Purified Factor IX Using Monoclonal Immunoaffinity Technique: Clinical Trials in Hemophilia B and Comparison to Prothrombin Complex Concentrates

By Hugh C. Kim, Campbell W. McMillan, Gilbert C. White, Garrett E. Bergman, Michael W. Horton, and Parvin Saidi

Replacement therapy for hemophilia B (factor IX deficiency) using prothrombin complex concentrate (PCC) has been associated with serious complications of thromboembolic events and transmission of viral infections. Monoclonal antibody-purified factor IX (Mononine) provides a highly purified factor IX concentrate, while eliminating other vitamin K-dependent factors (II, VII, and X). Mononine was evaluated for in vivo recovery, half-life, and for its safety and efficacy in 10 patients with hemophilia B. The in vivo recovery of factor IX with Mononine was a 0.67 ± 0.14 U/dL (mean ± SD) increase per 1 U/kg of infused factor IX, and the biologic half-life (t½), determined using the terminal phase of elimination, was 22.6 ± 8.1 hours. Comparison of in vivo recovery of other vitamin K-dependent factors following a single infusion of either Mononine or PCC showed that, whereas Mononine infusion caused no changes in other vitamin K-dependent factors or in prothrombin activation fragment (F₁₋₃), PCC infusion was associated with significant increases of factors II (2.7 U/dL per 1 U/dL of IX increase) and X (2.2 U/dL for 1 U/dL of IX). Patients who used Mononine as their sole therapeutic material during the 12-month period showed an excellent response in hemostasis for their bleeding episodes. Their experience with long-term use of Mononine was at least equivalent to their previous experience with PCC in the frequency and amount of factor usage. No patients developed antibody against mouse IgG or an increase in IX inhibitor during the 12-month period. These results indicate that monoclonal antibody-purified factor IX concentrate provides hemostatically effective factor IX replacement while avoiding extraneous thrombogenic substances.

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MATERIALS AND METHODS

Patients. Ten patients with factor IX deficiency from two hemophilia treatment centers (New Jersey Regional Hemophilia Program at Robert Wood Johnson Medical School, New Brunswick, NJ, and the Comprehensive Diagnostic and Treatment Center at University of North Carolina at Chapel Hill, NC) entered the study after signing consent forms approved by the institutional review board. Their ages ranged from 11 to 60 years, with a mean age of 26. The baseline factor IX level ranged from less than 1.0% to 5.0%, with a mean of 2.8% ± 1.3% (±SD). All study patients were positive for HIV antibody and also for antibody to hepatitis B surface antigen. The study participants underwent an in vivo recovery and half-life study with a single infusion of Mononine at entry and at 6 months. For the long-term efficacy and safety study, participants were supplied with Mononine for home treatment of their bleeding episodes on a on-demand basis with careful recording of home care logs indicating the amount, frequency, and reasons for factor infusions over the 12-month period. An additional study was performed in six patients from one center (RWJ) with a single infusion of PCC with comparison to monoclonal antibody-purified IX at 6 months.

Concentrates. The monoclonal antibody-purified factor IX concentrate (Mononine) used in this study was provided by the manufacturer (Armour Pharmaceuticals). The steps for purification of the factor IX molecule included application of PCC to the immunoaffinity chromatography column in which anti-factor IX monoclonal antibody was covalently bound and eluted with 3 mol/L sodium thiocyanate. The monoclonal antibody was from a hybridoma clone designated AG.1 (obtained from Dr T. S. Zimmerman of Scripps Clinic), which produces IgG, antibody against factor IX. The manufacturing process incorporates multiple steps for virus reduction, including the immunoaffinity chromatography itself, incubation with 3 mol/L sodium thiocyanate, and virus retentive membrane ultrafiltration. The product is also passed through aminohexyl-sepharose chromatography to remove any mouse protein. According to the manufacturer, the in vitro characterization of Mononine showed that the specific activity was, on average, 180 to 200 U/mg of protein, and analysis by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot show a single band with a molecular weight of 71,000, and the absence of other vitamin K-dependent factors (II, VII, and IX) by direct assay. Animal thrombogenicity tests using the Wessler’s rabbit stasis model showed no induction of thrombosis with Mononine infusion of up to 400 U/kg, whereas conventional PCCs would induce fibrin thrombosis with as little as 50 U/kg in the same rabbit model. Mononine concentrate does not contain an exogenous protein stabilizer in the final formulation and is reconstituted into a clear solution within seconds.

