Thrombin Generation Is Not Increased in the Blood of Hemophilia B Patients After the Infusion of a Purified Factor IX Concentrate

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Prothrombin complex concentrates (PCC), licensed for the treatment of hemophilia B, are known to carry a significant risk of thromboembolic complications. Although the reasons for thrombogenicity are not completely understood, several manufacturers have developed purified factor IX concentrates that contain negligible amounts of the other vitamin K-dependent factors. To evaluate whether or not the infusion of such a factor IX concentrate is followed by lesser activation of the hemostatic system than by the infusion of a PCC, we performed a series of coagulation assays on 11 hemophilia B patients before and after the administration of these two types of concentrate using a randomized cross-over design. The levels of prothrombin fragment F1+2, a sensitive measure of the in vivo cleavage of prothrombin by factor Xa, was significantly increased in plasma after PCC, but not after factor IX concentrate. Plasma fibrinopeptide A, a sensitive index of the enzymatic activity of thrombin on fibrinogen, also increased significantly after PCC but not after factor IX concentrate. The fragment Bβ 15-42, a sensitive index of the enzymatic action of plasmin on fibrin II, did not change after either concentrate. There were also no differences in less sensitive coagulation measurements, such as plasma fibrinogen, antithrombin III, and fibrin monomers, nor in indices of platelet activation, such as β-thromboglobulin and platelet factor 4. These findings show that the infusion of a purified factor IX concentrate can result in substantially less activation of the coagulation cascade than may be seen with PCC.

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THROMBIN GENERATION AFTER PURIFIED FACTOR IX

PATIENTS, MATERIALS, AND METHODS

Study design. This was a randomized, cross-over, open-label study. Patients with severe hemophilia B were each infused with the same dose (50 U/kg of factor IX coagulant activity, calculated according to the concentrations declared on the labels by manufacturer) of either factor IX concentrate or PCC, in a randomly determined order. Concentrates were given in hospital as replacement therapy for joint bleeding. Blood samples were scheduled before and at regular intervals for up to 4 hours after concentrate, and plasma levels of coagulation and fibrinolysis reaction products and of the more conventional hemostasis parameters were measured. An interval of at least 10 days, during which no treatment was given, had to elapse before each patient was eligible to receive the alternate product. Within- and between-concentrate changes of the measurements were then evaluated statistically.

Concentrates. The PCC (Profiline, heat-treated), was provided by the manufacturer (Alpha Therapeutic Corporation, Pasadena, CA) as a single lot (CW8026A) in vials containing 55 U/mL factor IX after reconstitution in 10 mL of distilled water. The PCC contained substantial amounts of factors II and X: the factor II level was 160 U/100 factor IX units; the factor X level was 11 U/100 factor IX units; the factor VII level was 11 U/100 factor IX units.

The coagulation factor IX concentrate (AlphNine) was provided in vials containing 49 U/mL factor IX coagulant activity after reconstitution in 10 mL. The concentrate was produced from the PCC by barium citrate precipitation and an affinity chromatography step (not disclosed in detail by the manufacturer) designed to minimize the amounts of factors II, VII, and X contained in the concentrate. No factors II and VII were detected in the lot of concentrate used in this study (CP8004A), whereas the factor X level was 5 U/100 factor IX units. No significant levels of activated forms of clotting factors were contained in the concentrate, as judged by the fact that the nonactivated partial thromboplastin time was in excess of 150 seconds with a control clotting time in excess of 200 seconds. Heparin (no more than 2 U/50 factor IX units) was added to the factor IX concentrate, but not to the PCC. We found that the two concentrates contained similar amounts of factor IX antigen as determined by radioimmunoassay (RIA). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the manufacturer on the factor IX concentrate and PCC. The PCC consisted mainly of one polypeptide with a molecular weight (mol wt) of 84,000 (which most likely is prothrombin) and several other bands. The major component of the factor IX concentrate was a polypeptide with a mol wt of 71,000, which migrated at a position identical to that of factor IX. Major contaminants were polypeptides with mol wts of 53,000 and 23,000, which migrated at positions identical to those of the heavy and light chains of factor X. The 84,000-mol wt polypeptide prevailing in the PCC was present in trace amounts.

