Coagulation Factor IX Concentrate: Method of Preparation and Assessment of Potential In Vivo Thrombogenicity in Animal Models

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Thrombosis and/or disseminated intravascular coagulation (DIC) are complications specifically associated with the use of factor IX complex in some patients. Assuming that these complications might result from zymogen overload, we have produced, using diethylaminoethyl (DEAE)-Sephadex (Pharmacia, Piscataway, NJ) and sulfated dextran chromatography, a factor IX concentrate (coagulation factor IX) that is essentially free of prothrombin, factor VII, and factor X. Factor IX specific activity is at least 5 U/mg protein, a 250-fold purification compared to plasma. Amounts of factors II, VII, and X are less than 5 units each per 100 units of factor IX. The concentrate is essentially free of activated clotting factors and contains no added heparin. In the rabbit stasis model, a dose of 200 factor IX U/kg was less thrombogenic than 100 factor IX U/kg of the DEAE-Sephadex eluate from which the concentrate was derived. Infusion of 200 factor IX U/kg did not induce DIC in the nonstasis rabbit model, whereas 100 factor IX U/kg of the DEAE-Sephadex eluate resulted in DIC in this model. Several factor IX lots were found to have shortened nonactivated partial thromboplastin times (PPTs), but were nonthrombogenic in both animal models. These data indicate that coagulation factor IX concentrate is less thrombogenic than factor IX complex.

PURIFICATION METHODS used to prepare factor IX complex concentrates for replacement therapy in hemophilia B patients take advantage of the common adsorption and elution properties of all the vitamin K-dependent proteins on anion exchangers. As a result, all currently available products designated in the United States by the generic term, factor IX complex, contain, in addition to factor IX, relatively large amounts of prothrombin and factor X on an activity basis.1-3 The factor VII content varies according to the adsorbent selected for the manufacture of a specific product. Thus, while some concentrates contain higher (eg, Proplex, Hyland, Glendale, Calif) or similar (eg, Konyne, Cutter, Berkeley, Calif) ratios of factor VII to factor IX, others contain only minimal amounts of factor VII (eg, Prothromplex, Immuno, Vienna). Protein C is also present in these products.4-7

Although factor IX complex concentrates have been used in a variety of pathologic conditions, including acquired combined deficiencies of the vitamin K-dependent clotting factors and, more recently, in hemophilia A patients with antibodies to factor VIII, their major clinical indication remains replacement therapy in patients with congenital factor IX deficiency.

The clinical effectiveness of these concentrates in hemophilia B patients, whether to treat or to prevent hemorrhagic episodes, is well established and indisputable. However, in addition to the transmission of viral hepatitis, the occurrence of thrombotic episodes and/or DIC in some patients is a complication specifically associated with the use of factor IX complex.8-10

The nature of the material(s) present in factor IX complex that is responsible for inducing such thrombogenic complications is unknown. Some studies have implicated factors Xa, IXa, VIIa, or the contact phase factors,11-18 while others have shown that the thrombogenicity of some concentrates is mediated by a combination of coagulant-active phospholipid and activated clotting factors.17 Another assumption derived from experiments conducted in mice is that the thrombogenicity elicited by these products is due to an induced zymogen overload, particularly of prothrombin and factor X, which have relatively long half-lives and could therefore accumulate in the circulation.18

With the availability of methods for the separation of human factor IX from the other vitamin K-dependent coagulation factors,19-21 it has become feasible to produce a factor IX concentrate essentially free of prothrombin, factor X, and factor VII. In this study, we report the preparation of such a factor IX concentrate, which appears to be nonthrombogenic as judged by the results observed in two animal models. This product carries the name of coagulation factor IX and is intended solely for use in the treatment of hemophilia B patients.

MATERIALS AND METHODS

Russell's viper venom in cephalin, soybean trypsin inhibitor, factor X-, factor V-, factor VII-, and prothrombin-deficient plasmas were obtained from Sigma Chemical Company, St Louis. Factor IX-deficient plasma was obtained from George B. King.
Biomedical, Inc, Overland Park, Kan. Factor VIII-deficient plasma was obtained from Dr John Penner, American Red Cross Blood Services, Lansing, Mich. Rabbit brain thromboplastin was a product of Dade Diagnostics, Inc, Miami. Male New Zealand rabbits were obtained from the B & H Rabbitry, Rockville, Md. Cryosupernatant plasma was prepared by the Michigan Department of Public Health (MDPH) from plasma frozen within 15 hours after collection. All plasma was obtained from volunteer blood donors and supplied by the Michigan Regional Blood Centers of the American Red Cross Blood Services.