Factor IX infusions and blood samples. Ten patients with hemophilia B underwent a pharmacokinetic in vivo recovery study with a single infusion of Mononine at entry into the protocol and 6 months later. The subjects were given approximately 25 U/kg of Mononine concentrate. Blood was drawn 15 minutes before the infusion, then at 0 time (at the completion of infusion), 30 minutes, 1, 2, 4, 6, 8, 24, and 48 hours postinfusion, and factor IX activity levels were determined at each time point. For comparison of in vivo recovery of coagulation factors, single infusions of PCC were administered to 6 of the 10 patients at least 2 weeks after the 6-month Mononine infusion, and levels of factors IX, II, VII, and X, protein C and S, antithrombin III, and prothrombin activation fragment (F1+2) were measured at specified intervals. Assays for factor IX inhibitor were performed at entry, and 6 and 12 months, using the modified method of Kasper et al. Antibodies (IgG, IgM, and IgE class) against mouse IgG were measured at entry, and 6 and 12 months of study.

Pharmacokinetic analysis. The baseline factor IX activity obtained before the infusion of factor IX concentrates was assumed to represent steady-state factor IX. Therefore, all pharmacokinetic parameters were calculated by subtracting the baseline factor IX level from the observed IX activity after the administration of exogenous factor IX concentrate. Each single-dose pharmacokinetic curve of factor IX was analyzed using both compartmental and noncompartmental methods.

In the compartmental method, factor IX activity level data were analyzed using a nonlinear least-squares regression program. Parameters were derived by fitting the data to the biexponential equation: \( C = A e^{-a t} + B e^{-b t} \), where \( C \) is the factor IX activity level at time \( t \); \( A \) and \( B \) are the \( y \)-intercepts; and \( a \) and \( b \) are the disposition rate constants obtained from the first and second linear phases, respectively. The \( \beta \)-phase half-life \( (t_{1/2}) \) was calculated using the equation \( t_{1/2} = -\ln(0.5) / b \). The \( \alpha \) (distribution)-phase half-life was not determined due to insufficient blood samplings in the immediate postinfusion period. In vivo recovery (K) in units per deciliter was calculated by the formula: \( K = \text{body weight (kg) \times factor IX increase (U/dL)/dose (U)} \) factor IX concentrate administered.

In the noncompartmental (model-independent) method, parameters were calculated by the use of statistical moment methods. The area under the factor IX activity level curve (AUC) was calculated using the linear trapezoidal method from hour 0 to the last activity level time point. The area under the activity level curve from hour 0 to infinity (AUC∞) and the area under the first moment of the activity time curve (AUMC) were calculated by the linear trapezoidal method from hour 0 to the last activity level time point (Ct). The area under the activity level time curve from hour 0 to infinity (AUC∞) and the area under the first moment of the activity time curve from hour 0 to infinity (AUMC∞) were calculated as follows: \( AUC∞_t = AUC_{t-0} + C/\beta \) and \( AUMC∞_t = AUMC_{t-0} + (t \cdot C)/\beta + C/\beta^2 \), where \( \beta \) is the rate constant of the terminal disposition phase and \( t \) is the time of the last activity level time point. The total body clearance (CL), volume of distribution at steady-state (\( V_s \)), and mean residence time (MRT) were calculated using the following equations: \( CL = Dose / AUC_{t-0} \), \( V_s = (Dose \cdot AUMC_{t-0}) / AUC_{t-0} \), and \( MRT = AUMC_{t-0} / AUC_{t-0} \).