Each unit of plasma used by the manufacturer to prepare the concentrates was nonreactive for hepatitis B surface antigen and for antibody to human immunodeficiency virus type 1 (HIV-1), and had alanine aminotransferase levels less than twice the upper limit of normal for the assay method used. Both concentrates were heated for 20 hours in a solvent suspension of n-heptane to reduce the risk of transmitting viral infections.

Patients. Eleven adults (median age 26 years; range, 18 to 60) with severe hemophilia B (factor IX levels: 1 U/dL or less), previously treated with multiple infusions of PCC from several commercial manufacturers, agreed to take part in the study. No patient had symptomatic HIV-1 infection although nine were anti–HIV-1 seropositive, nor did they have clinical evidence of severe kidney or liver disease (though most patients had mild and transient elevations of alanine aminotransferase). At the time of each treatment, all the patients (who had received no infusion of a blood product for at least 10 days) had joint bleeding (in the knee on nine occasions, in the elbow on eight occasions, and in the ankle on five occasions). The order in which the two concentrates were given was determined by a series of random numbers placed in sealed envelopes to be opened before the initial infusion. Blood samples for tests were scheduled before each concentrate infusion and 15, 60, 90, 120, and 240 minutes thereafter. In a few instances samples were not obtained at all time points (see figures). Approval for these studies was obtained from the Institutional Review Board. Patients were informed that their privacy would be protected.

Measurements and methods. Fibrinopeptide A (FPA) is an indicator of thrombin activity, being released from the β-chain of fibrinogen when thrombin converts fibrinogen to fibrin. FPA was measured by enzyme immunoassay in plasma extracted twice with bentonite to remove fibrinogen, using a kit from Boehhringer (Mannheim, West Germany). Prothrombin fragment F1+2 (F1+2) is a measure of the cleavage of prothrombin by activated factor X (factor Xa), being released from the amino terminal portion of the molecule during its conversion to thrombin. F1+2 was measured in plasma by a previously described double antibody RIA. Fragment Bβ 15-42 (Bβ 15-42) is an indicator of plasmin activity, being released by the enzyme from the β chain of fibrin II. It was measured by enzyme immunoassay in plasma heated at 56°C for 35 minutes, using reagents (fibrin monomer, mouse monoclonal antibody [MoAb] to Bβ 15-42, peroxidase-conjugated antimouse immunoglobulin G [IgG], Bβ 15-42 standard) from the New York Blood Center (New York, NY). The within-day and between-day coefficients of variation were 4%, 8%, and 7% or lower for the F1+2, FPA, and Bβ 15-42 assays, respectively. β-thromboglobulin and platelet factor 4 are platelet proteins released into plasma when platelets are activated by thrombin or other aggregating agents, and are, therefore, indirect indices of the presence in vivo of agonists that are capable of inducing platelet activation. They were measured with kits from Amersham International (Amersham, UK) for β-thromboglobulin and from Abbott Laboratories (North Chicago, IL) for platelet factor 4. Other hemostasis parameters included the platelet count (by electronic counting), plasma fibrinogen (by a fibrin polymerization assay, using a kit from Boehringer), antithrombin III (by amidolytic assay, using the chromogenic substrate S-2238 assembled into a kit by Kabi, Stockholm, Sweden), and fibrin monomers (by amidolytic assay, using the chromogenic substrate S-2390 assembled by Kabi into a kit based on the property of fibrin monomers contained in plasma to accelerate the conversion of plasminogen into plasmin by tissue plasminogen activator). The coagulant activities of factors II, VII, IX, and X were measured by standard one-stage assays using congenitally-deficient plasmas containing less than 1% coagulant activities.

Collection and processing of blood samples. Because many of the assays used in this study, particularly the FPA assay, can lead to artificially high results if blood collection and processing are not performed under the strictly standardized and atraumatic conditions described below, all the samples were collected and processed by the same experienced technician. Each of the six blood samples was obtained through a separate venipuncture using 19-gauge needles and a multiple-syringe technique. The first 3 mL of blood collected in a syringe containing no anticoagulant was placed in an EDTA tube for platelet counting. Blood, 2.7 mL, was collected for FPA and Bβ 15-42 into a plastic syringe containing 0.3 mL of the special anticoagulant (containing a thrombin inhibitor, EDTA, and aprotinin) made available by the manufacturers of the FPA kit (Byk-Sangtec, Dietzenbach, West Germany); then, 1.5 mL for F1+2, were collected into a syringe containing 0.3 mL of the special anticoagu-
lant described by Bauer et al\textsuperscript{2} (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/mL heparin). A fourth blood sample of 4.5 mL for $\beta$-thromboglobulin and platelet factor 4 assays was collected in a syringe containing 0.5 mL of the anticoagulant described by Ludlam and Cash\textsuperscript{3} (containing EDTA, theophylline, and prostaglandin E\textsubscript{1}). Finally, an additional sample of 4.5 mL, to be used for the remaining tests, was collected into a syringe containing no anticoagulant and added immediately to a plastic tube containing trisodium citrate (0.129 mol/L). The total time used to obtain blood was between 45 and 60 seconds. All samples were immediately centrifuged at 3,000 g for 30 minutes at 4°C, and the platelet-poor plasma was snap-frozen and stored at -80°C before analysis.

