Activated Clotting Factors in Factor IX Concentrates

By Mae B. Hultin

The precise quantitation of activated factors in human factor IX concentrates has been accomplished with the use of recently developed, specific assays for factors IXa, Xa, and thrombin. The assay for factor IXa, which measures the initial rate of $^3$H-factor-X activation, was shown to be specific for factor IXa in the concentrates. Activated factor IX concentrates contained 1.0–2.3 μg/ml of factor IXa; whereas the assays of unactivated concentrates were negative (<0.2 μg/ml). The assays of factor Xa and thrombin, which measure the initial rate of p-nitroaniline release from S-2222 and S-2238, respectively, showed similar small amounts of factor Xa (4–34 ng/ml) and thrombin (12–76 ng/ml) in the activated and unactivated concentrates. The nonactivated partial thromboplastin time of the concentrates correlated significantly with the factor IXa content, but not with factor Xa or thrombin. Antithrombin III antigen in 3 of 4 concentrates was several-fold higher than antithrombin III activity, suggesting the presence of antithrombin III complexed with activated factors. These results support the hypothesis that the degree of activation of factor IX concentrates is related primarily to the concentration of factor IXa, which may be responsible for the thrombogenicity of these concentrates in some clinical settings.

Human factor IX concentrates are partially purified plasma fractions that are commercially prepared for the transfusion therapy of patients with isolated or combined deficiencies of the vitamin-K-dependent clotting factors II, VII, IX, and X.1,2 Recently, these concentrates, including experimental activated preparations, have also been used in factor-VIII-deficient patients with factor VIII inhibitors.3,4 Thrombotic complications have occurred following therapy with these concentrates in patients with hemophilia B5 or liver disease,6 and signs of disseminated intravascular coagulation have been reported in two factor-VIII-inhibitor patients who received activated preparations of concentrates.7,8

Previous studies using clotting assays and immunologic techniques suggested that some lots of these concentrates contain not only the zymogens but also varying amounts of the activated enzymatic forms of the vitamin–K-dependent factors.9–11 Since factor IXa, in particular, as well as factor Xa and thrombin, is thrombogenic in animal models,12 this study was designed to quantitate these enzymes in both the unactivated and the activated concentrates. A new specific assay for factor IXa, recently reported by this laboratory,13 was adapted for the assay of the concentrates. Specific assays for factor Xa and thrombin, using chromogenic substrates, were also performed on the concentrates. The results of these specific quantitative assays were compared to the nonactivated partial thromboplastin time of the

From the Division of Hematology, Department of Medicine, the State University of New York at Stony Brook, and the Veterans Administration Medical Center, Northport, New York.

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Address reprint requests to Mae B. Hultin, Division of Hematology, Health Sciences Center, T-15-040, Stony Brook, N.Y. 11794.

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concentrates, since this less specific assay has been used widely to detect activated material in the concentrates. In order to clarify discrepant data on the importance of the antithrombin III content of the concentrates and the possible value of added heparin, the antithrombin III content of the concentrates was measured both immunologically and functionally.

MATERIALS AND METHODS

Materials

Chromogenic substrates S-2222 (N-Benzoyl-Ile-Glu-Gly-Arg-p-nitroanilide HCI), S-2238 (H-D-Phe-Pip-Arg-p-nitroanilide HCI), and S-2251 (H-D-Val-Leu-Lys-p-nitroanilide HCI) were purchased from Ortho Diagnostics (Raritan, N.J.), reconstituted in water, and stored at 4°C. M-Partigen radial immunodiffusion kits for antithrombin III were purchased from Behring Diagnostics (San Diego, Calif.). Factor VIII concentrate (Hemofil, Method IV) was kindly supplied by Hyland Laboratories, Costa Mesa, Calif. Disopropylfluorophosphate (DFP) was purchased from Sigma, St. Louis, Mo. Other chemicals and reagents for protein purifications and assays were obtained as previously reported.

Factor IX Concentrates

Proplex (Lots 0581D137 AA, 0581D153 AA) and Konyne (lot NC 9007) were obtained from Hyland Laboratories and Cutter Laboratories, Berkeley, Calif., respectively. Activated factor IX concentrates were obtained from two manufacturers: Auto-IX (lot 650D023) was a gift of Hyland, and FEIBA (lots a-d: 05A2477, 05A1877, 05A2377, 05A2277) was a gift of Immuno. AG., Vienna, Austria. All concentrates were freshly reconstituted with 10–20 ml of sterile water within 1 hr of assay, kept on ice, and diluted in buffer prior to assay. In order to facilitate comparison between concentrates, all assays on concentrates reported here are expressed as the concentration in a total volume of 20 ml, i.e., concentration x 20 = total content of one vial. Proplex and Auto IX both contain heparin (~1–1.5 U/ml); whereas Konyne and FEIBA do not.