Coagulation Factor Assays

Factors IX and VIII were assayed by a one-stage assay (activated partial thromboplastin time) as described in Biggs, using the appropriate deficient substrate plasma. The factor IX content of the concentrate was calculated using an in-house factor IX concentrate standard calibrated against the WHO reference standard provided by the National Institute of Biological Standards and Control, London. The in-house standard was prepared by MDPH from a 500-L pool of plasma. Factor X was assayed by the method of Bachman et al., factor V by the method of Lewis and Ware, prothrombin by the method of Hjort et al., and factor VII by the method of Nemerson and Clyne. Fibrinogen was determined as described by Huseby and Bang. The nonactivated PTT was performed by the modified method of Kingdon et al.

Assays for activated clotting factors were performed by Dr Mae Hultin, SUNY, Stony Brook, N.Y. Factor VIIa was measured using both the coupled amidolytic assay and a clotting assay described by Seligsohn et al. Factor IXa was measured by both a clotting assay and by the specific 1\H-factor X activation method developed by Hultin and Nemerson and adapted specifically for assaying concentrates. Factor Xa was measured using a clotting assay.

Assays to determine the factor IX antigen to coagulation activity ratios were performed by Dr Arthur R. Thompson, Pacific Medical Center, Seattle. The immunoradiometric assay was performed using a polyclonal antibody as previously described.

Animal Models

**Rabbit stasis model.** The method of Wessler et al. was used to test the thrombogenicity of the various concentrates that were infused manually in a total volume not to exceed 5 mL over a period of 30 seconds. One jugular vein (either right or left) was tied off exactly 60 seconds after the start of the infusion. The formation of thrombi in the isolated vein segment was determined by visual inspection after ten minutes and scored from 0 to 4, with a score of 4 indicating complete occlusion of the vein segment.

**Rabbit nonstasis model.** A modification of the nonstasis models of Triantaphyllopoulos and Prowse and Williams was used as an in vivo test of thrombogenicity. In this procedure, 3.6 to 4 kg male white rabbits were sedated with Nembutal (Abbott Laboratories, Chicago, Ill) (27 mg/kg). A polyethylene cannula was introduced into the carotid artery. Blood samples were obtained from this cannula 30 minutes prior to and immediately before (0 time) the test material was infused into the marginal vein of the ear. The material (1.8 mL/kg) was infused manually at a rate of 2 mL/min. Blood samples were taken at various times up to two hours after test sample infusion. The blood samples (7 mL) were collected into plastic tubes containing 0.15 mL 1 mol/L citrate. Three milliliters of the citrated blood was then placed into a second tube containing 6 mg of soybean trypsin inhibitor. The citrated sample was used for the coagulation factor assays and platelet count, while the soybean trypsin inhibitor-treated blood was used to measure fibrinogen. Platelet counts were performed using a Coulter Counter (Hialeah, Fla).

RESULTS

**Preparation of Coagulation Factor IX Concentrate**

For each liter of human cryosupernatant plasma, 1.7 g of DEAE-Sephadex A-50 (previously swollen in 0.07 mol/L Na-citrate, pH 6.0) was added. The mixture was stirred gently for one hour, the supernatant decanted, and the resin collected in a polyethylene Buchner funnel (35 μm porosity) and washed with 0.07 mol/L Na-citrate, pH 6.0, until the A absorbance reached a plateau value of about 0.2 and the color of the resin changed from deep green to light blue (approximately 0.64 L/L original plasma). Elution was performed with 0.2 mol/L Na-citrate, pH 6.0 (approximately 0.08 L/L original plasma). This fraction was dialyzed against 0.08 mol/L NaCl, 0.02 mol/L Na-citrate, pH 6.0, to a final volume of 15 mL/L original plasma and loaded onto a column of sulfated dextran, which was prepared by the method of Miletich et al. After the breakthrough protein eluted from the column, the NaCl concentration was increased to 0.25 mol/L to elute the prothrombin. Factor X was eluted with 0.45 mol/L NaCl and factor IX with 0.8 mol/L NaCl. The factor IX, factor X, and prothrombin that were obtained by this procedure were used in the experiments described below.

