, X, and VII were assayed by using deficient plasmas from Dade and human brain thromboplastin.15 A nonactivated PTT12 used normal pooled plasma and rabbit brain cephalin.

Samples from rabbits were drawn in vacuum tubes with sodium citrate or EDTA or in polystyrene tubes to which thrombin (10 U/mL) was added. Rabbit antithrombin III antigen was measured by using goat antirabbit antithrombin III by immunonephelometry.13 The goat antiserum was provided by Dr Timothy Carlson, Albuquerque. Fibrinogen levels were determined by using a kinetic method14 with bovine thrombin from Parke-Davis, Detroit. Fibrinogen/fibrin degradation products were determined by staphylocoocal clumping15 with lyophilized staphylocoocal from Sigma. Platelet counts were done on a Coulter S Plus IV, Hialeah, FL.

Values for coagulation tests were expressed as percentages of preinfection values.

Factor IX antigen. Peroxidase was coupled to 1 mg of the A-7 antibody after periodate oxidation of enzyme and reduction of Schiff base with NaBH4.16 Coupled antibody was separated from free A-7 by size-exclusion chromatography on a Zorbax GF-250 HPLC column (DuPont, Wilmington, DE) in 0.05 mol/L sodium phosphate, pH 6.5.

Double-antibody-enzyme-linked immunosorbent assays (ELISA) were performed by using a 0.1-mL reaction volume in microtiter plates (Falcon Pro-Bind, Becton Dickinson, Oxnard, CA) coated with 0.1 mL of a 25-μg/mL solution of antibody A-5 (anti-heavy chain of factor IXa) in 0.1 mL NaHCO3/Na2HCO3 (pH 8.6) overnight at 4°C. The plates were washed three times with 0.15 mol/L NaCl/0.02 mL Tris-HCl (pH 7.2)/0.005 mol/L CaCl2/0.001 mol/L MgCl2, 0.1% Tween 20 (wash buffer) and then blocked for one hour at 37°C with 3% crude ovalbumin suspension (grade II) in TBS with 0.1% sodium azide. The plates were then washed three times, and 0.1-mL vol of normal plasma (one to 100 to one to 3,200) and samples diluted in 0.15 mol/L NaCl/0.02 mL Tris-HCl (pH 7.2)/0.005 mol/L CaCl2/0.001 mol/L MgCl2 with 0.1% ovalbumin (grade III) (sample buffer) were added in triplicate and incubated for three hours at 37°C. The plates were then washed six times, and peroxidase-coupled A-7 was added at a concentration of approximately 1 nmol/L in sample buffer and incubated for two hours at 37°C. The plates were washed six times before the addition of 1 mL/mL of o-phenylene diamine in 0.1 mol/L sodium citrate, pH 4.5, with 0.03% H2O2. The reaction was stopped after approximately 15 minutes with 0.1 mL of 2.5 mol/L H2SO4, and the absorbance was read in an ELISA reader (Dynatech, Santa Monica, CA) at 490 nm. A standard curve was determined graphically or by curve fitting.17

Assays for the antigen content of purified factor IX used a standard preparation of purified factor IX for reference.

Factor IXa assay. This assay is based on antithrombin III incorporation into immobilized IXa.18 Affinity-purified rabbit anti-human antithrombin III was prepared by the passage of antisem from Dr Timothy Carlson over Affigel 15–linked human antithrombin III from Dr Carlson19 coupled at 1 mg/mL gel. The bound antibody was eluted with 0.1 mol/L glycine-HCl (pH 2.6) and neutralized with 0.5 mol/L K2HPO4. One milligram of antibody was dialyzed against 0.1 mol/L NaHCO3 (pH 8.2) and coupled to 120 μg of N-hydroxysuccinimide ester of bovine in dimethyl sulfoxide (1 mg/mL). After coupling, antibody was dialyzed against 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2).