Protein Purification

Human factor VIII and bovine factors IX, IXa, X and tritiated X were prepared as previously reported. Bovine factors Xa and thrombin (kindly provided by Dr. Jolyon Jesty) were purified as previously reported.

Coagulation Assays

Assays for factors VIII, IX, Xa, thrombin, and antithrombin III (heparin cofactor activity) were performed as previously reported. Factor X was measured by the method of Denson, using barium-sulfate-adsorbed bovine plasma to which purified prothrombin was added. The unactivated partial thromboplastin time test (NAPTT) was performed according to Kingdon et al. Results are expressed as the ratio of the NAPTT to a buffer control, since the buffer control varied among batches of nonactivated platelet-poor plasma (200–250 sec). The results reported here are the NAPTTs of a 1/80 dilution of the activated concentrates, rather than a 1/10, since heparin present in Auto IX interfered with the assay at dilutions 1/10–1/50. The assay is considered negative if the NAPTT of all dilutions tested (1/10–1/80) differed from the buffer control by less than 20 sec. or a ratio >0.9.

Specific Factor IXa Assay

As previously reported, this assay is performed by combining a test sample, diluted in 0.1 M NaCl, 0.05 M Tris, pH 7.5, with tritiated bovine factor X (50 μg/ml), cephalin, thrombin-activated human factor VIII (30–50 U/ml after activation), CaCl₂ (8 mM), and 2 mM benzamidine-HCl in a polystyrene tube at 37°C. The initial rate of factor X activation is quantitated by scintillation counting of the trichloroacetic acid-soluble tritiated activation peptide in timed serial subsamples over 3–5 min. The amount of factor IXa in a test sample is calculated by reference to the calibration curve of initial rate versus factor IXa concentration for purified bovine factor IXa, performed on the same day. Bovine factor IXa was calibrated against purified human factor IXa (a gift of Dr. David Aronson) and found to have 100% of the specific activity of human factor IXa in the assay; therefore, the reported data were calculated relative to the bovine factor IXa reference without correction.
Preliminary experiments were performed to determine the specificity of the assay for factor IXa when the test sample contained a mixture of proteins, such as the factor IX concentrates. Varying mixtures of factors Xa, IXa, and thrombin were assayed as described in the presence of 2 mM-benzamidine-HCl, which has previously been shown to inhibit thrombin and factor Xa much more than factor IXa. Factor IXa could be accurately assayed over the range of 0.1–3.0 μg/ml in the presence of as much as 0.1 μg/ml of thrombin and 0.2 μg/ml of factor Xa. These levels are greater than the levels of factor Xa and thrombin found in the concentrates. The assays on the concentrates were also performed in several other ways to confirm the specificity of the assay: (1) with and without activated factor VIII, (2) posttreatment with 10 mM DFP x 16 hr. and (3) with and without a specific human inhibitor of factor IX. Treatment with 10 mM DFP under identical conditions completely inhibited purified factor Xa and thrombin but had no effect on factor IXa.

Since factor IX and X in the concentrates might inhibit the factor IXa assay, the sensitivity of the assay on the concentrates was tested by assaying mixtures of factor X, or factor IX, with factor IXa. Varying concentrations of bovine factor IX in excess were combined with a constant concentration of bovine factor IXa and the assay performed on the mixtures. As shown in Fig. 1, increasing concentrations of factor IX caused a proportional decrease in the apparent factor IXa activity at a constant concentration of factor IXa. Below 50 μg/ml, factor IX gave <10% inhibition of the factor IXa assay. Similarly, varying concentrations of bovine or human factor X (a gift of Dr. Philip Majerus) were added to a constant concentration of bovine factor IXa, and the factor IXa assay performed on the mixtures. Increasing concentrations of unlabeled factor X showed a proportional decrease in the apparent factor IXa activity at a constant concentration of factor IXa and tritiated factor X. This inhibition showed a stoichiometry of approximately 1:1; that is, the assay of factor IXa, 1 μg/ml, with 50 μg/ml of tritiated factor X, was reduced 50% by the additional presence of 50 μg/ml of cold factor X.