A summary of the purification is shown in Table 1. In this specific preparation, the overall yield of factor IX is 18%, with an 880-fold increase in specific activity. Based on the specific activity of pure human factor IX of 325 or 275 U/mg protein, this factor IX

<table>
<thead>
<tr>
<th>Table 1. Purification of Factor IX (Lot 301)</th>
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<tr>
<td><strong>Total Units</strong></td>
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<td>(x 10^3)</td>
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<tr>
<td>Plasma</td>
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<td>DEAE eluate</td>
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<td>Sulfated-dextran fractions</td>
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<td>Prothrombin</td>
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<td>Factor X</td>
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*Ratio in terms of plasma equivalent units.
†Factor IX.
preparation is between 5% and 6% pure in terms of protein, and on an activity basis, has less than 1% of either prothrombin or factor X contamination. Factor VII could not be detected in this preparation.

To date, 12 lots of factor IX concentrate for both animal testing and clinical investigation have been prepared at MDPH, Lansing, Mich. In order to be able to process up to 800 L of plasma at one time, the procedure was modified by eluting the prothrombin and factor X from the dextran sulfates in one step rather than two. The factor IX eluate was sterilized by membrane filtration, dispensed in 10 mL aliquots into 50 mL bottles, and freeze-dried. For the last eight lots prepared using 300 to 800 L of plasma, the average factor IX recovery was 269 ± 74 U/L of plasma; the average specific activity was 9.2 ± 1.2 factor IX U/mg protein. Depending on the planned dosage for each lot, the final freeze-dried container material contained 360 to 1,100 factor IX units. The average contamination was less than 2 units of either factor X or prothrombin per 100 units of factor IX. The factor IX antigen to coagulation activity ratios for five lots tested was 1.9 ± 0.3 (A.R. Thompson, Pacific Medical Center, Seattle, personal communication, February 1984).

Thrombogenicity Testing

Rabbit stasis model. Preliminary experiments with the Wessler stasis model33 indicated that the factor IX concentrate was much less thrombogenic than the DEAE-Sephadex eluate from which it was derived, the purified prothrombin, or the factor X. We therefore used a dose of 200 U/kg for the factor IX concentrate (lot 301) and a dose of 100 U/kg for prothrombin (lot 301) and factor X (lot 301). For the DEAE-Sephadex eluate (lot 301), a dose of 100 U/kg was used based on the factor IX content. At these doses, the DEAE-Sephadex eluate that contained prothrombin, factor X, and factor IX in a ratio of 1:0.5:1 (ie, for each unit of prothrombin, 0.5 unit of factor X and 0.9 unit of factor IX) was significantly more thrombogenic than the other concentrates (P < .01, Student's t test). When infused at twice the dosage of either prothrombin or factor X, factor IX was not significantly different than these concentrates. Thus, factor IX by itself is less thrombogenic than either factor X or prothrombin in this model. The results are summarized in Fig 1.

Rabbit nonstasis model. Three rabbits each were infused with either factor IX concentrate (lot 301) at 200 factor IX U/kg, DEAE-Sephadex eluate (lot 301) at 100 factor IX U/kg, prothrombin (lot 301) at 100 U/kg, or factor X (lot 301) at 100 U/kg, as described in Materials and Methods. The results of these infusions on the platelet count and levels of fibrinogen, factor V, and factor VIII are shown in Fig 2. As in the stasis model, the DEAE-Sephadex eluate is significantly more thrombogenic than the other concentrates. Although the factor IX concentrate was infused at twice the dosage of the other preparations, the only significant changes observed after infusion were a drop in the platelet count at 30 minutes, with a return to the control value at 60 minutes and a second drop at 120 minutes (Fig 2A), together with an increase in the factor VIII level at 30 minutes, with a subsequent return to normal at 60 minutes (Fig 2D). In contrast, the animals treated with DEAE-Sephadex eluate showed significant decreases in platelet count at 30 minutes, in fibrinogen level at 60 minutes, factor VIII at 90 minutes, and in factor V at 120 minutes (Fig 2, A through D).