Coagulation factor assays. Factor IX coagulant activity was determined by a one-stage partial thromboplastin time (PTT) assay using the appropriate deficient substrate plasma and compared with a normal pooled plasma that had been calibrated against the World Health Organization (WHO) reference standard. Multiple blood samples from each infusion study for each patient were assayed in one batch using the same reagents. Factors II, VII, and X were assayed by the methods of Hjort et al., Nemerson and Clyne, and Bachman et al., respectively, on an ACL-300 automated coagulation machine (Instrumentation Laboratory, Lexington, MA). Antithrombin III was measured by the chromogenic substrate assay using an IL commercial kit. Protein C was measured by the chromogenic substrate method using a Stachrom protein C kit (American Bioproducts, Parsippany, NJ). Free protein S antigen was measured by the enzyme-linked immunosorbent assay (ELISA) method using the ABC kit. Blood for prothrombin activation fragment (F1+2) was collected in plastic syringes loaded with anticoagulants containing 38 mmol/L citric acid, 75 mmol/L sodium citrate, 136 mmol/L dextrose, 6 mmol/L EDTA, 6 mmol/L adenosine, and 25 U of heparin per milliliter and assayed by radioimmunoassay by Dr Ken Bauer of Beth Israel Hospital, Boston, MA.

Statistical methods. Mean values of the preinfusion and postinfusion levels for selected coagulation parameters were compared using Student’s paired \( t \) test, and Mononine was compared with
PCC using the two-sample t test. Significance levels expressed are for two-tailed comparisons.

RESULTS

Factor IX in vivo recovery and half-life of Mononine. Table 1 summarizes factor IX pharmacokinetic parameters following the administration of Mononine, at the time of entry into the protocol and after 6 months, in 10 patients with moderate to severe hemophilia B from two treatment centers using two lots of Mononine. The mean factor IX activity levels versus time curve is shown in Fig 1. The baseline factor IX activity level ranged from 1.0% to 5.0% (2.8% ± 1.3%, mean ± SD). Within 30 minutes of a single infusion of Mononine at 24 ± 0.97 U/kg, factor IX levels maximized to between 12% and 26% (mean, 19 ± 4.0%). In vivo recovery (K) of the infused factor IX was a 0.67 ± 0.14 U/dL increase per 1 U/kg of factor IX infused. Beta half-life representing the elimination phase of factor IX by 0.14 U/dL increase per 1 U/kg of factor IX infused. Beta entry into the protocol and after 6 months, in 10 patients Table 1 summarizes factor IX pharmacokinetic parameters following the administration of Mononine, at the time of maximized to between 12% and 26% (mean, 19%

Table 2. Comparison of Pharmacokinetics of Monine and PCC

<table>
<thead>
<tr>
<th></th>
<th>Initial (n = 10)</th>
<th>At 6 Months (n = 9)</th>
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<tbody>
<tr>
<td>Dose infused (U/kg)</td>
<td>24.0 ± 0.97</td>
<td>29.6 ± 7.6</td>
</tr>
<tr>
<td>Peak (U/dL)</td>
<td>19 ± 4</td>
<td>22.9 ± 7.9</td>
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<tr>
<td>In vivo recovery (K)</td>
<td>0.67 ± 0.14</td>
<td>0.88 ± 0.16</td>
</tr>
<tr>
<td>Compartmental model</td>
<td></td>
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<tr>
<td>β half-life (h)</td>
<td>22.6 ± 8.1</td>
<td>25.3 ± 11.6</td>
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<tr>
<td>Noncompartmental model</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC&lt;sub&gt;inf&lt;/sub&gt;</td>
<td>414.6 ± 124.5</td>
<td>528.5 ± 263.9</td>
</tr>
<tr>
<td>Clearance (mL/kg/h)</td>
<td>6.40 ± 2.27</td>
<td>6.51 ± 2.33</td>
</tr>
<tr>
<td>V&lt;sub&gt;ss&lt;/sub&gt; area (mL/kg)</td>
<td>184 ± 62</td>
<td>190 ± 34</td>
</tr>
<tr>
<td>Mean residence time (h)</td>
<td>30.9 ± 11.4</td>
<td>33.4 ± 16.0</td>
</tr>
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Values are mean ± SD. In vivo recovery (K) is calculated by the formula: Body weight (kg) × factor IX (U/dL) increase/dose (U) administered.