Since a similar inhibition of the assay of human IXa would be likely in the presence of a large excess of human factor IX, or lesser amounts of factor X, it was necessary to quantitate the factor IX and X content of the concentrates in order to determine the dilution necessary to perform the factor IXa assay. The factor IX content of the concentrates was estimated by antibody neutralization assay with a specific human factor IX antibody and compared to pooled normal human plasma, which was assumed to contain 5 μg/ml of factor IX antigen. Auto IX, Proplex, and Konyne contained 40-50 μg/ml, while FEIBA-1000 contained 150 μg/ml. The small fraction of factor IX antigen, which may be factor IXa, was disregarded, so that the factor IX content may be slightly overestimated by this method. The factor X content of the concentrates was estimated by a clotting assay in which factor X is converted to factor Xa by Russell's viper venom; the assay is calibrated by comparison to pooled normal human plasma, which was assumed to contain 10 μg/ml of factor X. The factor X content of the four types of concentrates was 50–200 μg/ml. Since this assay measures the sum of factors X and Xa in the concentrates, it may slightly overestimate the factor X content. On the basis of these factor IX and X estimates, a 1/5 or 1/4 dilution of these concentrates would be sufficient in every case to reduce the factor IX level below 50 μg/ml, while a 1/5 to 1/10 dilution would be necessary to reduce factor X below 10 μg/ml (<20% inhibition of the factor IXa assay). Therefore, the concentrates were assayed at a final dilution of 1/5 or 1/4, and in some cases, repeated at higher dilutions. The reported data are the average of at least 2
independent assays. The coefficient of variation of the assay on the concentrates was determined to be 8% by 5 independent assays on Auto IX; thus, the reproducibility of the assay is comparable to that for purified factor IXa.13

Assay of Factor Xa on S-2222

Concentrates were diluted in 50 mM phosphate, 0.2% bovine serum albumin pH 7.5. Assays were performed by placing duplicate 250-µl diluted samples in microcuvettes, which were prewarmed and transferred to a 37°C cuvette holder in the Beckman Acta CIII recording spectrophotometer. The reaction was started by the addition to each cuvette of 50 µl of prewarmed S-2222 (final concentration 0.16 mM), and the initial rate of p-nitroaniline release (linear change in absorbance, ∆A, at 405 nm) recorded over 5-25 min. The assay was calibrated with purified bovine factor Xa, which gave a linear reproducible plot of ∆A/sec versus concentration over a wide range. The usual concentration range used was 0.5–10 ng/ml, and test samples were diluted to fall within this range. Purified bovine factor IXa had negligible activity on this substrate (0.5 ng/ml of apparent factor Xa in 24 µg/ml factor IXa). Dilutions of freshly reconstituted concentrates were made just prior to assay, since control experiments showed that factor Xa in diluted concentrates increased twofold after 1–2 hr at 4°C and as much as tenfold after freezing and thawing.

Assay of Thrombin on S-2238

Assays were performed as described above for factor Xa, with the exception that S-2238 at a final concentration of 0.05 mM was used. The assay was calibrated with purified bovine α-thrombin (3500 NIH U/mg, 95% active by NPGB titration) over the range of 0.5–5 ng/ml. When this bovine thrombin was compared to purified human α-thrombin (2914 NIH U/mg, 97% active by NPGB, a gift of Dr. John Fenton), it was found to have 85% of the specific activity of human thrombin on S-2238. The assay results reported here were calculated relative to the bovine thrombin reference without correction; therefore, an assay reported as 1 ng/ml is the equivalent of 3.5 mU/ml. Purified bovine factor IXa at 6 µg/ml had no activity (<0.2–0.3 ng/ml) on this substrate, while purified bovine factor Xa at 1 µg/ml had 12 ng/ml apparent "thrombin" activity.

Assay for Plasmin on S-2251

Since the presence of plasmin might cause false negative factor IXa assays by indicating factor VIII, the concentrates were assayed for plasmin as described above for factor Xa but with the substitution of substrate S-2251 at a final concentration of 0.6 mM. The assay was calibrated with a purified plasmin reference (a gift of Dr. John Finlayson), compared to the manufacturer’s stated specific activity for purified plasmin. Purified factors IXa, Xa, and thrombin had negligible activity on S-2251.

Antithrombin III Assays

Antithrombin III was measured in two ways. (1) Heparin cofactor activity was measured in the presence of added thrombin and excess heparin, as previously described.13 Concentrates were diluted sufficiently for assay so that traces of thrombin or heparin in the concentrates would not affect the antithrombin III assay. The assay was calibrated with pooled normal human plasma (PNP) over a range of 0.4–1.7 µg/ml (1/4–1/2 of PNP, 250 µg/ml). (2) Antithrombin III antigen was measured by radial immunodiffusion using M-Partigen plates. Pooled normal human plasma gave a reproducible assay over a range of 15–250 µg/ml (undiluted to 1/4); the presence of 1–2 U/ml heparin had no effect on the assay of PNP. In order to measure the small amount of antithrombin III in the concentrates, vials of concentrates were reconstituted to 10–15 ml and assayed undiluted and diluted 1/2 with normal saline. Thus, the assay could detect as little as 150 µg/vial (15 µg/ml x 10 ml). The results are reported as the concentration for a 20-ml total volume per vial in order to allow direct comparison with the activated factor assays.