Nonactivated partial thromboplastin time. The experiments described above were performed using the DEAE-Sephadex eluate and factor IX concentrate (lot 301) whose characteristics are listed in Table 1. While preparing large-scale lots of factor IX concentrate, we noticed that some of these lots had shortened nonactivated PTTs. Three lots, 309, 310, and 311, when diluted 1/8, had nonactivated PTTs of 62.5, 62.2, and 52.2 seconds, respectively, compared to buffer controls of > 190 seconds. These three lots were tested for their activated clotting factor content and compared with that of lot 307 (nonactivated PTT 151 seconds; buffer control 200 seconds). Factor IXa was undetectable (< 20 ng/mL) in all four samples when using the
specific \(^3\)H-factor X activation method. When using a clotting method for detecting factor IXa, the results were not significantly different in the presence or absence of factor IX inhibitors (without inhibitor: 0.14 to 0.22 ng/mL; with inhibitor: 0.14 to 0.30 ng/mL), indicating again that the IXa content is no more than 0.0001% of the factor IX content. The factor Xa content ranged from 1.4 to 3.6 ng/mL (control Lot 307: 2.6 ng/mL). This amount of factor Xa is less than 0.001% of the factor IX content and is therefore insignificant. The factor VII content of these samples was less than 0.2% of that of factor IX (less than 2 units of factor VII per 1,000 units of factor IX) (M.B. Hultin State University of New York, Stony Brook, personal communication, March 1983).

Since a correlation between the nonactivated PTT test and in vivo thrombogenicity in animal models has been reported for factor IX complex, the lots of factor IX concentrate that exhibited short nonactivated PTTs were tested in animal models. Lot 311, which had the shortest nonactivated PTT, was tested in the nonstasis rabbit model. The results are shown in Table 2. Also listed for comparison are the values used for constructing Fig 2, obtained with factor IX concentrate (lot 301) and the DEAE-Sephadex eluate of lot 301. In none of the three rabbits infused with lot 311 did the level of factor VIII fall below 80% of the pretreatment value during the first two hours after infusion. In contrast, in each of the rabbits infused with the DEAE-Sephadex eluate of lot 301, the levels of factor VIII fell below 37%. One rabbit infused with lot 311 had only 34% of the pretreatment value of factor V after two hours, but the other five rabbits given lot 301 or lot 311 had levels of factor V above 75% of the pretreatment value. In contrast, none of the three rabbits infused with the DEAE-Sephadex eluate had factor V levels...
greater than 41% of the pretreatment factor V level. Thus, it can be seen from these data that lots 301 and 311 are not significantly different from each other and that by the criteria of this model, the factor IX concentrate is less thrombogenic than a DEAE-Sephadex eluate of lot 301.

Lot 309 (nonactivated PTT of 62.5 seconds) was tested in two rabbits at a dose of 100 factor IX U/kg using the Wessler stasis model. The results are given in Table 3, which also presents the scores of the five rabbits given 100 factor IX U/kg of the DEAE-Sephadex eluate of lot 301, one rabbit given 50 factor IX U/kg of Konyne, and one rabbit given 100 factor IX U/kg of Proplex. As in the nonstasis model, the results in this in vivo stasis model indicate that for our factor IX concentrate, a short nonactivated PTT is not predictive of a thrombogenic tendency.

**DISCUSSION**

Since the first documentation of DIC following the administration of factor IX complex to a patient with liver disease, several reports have substantiated the relationship between factor IX complex infusion and the occurrence of thrombohemorrhagic complications. These complications have been observed in patients with combined deficiencies of the vitamin K-dependent clotting factors, particularly in patients with liver disease, as well as in patients with congenital deficiencies of factor X and in hemophilia A patients with antibodies to factor VIII. These case reports describe the occurrence of DIC, superficial vein thrombosis, deep vein thrombosis, pulmonary embolism, or, in some cases, myocardial infarction. Of interest is the occurrence of myocardial infarction following a high-dose regimen of approximately 300 factor IX U/kg administered for several days in four young hemophilia A patients with antibodies to factor VIII.

Although the factor(s) responsible for inducing such thrombogenic complications is unknown, attempts have been made to improve the safety of factor IX complex. On the assumption that activated clotting factors are the cause of thrombogenicity, natural inhibitors of coagulation, such as heparin and serum, or partially purified antithrombin III with or without heparin, have been added to these concentrates. However, products containing heparin are known to have induced thrombosis in some patients. On the assumption that the adverse effects of factor IX complex may result from zymogen overload rather than...
from the small amounts of active enzymes present in factor IX complex,\textsuperscript{18} we have produced and tested a factor IX concentrate essentially free of prothrombin and factors X and VII. This concentrate is therefore intended solely for intravenous replacement therapy in patients with congenital factor IX deficiency (hemophilia B).