Microtiter plates were coated and blocked as described earlier but with A-7 (anti-light chain of factor IXa). Samples containing factor IX and factor IXa were diluted in sample buffer to a final concentration of 10 μg/mL total factor IX. Factor IX/IXa mixtures were added to coated wells in sample buffer. A standard factor IXa

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FACTOR IX PURIFICATION

Preparation was added at IXa concentrations of 2.8 to 0.045 nmol/L (160 to 2.5 ng/mL) diluted in solution containing 10 µg/mL factor IX. The plates were incubated for two hours at 37°C and then washed six times before the addition of human antithrombin III at 0.5 µg/mL in sample buffer with 2 U/mL heparin. After the plates were incubated for one hour at 37°C, they were washed six times before the addition of affinity-purified biotinylated antibody to human antithrombin III at 1 µg/mL. After one hour at 37°C, the plates were washed six times, and then a 1:1,000 dilution of streptavidin-peroxidase in sample buffer was added for 30 minutes at 37°C. The plates were washed six times, substrate was added, and the results were quantitated as described earlier.

Gel electrophoresis and densitometry. Preparations underwent electrophoresis on 10% polyacrylamide gels for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and were scanned on a Zeineh laser densitometer (Biomed Instruments, Inc., Fullerton, CA). Western blots for factor IX in concentrates were performed with 125I–staphylococcal protein A and A-7 anti–factor IX as previously described.

ELISA for mouse IgG. Microtiter plates were coated with goat antimouse IgG (Meloy Laboratories, Springfield, VA) at 25 µg/mL and blocked as described earlier for factor IX ELISA. The A-7 antibody in 0.15 mol/L NaCl/0.02 mol/L Tris-HCl (pH 7.2) with 1 mg/mL bovine albumin (fatty acid free, Boehringer Mannheim), was added to wells at concentration of 1 µg to 10 ng/mL for a standard curve. Test samples were added after dilution in buffer and incubated for two hours at 37°C. The plates were washed with 0.15 mol/L NaCl/0.02 mol/L Tris-HCl/0.1% Tween 20 six times and then incubated with a 1:1,000 dilution of peroxidase-coupled goat anti-mouse IgG (Sigma) for two hours at 37°C. The plates were washed six times, and then substrate was added as described for the factor IX ELISA.

Statistical methods. Means for postinfusion coagulation parameters were compared with preinfusion values by t distribution. Group means were compared by a two-sample t test. Significance levels expressed are for two-tailed comparison.

RESULTS

Purification. Purification of factor IX achieved with immunoaffinity chromatography is shown in Figs 1 and 2 and Table 1. The single-step procedure gave a 99- and 87-fold purification of factor IX clotting activity in the crude concentrate for Armour and Cutter preparations, respectively. In addition to native factor IX, the starting and the final product contained a previously described immunoreactive factor IX degradation product with an apparent molecular weight (mol wt) of 50 Kd on SDS-PAGE and a peptide with an apparent mol wt of 14 Kd. The 50-Kd species reacted with monoclonal antibodies to the heavy and light chains of factor IXa, which react with thrombin-degraded factor IX but do not recognize the C-terminal peptide cleavage product from thrombin degradation of factor IX (data not shown). Degraded factor IX (50,000 and 14,000 daltons) accounted for 29% and 20% of the protein on densitometry of stained gels of factor IX prepared from Armour and Cutter concentrates, respectively. Western blots with A-7 anti–factor IX showed that similar amounts of degraded factor IX were present in the crude concentrate and immunoaffinity-purified factor IX, which suggests that degradation did not result from the purification process. Also, ratios of factor IX antigen to factor IX clotting activity did not change after immunoaffinity purification, and the Armour concentrate, which showed 29% degraded factor IX in the final product, had a higher antigen/activity ratio (1.4 for the Armour concentrate and 1.1 for the Cutter concentrate).