**Statistical analysis.** Before statistical analysis, data were transformed in several ways in an attempt to normalize their distribution when skewness was greater than 0.71 ($P < .05$). For measurements that yielded normally distributed values, results were expressed as means ± one standard deviation. For each measurement, the statistical significance of within-concentrate differences (ie, the differences between baseline values and values obtained at the scheduled times after each concentrate) was evaluated by the Student’s $t$-test for paired data. Between-concentrate differences (ie, the difference between PCC and factor IX concentrate at each postinfusion time) were evaluated by the Student’s $t$-test for unpaired data. For measurements that did not yield normally distributed values even after an array of data transformations, nonparametric tests were used (the Wilcoxon sum test to evaluate between-concentrate difference, the paired signed-ranks test to evaluate within-concentrate differences) and results were expressed as medians and ranges.

**RESULTS**

Figure 1 and Table 1 show that in patients with hemophilia B, factor IX levels increased in plasma after both factor IX concentrate and PCC. At each postinfusion time, factor IX was significantly higher after factor IX concentrate than after PCC ($P < .01$). For factor VII there was no between-concentrate difference at any postinfusion time, but at all postinfusion times factors II and X were significantly higher after PCC than after factor IX concentrate ($P < .001$) (Table 1).

Figures 2 and 3 and Table 2 show the plasma levels of the reaction products of the enzymatic activity of thrombin on fibrinogen (FPA), factor Xa on prothrombin ($F_{\alpha 2}$), and plasmin on fibrin II (B$\beta$ 15-42). Although FPA levels were increased in a few patients after either of the two concentrates, these increments were more frequent and more marked after PCC than after the factor IX concentrate (Fig 2). Accordingly, within-concentrate differences, never significant after factor IX concentrate, were statistically significant immediately after the infusion of PCC (at 15 minutes, $P < .05$) and at late postinfusion times (at 120 minutes, $P < .01$, and at 240 minutes, $P < .05$) (Table 2). The only between-concentrate difference occurred 15 minutes after PCC, with FPA levels slightly higher ($P < .05$) (Table 2). $F_{\alpha 2}$ remained at baseline levels after the factor IX concentrate, whereas after PCC there was a highly significant increase in $F_{\alpha 2}$ ($P < .001$ at each postinfusion time as compared with baseline values) (Fig 3). Between-concentrate differences in $F_{\alpha 2}$ were highly significant throughout the postinfusion period ($P < .001$) (Table 2). There was one patient who had $F_{\alpha 2}$ values clearly above those of the other patients before either concentrate; after factor IX concentrate his high values did not change, whereas after PCC they increased further to about twice the baseline values at each postinfusion time (Fig 3). Table 2 shows the other measurements of hemostasis. B$\beta$ 15-42 did not change after either concentrate, with no significant within- or between-concentrate difference at any postinfusion time (Table 2). Plasma levels of platelet release products ($\beta$-thromboglobulin and platelet factor 4) showed no significant within- or between-concentrate differences nor were there any differences in platelet count, plasma fibrinogen, antithrombin III, and fibrin monomer.

To evaluate whether or not reaction products formed in vitro during the manufacturing of the concentrates might have affected the postinfusion plasma levels, the amount of such products in the two batches of concentrates used in this study was measured. The levels of $F_{\alpha 2}$ and B$\beta$ 15-42 were approximately eight and six times more in the PCC than in the factor IX concentrate (118 $\times$ 13.7 mmol/L and 30 $\times$ 4.6 mmol/L, respectively). The PCC contained 97 nmol/L of FPA, while the factor IX concentrate did not contain detectable amounts of this species.