Currently available factor IX complex products have a factor IX specific activity of less than 2 U/mg protein and contain factors II, VII, and X in concentrated form and in approximately the same amounts on an activity basis as that of factor IX. In contrast, the factor IX concentrate described in this article has a factor IX specific activity of at least 5 U/mg protein, representing a 250-fold purification in terms of plasma proteins, while the amounts of factors II, VII, and X are less than 5 units each per 100 units of factor IX on an activity basis. The factor IX coagulant to antigen ratio of 1.9 ± 0.3 is quite similar to that found previously in factor IX complex (range, 1.3 to 2.1) when using a rocket technique to measure factor IX antigen.\textsuperscript{40} In addition, this factor IX concentrate is free of detectable thrombin and has no added heparin.

Methods to evaluate the potential thrombogenicity of factor IX complex have previously been devised. Studies using several animal models have provided evidence for thrombohemorrhagic complications resulting from the infusion of factor IX complex. These animal models have been used to screen for the presence of thrombogenic material(s), as well as to elucidate the mechanism by which this material(s) may trigger in vivo coagulation. Using the rabbit stasis model,\textsuperscript{33} thrombotic doses have been found to range between 5 and 100 factor IX U/kg, depending on the lot or product tested.\textsuperscript{28,35,61} Heparin has been found to reduce or suppress the thrombotic effect.\textsuperscript{11,28} Using an in vivo nonstasis rabbit model,\textsuperscript{34,35} the infusion of factor IX complex into healthy rabbits at a dose of 50 to 100 factor IX U/kg has resulted in a decrease in platelet count and in levels of factor VIII, factor V, and fibrinogen, which is indicative of DIC. The maximum dose tolerated by rabbits without producing appreciable changes in the coagulation parameters was found to be 100 factor IX U/kg.\textsuperscript{35} In these experiments, it was also thought that from the kinetics of the coagulation system in the rabbit in vivo, at least two mechanisms were involved. The first resulted in immediate activation such that maximal effects were seen immediately after infusion of the concentrate. The second was characterized by a slow activation, so that maximal effects occurred two hours after infusion.\textsuperscript{35} A similar lag phase has been observed in dogs,\textsuperscript{62,63} with maximal coagulation changes occurring after up to four hours following the infusion of factor IX complex. Severe DIC occurred in animals receiving factor IX complex at a dose of 100 factor IX U/kg in a sensitive, in vivo nonstasis porcine model developed by Harrison et al.\textsuperscript{84}

Using the two rabbit models described above, we have evaluated the thrombogenicity of our factor IX concentrate and compared it with the DEAE-Sephadex eluate from which it was derived, as well as with purified prothrombin and factor X. In the rabbit stasis model, a dose of factor IX concentrate was less thrombogenic than half the dose of the DEAE-Sephadex eluate, purified prothrombin, or factor X (Fig 1). In the rabbit nonstasis model (Fig 2), high doses of factor IX concentrate did not induce DIC, whereas the DEAE-Sephadex eluate infused at half the dose resulted in coagulation changes indicative of DIC.

The nonactivated PTT test is widely used by manufacturers of factor IX complex to assess the potential thrombogenicity of each lot produced. Some investigators have found a correlation between short nonactivated PTT and the occurrence of thrombosis in animal models.\textsuperscript{28,35} However, thromboses have occurred in patients following the infusion of factor IX complex with long nonactivated PTTs. Of interest is the fact that our factor IX lots exhibiting short nonactivated PTTs did not induce thrombosis or DIC in the two animal models tested.

Our results indicate that this factor IX concentrate is significantly less thrombogenic than factor IX complex. The various hypotheses put forward thus far in an effort to identify the cause(s) of thrombogenicity in factor IX complex have been (1) in vivo zymogen (prothrombin, factor X) overload, (2) presence of activated clotting factors, or (3) coagulant-active phospholipid-activated clotting factor interactions. The purification procedure we have chosen yields a product that cannot induce an overload in the other vitamin K-dependent clotting factors. However, the product is also essentially free of activated clotting factors. It might well be that both these properties increase the animal models’ tolerance to the product. Only wide clinical use will substantiate the inferred greater safety of this product.

Preliminary results in hemophilia B patients indicate that our factor IX concentrate is well tolerated and that the factor IX recovery and half-life is comparable to that of factor IX contained in factor IX complex. These results, as well as the clinical effectiveness of this factor IX concentrate, are currently being analyzed.

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