Abbreviations: AUC, area under the curve; V<sub>ss</sub>, volume of distribution at steady-state.

underwent an additional infusion of PCC using one lot of Konyne (Cutter, Berkley, CA) in the same amount of factor IX (25 U/kg) as that used in the 6-month Mononine infusion. Blood samples for each phase of Mononine and PCC infusion were assayed for factors IX, II, VII, and X, protein C, protein S, and antithrombin III. In three subjects, levels of prothrombin activation fragment (F<sub>1+2</sub>) were also measured. Comparison of factor IX pharmacokinetic parameters following the administration of Mononine and PCC obtained in six patients with hemophilia B are summarized in Table 2. In vivo recovery of factor IX following the infusions of Mononine and PCC was equivalent, yielding a 0.65 ± 0.17 and 0.78 ± 0.08 U/dL increase per 1 U/kg, respectively. There were no significant differences between the two products in factor IX pharmacokinetic parameters. Although Mononine yielded a somewhat longer β half-life than that of PCC, the values did not achieve statistical significance. While the Mononine infusion was associated with virtually no change in the levels of factor II, VII, and X, the PCC infusion caused an increase in factor II activity from the preinfusion level of 106% ± 26% to the 30-minute

Fig 1. Factor IX activity levels versus time curve following monoclonal factor IX infusion in 10 hemophilia B patients. Each point represents the mean (±SD) factor IX activity.
postinfusion level of 159% ± 26% (P = .0001) and factor X from 94% ± 18% to 137% ± 13% (P = .0005). These increments are equivalent to 2.7 U/dL for factor II, and 2.2 U/dL for factor X for 1 U/dL of factor IX increased by the infusion of PCC. Disappearance of factors II and X after PCC infusion was slower than that of factor IX during the 48-hour postinfusion period, reflecting the longer half-lives of II and X compared with that of IX (Fig 2). The factor VII increase with PCC was relatively small compared with factors II and X, reflecting a small amount of factor VII contained in the PCC concentrate. Protein C, also vitamin K-dependent but a natural anticoagulant and, therefore, concentrated in the PCC preparation, increased significantly after the PCC infusion, but not after the Mononine infusion (Fig 3). Protein S levels and antithrombin III levels did not appreciably change following the infusion of either Mononine or PCC (Table 3).

To assess the degree of hemostatic system activation during the infusion of Mononine and PCC, F$_{1+2}$ levels were measured on blood samples from three subjects at preinfusion, 1 and 4 hours after the infusions (Fig 4). Levels of F$_{1+2}$ following Mononine infusion remained the same in preinfusion and postinfusion samples at 1 and 4 hours. However, the infusion of PCC was associated with a significant elevation in F$_{1+2}$ at 1 hour postinfusion, reaching 8.6-fold of the preinfusion value (P < .01), and somewhat declining at 4 hours, but the level remained at more than four times the preinfusion level.

Clinical efficacy of Mononine during long-term usage. All 10 patients who entered the study were supplied with Mononine for on-demand use as their sole replacement therapeutic material for their bleeding episodes during a 12-month trial period. All patients were requested to complete home care logs indicating the amount, frequency, and indication for factor infusions. All patients reported excellent response to Mononine in controlling their bleeding episodes, and the responses were at least equivalent to those previously achieved with the use of commercially available PCCs. Home care logs of PCC treatment during the 12 months preceding this trial were available for six patients from one treatment center, and these logs were compared with those of the 12-month Mononine trial period. The frequency of infusions and total amount of factor usage were comparable for the preceding 12 months of PCC use and the 12-month trial of Mononine (Table 4).