**DISCUSSION**

As expected, infusions of both the PCC and factor IX concentrate were followed by the attainment of measurable plasma levels of factor IX coagulant activity in patients with severe hemophilia B. Less expected was the finding that higher levels were obtained after factor IX concentrate than after PCC, despite the fact that the same dose of factor IX coagulant activity (50 U/kg) was administered to patients, as calculated on the basis of the concentrations declared on the labels by the manufacturer. Possible explanations for this finding include inaccurate assays of either concentrate by the manufacturer, or substantial differences in their plasma.
Table 1. Changes in Vitamin K-Dependent Coagulation Factors in 11 Patients With Hemophilia B Treated With Factor IX Concentrate or With Prothrombin Complex Concentrate (50 U/Kg)

<table>
<thead>
<tr>
<th>Measurement</th>
<th>Treatment</th>
<th>Baseline (time 0)</th>
<th>After 15 min</th>
<th>After 60 min</th>
<th>After 90 min</th>
<th>After 120 min</th>
<th>After 240 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Factor II, % of normal plasma</td>
<td>Factor IX</td>
<td>95 ± 21</td>
<td>100 ± 33</td>
<td>110 ± 35</td>
<td>109 ± 30$^+$</td>
<td>116 ± 38$^+$</td>
<td>113 ± 41$^+$</td>
</tr>
<tr>
<td></td>
<td>PCC</td>
<td>87 ± 17</td>
<td>194 ± 41$^+$</td>
<td>201 ± 36$^+$</td>
<td>199 ± 34$^+$</td>
<td>200 ± 29$^+$</td>
<td>203 ± 38$^+$</td>
</tr>
<tr>
<td>Factor VII, % of normal plasma</td>
<td>Factor IX</td>
<td>92 ± 19</td>
<td>95 ± 17</td>
<td>95 ± 19</td>
<td>98 ± 18</td>
<td>102 ± 22</td>
<td>103 ± 21</td>
</tr>
<tr>
<td></td>
<td>PCC</td>
<td>90 ± 26</td>
<td>98 ± 30</td>
<td>95 ± 25</td>
<td>94 ± 24</td>
<td>95 ± 22</td>
<td>94 ± 31</td>
</tr>
<tr>
<td>Factor IX, % of normal plasma</td>
<td>Factor IX</td>
<td>&lt;1</td>
<td>74 ± 20$^+$</td>
<td>72 ± 20$^+$</td>
<td>70 ± 20$^+$</td>
<td>66 ± 19$^+$</td>
<td>57 ± 17$^+$</td>
</tr>
<tr>
<td></td>
<td>PCC</td>
<td>&lt;1</td>
<td>52 ± 11$^*$</td>
<td>45 ± 10$^*$</td>
<td>45 ± 10$^*$</td>
<td>41 ± 10$^*$</td>
<td>37 ± 8$^*$</td>
</tr>
<tr>
<td>Factor X, % of normal plasma</td>
<td>Factor IX</td>
<td>110 ± 21</td>
<td>101 ± 23</td>
<td>104 ± 22</td>
<td>106 ± 20</td>
<td>107 ± 25</td>
<td>106 ± 26</td>
</tr>
<tr>
<td></td>
<td>PCC</td>
<td>106 ± 25</td>
<td>165 ± 26$^+$</td>
<td>164 ± 23$^+$</td>
<td>166 ± 22$^+$</td>
<td>160 ± 21$^+$</td>
<td>158 ± 24$^+$</td>
</tr>
</tbody>
</table>

Results are given as means ± one standard deviation.
Significant within-concentrate differences: $^*P < .01; ^{t}P < .001$.
Significant between-concentrate differences: $^{\#}P < .05; ^{\#\#}P < .01; ^{\#\#\#}P < .001$ as compared with baseline values.

distribution and clearance. Both concentrates contained similar amounts of factor IX, as measured immunologically. However, because our recovery studies were based on an assay of factor IX coagulant activity and the PCC may contain significant amounts of activated factor IX, it is possible that the functional assay used by the manufacturer to establish the factor IX potency of the PCC overestimated the coagulant activity as compared with that of the factor IX concentrate. After either concentrate there was no important increase in factor VII, in agreement with the fact that neither contains this factor in large amounts. After PCC, factors II and X increased well above the normal preinfusion values, whereas they increased minimally after factor IX concentrate, in agreement with the low in vitro content of these factors.