An unidentified contaminant is present in these preparations with a mol wt greater than factor IX. This contaminant amounted to less than 3% of the protein on stained gels by laser densitometry. ELISA for mouse IgG showed that the factor IX from the Armour and Cutter concentrates contained 4 and 1.6 µg/mL immunoreactive antibody, respectively. This could also be demonstrated by immunodiffusion for the factor IX purified from the Armour concentrate. Immunoreactive mouse IgG could be reduced 500-fold by TSK-DEAE (Bio-Rad) chromatography of factor IX–mouse IgG mixtures in separate experiments (data not shown). The loss of factor IX was 48% in two experiments with analytic-scale ion-exchange chromatography. To determine whether...
microgram amounts of mouse IgG were immunogenic, two rabbits were immunized with 1 mg of immunoaffinity-purified factor IX administered with complete Freund's adjuvant (Difco, Detroit) and boosted with 1 mg of factor IX at 3 weeks and 6 weeks in incomplete Freund's adjuvant (Difco). Antiserum obtained 1 week after the final immunization had no detectable antibody to mouse IgG by immunodiffusion or ELISA.

Levels of other vitamin K–dependent coagulation factor activities in the factor IX prepared from the Armour and Cutter concentrates were quite low. Levels of factor X were less than 0.005 U/mL, less than 0.01 U/mL for factor VII, and less than 0.01 U/mL for prothrombin in clotting assays. There was less than 0.2 U/mL protein S or protein C antigen by immunoelectrophoresis (data not shown).

In separate experiments the immunoaffinity procedure was also used to separate factor IX from detergent and lipid solvent mixtures, which could be used to inactivate envelope viruses,22 as shown in Fig 2. The EDTA eluate contained less than 0.005% Triton X-100 and less than 0.005% tributyl phosphate, which were the limits of measurement.

**Animal infusion studies:** In the stasis-thrombosis assay, the Armour concentrate, which contains heparin, was not thrombogenic at doses of 50, 100, and 100 U/kg in three rabbits (manufacturer's units). The clot scores were 1, 1, and 0, respectively. The Cutter concentrate, which does not contain heparin, caused large thrombi (3 or 4+) in rabbit external jugular vein segments at 106, 138, and 234 U/kg.

Since concentrates treated with heparin may have decreased activity in the stasis-thrombosis assay but still cause delayed coagulation factor consumption in dog infusions,23-24 the Armour concentrate (which was not thrombogenic in the stasis-thrombosis assay) and immunoaffinity-purified factor IX prepared from it were tested in rabbits administered bolus (1- to 3-minute) infusions. Rabbit platelet count, fibrinogen, factor V, factor VIII, antithrombin III antigen, factor IX antigen, and fibrin/fibrinogen degradation products were monitored. Four rabbits were administered 100 U/kg of the crude concentrate, and four rabbits were administered 214 to 243 U/kg of immunoaffinity-purified factor IX. Despite factor IX levels that were nearly three-fold greater than those achieved with concentrate, the group treated with purified factor IX did not show any significant changes in parameters tested, while the concentrate-treated group had generally lower values of all labile coagulation factors and lower antithrombin III antigen (Fig 3 and Table 2). When postinfusion values were compared with preinfusion levels, there was a significant change ($P < .05$) in platelet count at 1, 2, 4, and 5 hours after infusion. Fibrinogen was decreased at four hours after infusion, and factor VIII was decreased at four and five hours after infusion. Antithrombin III antigen was decreased at three and four hours after infusion. Changes in antithrombin III crossed immunoelectrophoresis25,26 were not seen in postinfusion samples, suggesting that few protease–antithrombin III complexes were circulating at the postinfusion times tested. Amounts of fibrin/fibrinogen degradation products were only modestly elevated in the concentrate-treated group as compared with the purified factor IX–