During the study period, one patient underwent a surgical procedure consisting of open synovectomy and osteotomy of the right radial head. One year previously, this patient experienced thrombosis involving the right femoral vein extending into the inferior vena cava while receiving PCC for the treatment of a hemothorax. While maintaining the factor IX activity level between 40% and 100% with daily infusion of Mononine during the 2-week period, the therapy provided excellent hemostasis without any significant perioperative bleeding or thrombotic complications, even though no heparin was added to the infusate.

Safety surveillance of Mononine. During the 12-month trial period, a total of 121 evaluable infusions of Mononine were administered to subjects with hemophilia B. Factor IX inhibitor levels were monitored at 6 and 12 months, and none of the participating subjects showed any increase in their inhibitor titers. Levels of anti-mouse IgG, IgM, and

![Fig 2. Factors IX, II, VII, and X levels following a single infusion of Mononine (○) or PCC (■), 25 U/kg. Values represent the mean ± SD of six patient studies.](image-url)
IgE, monitored on a monthly basis, were not significant during the 12-month trial period.

**DISCUSSION**

Factor IX concentrate, produced by monoclonal antibody immunoaffinity chromatography, has an advantage over currently available PCC for hemophilia B treatment in that it provides a highly purified factor IX concentrate without other potentially thrombogenic substances. Factor VIII concentrates prepared by a similar monoclonal antibody immunoaffinity technique, especially if pasteurized, have provided safe therapeutic material for patients with hemophilia A.38 The present clinical trial was conducted to evaluate the purified human factor IX (monoclonal antibody immunoaffinity purified; Mononine) with regard to its in vivo recovery and half-life in patients with factor IX deficiency, to compare Mononine and PCC for their in vivo recovery of other coagulation factors that may predispose to thrombosis, and to assess the clinical efficacy in controlling bleeding episodes on a long-term basis.

The results of in vivo recovery of factor IX (0.67 ± 0.14 U/dL increase per 1 U/kg of infused IX) and \( \beta \) half-life (22.6 ± 8.1 hours) with Mononine in the present trial are comparable to those of previous reports using various sources of PCC. The results of previous reports using PCC show in vivo recovery of factor IX activity ranging from 0.57 to 1.1 U/dL increase per 1 U/kg infusion and half-lives ranging from 23 to 31 hours.39-41 The repeat infusion study of Mononine performed 6 months later on the same subjects yielded essentially identical pharmacokinetic values as those observed at study entry, lending further reassurance that the purification procedure of monoclonal antibody affinity chromatography and virucidal steps, including thiocyanate treatment, do not cause an alteration of factor IX activity nor an unusual immune response in the host.

A comparison of in vivo recovery following a single infusion of Mononine and PCC in factor IX-deficient patients shows that both products were associated with comparable in vivo recovery and survival of factor IX activity. As expected, Mononine infusion did not affect the in vivo levels of \( \Pi \) and \( \mathbf{X} \). However, the PCC infusion caused a significant increase of these factors, raising more than 2 U/dL of \( \Pi \) and \( \mathbf{X} \) while raising 1 U/dL of factor IX. Such in vivo response to PCC infusion is not surprising, since PCC contains more factors \( \Pi \) and \( \mathbf{X} \) than IX itself. Factors \( \Pi \) and \( \mathbf{X} \) also have better in vivo recovery efficiency and longer half-lives than factor IX.24,25 Therefore, infusions of PCC in larger amounts and at more frequent intervals, such as in surgery or major bleeding, would induce a grossly overloaded state of zymogen in patients whose plasma levels of \( \Pi \) and \( \mathbf{X} \) are normal to begin with.42 It is conceivable that even a small triggering of the coagulation cascade by surgery or coexisting trauma during the zymogen-overloaded state with PCC infusion can lead to thrombosis. As expected with PCC infusion, protein C activity increased significantly, but protein S levels did not change. Since protein C exerts its anticoagulant activity after thrombin is generated, increased plasma protein C levels achieved by PCC infusion may not be much of a deterrent against thrombosis by PCC.