Conventional indices of intravascular coagulation, such as fibrinogen, antithrombin III, fibrin monomer, and platelet count, did not change after either concentrate. There was also no increase in the plasma levels of β-thromboglobulin and platelet factor 4, indicating that the platelet release reaction did not occur. The most striking finding after PCC, but not after factor IX concentrate, was the consistent and sustained increase in the prothrombin fragment \( F_{1+2} \) concentrations. Interpretation of this finding is complicated by the fact that the batch of PCC administered to patients contained substantial amounts of the fragment, which were probably formed during the manufacturing process. Because we had determined that the reconstituted PCC contained 118 nmol/L of \( F_{1+2} \), a dose of 50 U/kg of this product to a 70 kg hemophiliac (3,500 U in 70 mL) should result in the infusion of 8.3 nmol of \( F_{1+2} \). Assuming that immediate distribution of the fragment into the extravascular space does not occur, the plasma \( F_{1+2} \) level would be expected to increase by 3.0 nmol/L (8.3 nmol divided by 2.8 L, the plasma volume of a 70-kg person). This amount of exogenous fragment would account for most of the elevations seen at the early postinfusion time point (15 minutes) (Fig 3). However, at subsequent time points (60, 90, 120, and 240 minutes), the \( F_{1+2} \) concentrations were considerably more elevated than expected from PCC infusion alone. From metabolic turnover studies with \( ^{125}I \)-labeled \( F_{1+2} \), it is known that this fragment has a half-life of approximately 90 minutes.22,26,27 For exam-
ple, based on clearance considerations alone, we would have expected the mean $F_{1+2}$ level (1.66 mmol/L) at the 240-minute time point (almost three $F_{1+2}$ half-lives) to be no different from the mean preinfusion level (0.65 mmol/L). Therefore, the data suggest that there was increased in vivo prothrombin activation resulting from the administration of PCC. Because no patient had clinical and laboratory signs of severe liver or kidney disease nor symptomatic HIV infection, it is unlikely that the late elevation of $F_{1+2}$ is due to delayed in vivo clearance of the fragment. In contrast to the PCC, the infusion of a purified factor IX concentrate was not followed by increments in $F_{1+2}$ levels.

To evaluate whether or not heightened activation of prothrombin led to increased thrombin and plasmin action on fibrinogen after PCC, we also measured FPA and $\beta\alpha$ 15-42, respectively. The plasma half-life of FPA is short (approximately 3 to 5 min). The infusion of FPA contained in the PCC could account for the small increment in the levels that was demonstrated at the 15-minute time point. However, significant elevations in FPA measurements were observed at 120 and 240 minutes postinfusion of the PCC as compared with baseline. These changes parallel the persistent elevations in $F_{1+2}$ levels at these time points, providing further evidence that thrombin generation is increased. However, this augmentation in thrombin activity was not sufficient to lead to elevation in the levels of fibrin monomer or to evidence of enhanced fibrinolytic activity as measured by the $\beta\alpha$ 15-42 assay.

In summary, elevations in the levels of $F_{1+2}$ and FPA without evidence of increased fibrin formation were observed after the administration of PCC, but not after the infusion of a purified factor IX concentrate. This result shows a prothrombotic state in biochemical terms. The mechanisms responsible for the relatively late elevations in $F_{1+2}$ and FPA levels after the administration of PCC are not known. It is of interest that Hedner et al^2^ reported a series of animal experiments in which the most profound abnormalities in coagulation measurements occurred 4 hours after the infusion of large doses of PCC. One must be cautious in extrapolating our results to clinical practice inasmuch as the total dose and schedule of factor IX concentrates were somewhat different than those that are commonly used in clinical practice. Only a single infusion of concentrate was administered to our patients and the dose was higher than which is usually recommended for the treatment of minor bleeding episodes in individuals with hemophilia B (10 to 30 U/kg).^3^ On the other hand, while a dose of 50 U/kg of factor IX is recommended before surgery to prevent postoperative bleeding,^3^ these infusions are usually repeated for several weeks. We do not yet have any data regarding the effects of multiple doses of factor IX concentrate on activation of the hemostatic system. Substantial clinical experience will be required to conclusively establish that purified factor IX concentrates are less thrombogenic than PCC. Clinical trials with these new preparations have thus far been limited to a relatively small number of patients.^1^
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