### Table 1. Purification of Factor IX by Immunoaffinity From Commercial Concentrates

<table>
<thead>
<tr>
<th>Volume (mL)</th>
<th>Concentrate A (Cutter)</th>
<th>Pure Factor IX From Concentrate A</th>
<th>Concentrate B (Armour)</th>
<th>Pure Factor IX From Concentrate B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein concentration (mg/mL)</td>
<td>16.2</td>
<td>0.714</td>
<td>16.2</td>
<td>1.65</td>
</tr>
<tr>
<td>Protein (mg)</td>
<td>1,943</td>
<td>20.0</td>
<td>6,496</td>
<td>66.0</td>
</tr>
<tr>
<td>Factor IX clotting activity (U/mL)</td>
<td>25.3</td>
<td>111</td>
<td>25.3</td>
<td>221</td>
</tr>
<tr>
<td>Total activity (U)</td>
<td>3,030</td>
<td>3,108</td>
<td>10,100</td>
<td>8,840</td>
</tr>
<tr>
<td>Factor IX antigen (U/mL)</td>
<td>35</td>
<td>119</td>
<td>28</td>
<td>300</td>
</tr>
<tr>
<td>Total antigen (U)</td>
<td>4,200</td>
<td>3,332</td>
<td>11,200</td>
<td>12,000</td>
</tr>
<tr>
<td>Specific activity (U/mg)</td>
<td>1.56</td>
<td>155</td>
<td>1.55</td>
<td>134</td>
</tr>
<tr>
<td>Specific antigen (U/mg)</td>
<td>2.16</td>
<td>167</td>
<td>1.72</td>
<td>182</td>
</tr>
<tr>
<td>Fold purification (activity)</td>
<td>98.4</td>
<td>86.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fold purification (antigen)</td>
<td>77</td>
<td>105</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (activity) (%)</td>
<td>103</td>
<td>87.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Yield (antigen) (%)</td>
<td>79</td>
<td>107</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Factor IX activity and antigen were determined as described in Methods. Similar yield and specific activities of the final product were obtained in small-scale preparations from the same concentrates and when commercial concentrates from two other manufacturers were used as the starting material.
Fig 3. Rabbit infusion studies with crude and purified factor IX. The composite diagram shows postinfusion values of coagulation parameters as percentages of initial values. Results are corrected for blood removed in sampling by adjusting for decreased hemoglobin concentration. Parameters shown are A. platelet count; B, fibrinogen; C. factor VIII activity; D. factor V activity; E. antithrombin III antigen; and F. factor IX antigen. Values in four rabbits administered purified factor IX are shown in solid circles; open squares are values for crude concentrate. The (++) symbol indicates a difference in group means and the (+) symbol indicates a group mean decrease from the initial parameter value.
treated group, and the titers were not significantly different from preinfusion values.

**Human factor IX kinetics in rabbits.** Mean factor IX antigen values were used to determine the half-time for the disappearance of factor IX and apparent volume of distribution of factor IX in rabbits treated with immunoaffinity-purified factor IX and in rabbits treated with concentrate. The half-time for factor IX disappearance was 5.8 hours in the purified factor IX–treated group and 6.0 hours in the concentrate-treated group. The apparent volume of distribution for factor IX was 99 mL/kg in the purified factor IX–treated group and 111 mL/kg in the concentrate-treated group one hour after infusion. The calculated zero time volumes of distribution are 106 and 122 mL/kg in the purified factor IX– and the concentrate-treated groups, respectively.

**Factor IXa and in vitro measures of activation.** The purified factor IX prepared from both concentrates had detectable factor IXa by ELISA. The amount of factor IXa in purified factor IX from the Armour preparation was 0.4 μg/mL, and the Cutter concentrate had 0.1 μg/mL. Rabbits infused with purified factor IX in the stasis-thrombosis or infusion studies received approximately 0.5 to 1 μg of factor IXa. Concentrations of factor IXa were 0.029 and 0.015 μg/mL for Armour and Cutter crude materials, respectively. Animals received approximately 0.3 μg of factor IXa in Armour concentrate infusions and 0.1 or 0.2 μg in Cutter concentrate infusions. The nonactivated PTT results obtained with the concentrates were >200 seconds for the Armour and Cutter concentrates at a 100-fold dilution. The times for the factor IX preparations with approximately six-fold higher factor IX concentrations were 158 and >200 seconds, respectively, for the factor IX purified from Armour and Cutter preparations.