Prothrombin activation fragment (\( F_{1+2} \)) is a peptide released during blood clotting when prothrombin is activated to thrombin by the prothrombinase complex (factors Xa and Va, calcium, and phospholipid), and therefore, \( F_{1+2} \)
is a sensitive biologic marker of the in vivo activation of the prothrombin molecule.\(^7\) \(F_{1+2}\) levels after Mononine infusion remained essentially the same as the preinfusion levels. PCC infusion was associated with a significant increase in \(F_{1+2}\) levels for up to 4 hours as compared with the preinfusion level. This finding of increased levels of activation peptides following PCC as a single infusion of 25 U/kg in the steady and nonbleeding state suggests that PCC induces activation of the coagulation cascade in vivo. It is possible that the elevated plasma levels of \(F_{1+2}\) following the PCC infusion are a reflection of exogenously infused \(F_{1+2}\), which may have been contaminating the PCC, rather than the result of in vivo activation of prothrombin. However, in view of relatively rapid metabolic clearance of \(F_{1+2}\) with a half-life of less than 90 minutes,\(^{37}\) the persistently elevated \(F_{1+2}\), lasting more than 4 hours postinfusion, suggests that a portion of elevated \(F_{1+2}\) was due to in vivo prothrombin activation with PCC infusion.

Despite various measures to prevent PCC-induced thrombosis, such as the manufacturer's rigorous screening of PCC lots for thrombogenicity by in vitro testing,\(^42\) addition of heparin to the infusate,\(^43\) or use of a subtherapeutic dose of PCC, thrombotic complications have continued to occur in patients receiving PCC. It is more likely that a purer form of factor IX concentrate devoid of other contaminants would be less thrombogenic than the currently available PCCs. Several new factor IX concentrates, further prepared from PCC, have been developed using a sulfated-dextran method,\(^{15}\) affinity chromatography,\(^{16}\) and heparin sepharose chromatography\(^{17}\) with their reported specific factor IX activity ranging from 10 to 120 U/mg of protein and with relatively lower levels of factors II, VII, and X than PCC. These concentrates have been shown to be less thrombogenic in animal models than PCC. Factor IX purified by monoclonal antibody immunoaffinity technique has demonstrated a high degree of purity, with a specific activity of 180 to 200 U/mg, is devoid of other vitamin K-dependent factors, and is nonthrombogenic up to 400 U/kg in the Wessler's rabbit model.\(^{29}\) The present clinical trial using Mononine further supports the notion that highly purified factor IX effectively increases factor IX in vivo without affecting the levels of vitamin K-dependent factors and thereby may be less thrombogenic than PCC.

In the long-term, 12-month trial, the subjects used Mononine as their sole replacement hemostatic agent. Mononine was shown to be at least as effective as their previous experiences with PCC in controlling bleeding.
episodes. Long-term monitoring for antibody against murine IgG in patients receiving Mononine over a 12-month period has shown the absence of sensitization to murine protein.

Advantages of highly purified forms of factor IX concentrates, such as Mononine, include less thrombogenicity and decreased exposure to allogeneic proteins. Multiple steps of viral reduction, such as immunoaffinity chromatography, the virucidal property of sodium thiocyanate, and the ultrafiltration process, incorporated into the manufacturing process have been shown to be effective in viral reduction and elimination in vitro. Ultimate proof of the safety of such virucidal measures needs to be provided by results from the PUP (previously untreated patient) trials currently underway. Potential disadvantages of monoclonal antibody affinity purification of factor IX are the high cost of the product and the possibility of an adverse immune reaction to miniscule amounts of contaminating mouse proteins infused in the recipients. Clinical experience with monoclonal factor VIII concentrate, which uses similar immunoaffinity procedures, and immune surveillance for mouse antibody, indicate that this adverse reaction is an extremely infrequent event. The availability of highly purified factor IX concentrates, such as Mononine, may reduce the therapy-related complications including thrombosis in patients with hemophilia B.

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