**DISCUSSION**

Monoclonal antibody immunoaffinity-purified factor IX has two potential advantages over current factor IX concentrates. The first advantage is reduced in vivo thrombogenicity as shown in this report. The second advantage is that a high-specific activity, virus-inactivated factor IX concentrate would also be less likely to be implicated in the poorly understood immune defect of hemophilia B patients.

A product with less risk of therapy-related thrombosis would be important for improved patient care. The use of high doses of vitamin K–dependent coagulation factor concentrates is complicated by infrequent life-threatening venous thromboses, myocardial infarction, or hemorrhage due to coagulation factor consumption. Hemothilia B patients receiving concentrate therapy for surgical procedures have a substantial risk of developing clinical venous thrombosis when administered repeated infusions of commercial factor IX concentrates in the postoperative period. Thrombosis rates were 6 in 12, 1 in 10, and 1 in 6 in three reports.

Much of the effort in improving the safety of crude factor IX concentrates has focused on the identification of coagulation factor activation. Small amounts of activated coagulation factors have been shown to cause stasis-thrombosis, and careful studies have shown that the potency of small amounts of activated factor Xa in this assay can be dramatic...
cally increased by phospholipid micelles from disrupted platelets in factor IX concentrates. Concentrates that show activated coagulation factors usually have activity in the stasis-thrombosis assay and can cause coagulation factor consumption in animal infusions. Clinical observations suggest that screening concentrates for activated coagulation factors by in vitro testing has been successful in decreasing the thrombotic risk of intermittent replacement therapy. Since patients receiving high-dose, long-term therapy may still have thrombosis or coagulation factor consumption, activated coagulation factors may not be the only consideration for safety. Factor IX is more lethal in mice than is activated factor IX, and 100-U/kg doses of crude concentrates with minimal content of activated coagulation factors caused sustained generation of fibrinopeptide A in the dog model. These studies and others have suggested that overload with vitamin K–dependent coagulation factors in addition to the desired replacement with factor IX is responsible for therapy-related thrombosis and that activation of native coagulation factors occurs in the patient.

More highly purified factor IX preparations would be expected to have less potential for triggering thrombosis. Large-scale production of a more purified factor IX concentrate (9 U/mg) largely free of other clotting factors has been described. This concentrate did show shortening of the nonactivated PTT but did not cause large thrombi in the stasis-thrombosis assay when infused in rabbits at doses of 200 U/kg and did not cause consumption of rabbit coagulation factors and inhibitors, whereas stasis-thrombosis and rabbit coagulation factor consumption were seen with the crude concentrate from which the purified preparation was derived. This more purified concentrate also did not cause the functional antithrombin III deficiency in humans that can be seen with standard concentrates.

In the current report, the rabbit model was used for in vivo screening of crude concentrates and purified factor IX. The pharmacokinetic properties of human factor IX in rabbits and baboons seem similar to those seen in humans administered radiolabeled factor IX. The volume of distribution of human factor IX in all three species is larger than the predicted plasma volume, which suggests vascular binding sites. The half-time of disappearance in rabbits (six hours) is shorter than the 10.8-hour half-life found in humans but similar to that in baboons (5.1 hours) studied between one and five hours after infusion.

These observations suggest that vascular binding but not half-life are similar for humans and animals. The use of the animal model may not predict human thrombosis since differences in thrombogenic effect could be expected if catabolism is an important variable, for example.

Factor IX prepared by metal ion–dependent monoclonal antibody immunoaffinity chromatography with specific activities of 134 and 155 U/mg had little detectable effect on the rabbit coagulation system despite the presence of small amounts of factor IXa in the final product. The lack of in vivo thrombosis is more remarkable since the clinical concentrates that were the source of the purified preparations had a readily detectable effect in the stasis-thrombosis assay or the rabbit infusion model.

Further advantages of a high-purity product produced by immunoaffinity chromatography could be a decreased risk of viral transmission and decreased patient exposure to allo- geneic plasma proteins other than factor IX. The immunoaffinity purification procedure allows the factor IX product to be manipulated in ways that may have other advantages for therapy. Heat treatment or lipid solvent/detergent treatment of commercial concentrates may produce denaturation of a portion of the factor IX contained. Immunoaffinity purification may be valuable in selecting those molecules with the ability to undergo a conformational change in the presence of divalent metal ions and is a convenient means of separating the protein to be administered from virucidal reagents. Physical separation from viral contaminants is another likely benefit of the immunoaffinity purification procedure since the factor IX bound to the affinity column can be washed extensively.

The poorly understood immune defect of HIV-seronegative hemophilia B patients may be due in part to exposure to viruses, particularly the agents of non-A, non-B hepatitis, and in part due to exposure to allo- geneic proteins with chronic replacement therapy. Immune defects described in HIV-seronegative patients with hemophilia A and B include decreased mitogen response to phytohemagglutinin, pokeweed mitogen, and concanavalin A. T-cell subset abnormalities include increased numbers of CD8 cells and a decreased CD4/CD8 ratio. Specialized tests have shown a decreased response to dinitrochlorobenzene skin challenge, decreased autologous mixed lymphocyte response, decreased monocyte-dependent T-cell stimulation, decreased natural killer cell activity with β- or γ-interferon stimulation, and increased B-cell growth factor and B-cell differentiation factor production by hemophilic mononuclear cells. These abnormalities in immune function were determined in many cases by comparing responses of seronegative hemophiliacs to normals, and although means differ, there is considerable overlap for the two groups. The clinical significance of these findings in seronegative hemophiliacs is uncertain, yet increased susceptibility to tuberculosis has been suggested.

The dose of extraneous proteins is 635 and 638 mg for every 1,000 units of factor IX with the commercial concentrates in this study. If there is a deleterious effect of extraneous protein on the recipient immune system, it should be possible to compare immune function in patients receiving products that differ 100-fold in purity in future studies. It is also possible that the progression of the immune defect of HIV-seropositive patients could also be modified by therapeutic products of high specific activity. In a recent report, hemophilia A patients treated with factor VIII purified by monoclonal antibody immunoaffinity chromatography had less precipitous declines in CD4 cells than did control patients receiving standard treatment and improvements in the number of skin test antigens recognized when compared with prestudy results. Other reports, however, have not identified the intensity of treatment and hence protein load as a factor predicting progression to acquired immunodeficiency syndrome in seropositive hemophiliacs.

Disadvantages of monoclonal antibody immunoaffinity purification of factor IX are the costs of the antibody production and the possibility of adverse reactions to nano-
gram amounts of immunogenic mouse proteins infused in recipients. Procedures to minimize the amounts of contaminating mouse proteins will be required to produce a product suitable for clinical use.

Monoclonal antibodies to factor IX can be readily scaled up to produce amounts of factor IX needed for replacement therapy, whereas polyclonal antibodies are not easily produced in more than milligram amounts. The use of a monoclonal antibody to a metal ion-dependent epitope on factor IX allows gentle elution of bound factor IX with EDTA-containing buffers and will preserve column capacity longer than would be seen if harsh elution conditions were used. A further advantage of the A-7 monoclonal antibody used in the immunoaffinity purification procedure in this report is that it distinguishes between factor IX, which contains γ-carboxyglutamic acids, and dysfunctional factor IX, which does not. When recombinant factor IX can be produced in sufficient scale to provide replacement therapy, the monoclonal antibody method will be useful since carboxylation of recombinant factor IX may not be complete.

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