Protein Concentration

Each reconstituted vial of concentrate was assayed for protein content by the method of Lowry15 in order to verify that concentrates reconstituted to different volumes were comparable in total protein content. The concentration of purified proteins was estimated by absorbance at 280 nm, as previously reported.13
Statistical Methods

The results of the factor IXa, Xa, or thrombin assays were compared to the NAPTT results on the same concentrates using the rank correlation coefficient, rather than the product-moment correlation coefficient, since the former does not depend on the data having a normal distribution and a linear relationship.25

RESULTS

Factor IXa Assays

Factor IXa was detectable at 1.0–2.3 µg/ml in the activated concentrates but not in the unactivated concentrates (Table 1). The specificity of the assay for factor IXa in the concentrates was supported by several control experiments. First, it was shown that the factor IXa activity detected in the activated concentrates depended on the presence of thrombin-activated factor VIII, since in the absence of factor VIII, the assay was negative, i.e., no activation of 3H-factor-X occurred. In addition, a specific human inhibitor of factor IX, which also inhibited purified human factor IXa, completely inhibited the activity of Auto IX in the factor IXa assay; whereas an identically prepared fraction of normal human serum had no effect on the assay (Fig. 2). Finally, all of the concentrates were treated with 10 mM DFP to inactivate factors Xa and thrombin (and any other DFP-sensitive enzymes that might be present) but not factor IXa. The factor IXa assay on the DFP-treated concentrates, in the absence of BZA, gave very similar results (within 10%) to the assays on the untreated concentrates, which were performed in 2 mM BZA. Since the assay in the absence of benzamidine (BZA) can detect as little as 50 ng/ml of factor IXa,13 a negative factor IXa assay on a DFP-treated sample in the absence of BZA probably represents <50 ng/ml multiplied by the dilution of the concentrate. On this basis, the negative factor IXa assays in Table 1 are reported as less than the calculated lower limit of sensitivity of the assay, which varied with the dilution necessary to perform the assay. All of the concentrates tested showed no or minimal activity on S-2251. Thus, it is unlikely that plasmin or any DFP-sensitive enzyme in the concentrate depressed the factor IXa assay by inactivating the factor VIII in the assay.

Table 1. Activated Clotting Factors in Factor IX Concentrates

<table>
<thead>
<tr>
<th>Concentrate</th>
<th>Factor IXa (ng/ml)</th>
<th>Factor Xa (ng/ml)</th>
<th>Thrombin (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated concentrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Auto IX</td>
<td>2300</td>
<td>34</td>
<td>46</td>
</tr>
<tr>
<td>2a. FEIBA-1000*</td>
<td>1000</td>
<td>22</td>
<td>76</td>
</tr>
<tr>
<td>b. FEIBA-1000*</td>
<td>1200</td>
<td>19</td>
<td>26</td>
</tr>
<tr>
<td>c. FEIBA-1000*</td>
<td>1100</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>d. FEIBA-250*</td>
<td>&lt;270†</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Unactivated concentrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3. Proplex‡</td>
<td>&lt;200†</td>
<td>6</td>
<td>14</td>
</tr>
<tr>
<td>4. Konyne</td>
<td>&lt;150†</td>
<td>18</td>
<td>37</td>
</tr>
</tbody>
</table>

*Lots a–d correspond to the lot numbers of FEIBA listed in order in Materials and Methods.
†These negative assays are expressed as less than the calculated lower limit of sensitivity of the assay, which varies with the dilution necessary to perform the assay.
‡Lot number 0681D153AA.
Fig. 2. Inhibition of factor IXa by factor IX antibody. The factor IXa assay was performed on Auto IX in the presence (A) and absence (B) of a partially purified fraction of serum containing a high-titer specific human inhibitor of factor IX. The assay is plotted as the counts per minute of $^3$H-activation peptide versus the time of subsampling (minutes) from the assay mixture. An identically prepared fraction of normal human serum had no effect on the assay of factor IXa ($\bullet$).

Factor Xa Assays

Activated and unactivated concentrates contained similar small amounts of factor Xa by S-2222 assay, with a range of 4–34 ng/ml and 6–18 ng/ml, respectively (Table 1). Since factor IXa and thrombin have very low specific activities on this substrate, the factor IXa and thrombin content of the concentrates could account for only a minimal fraction (<5%) of the apparent factor Xa detected by this method. In contrast, the apparent factor Xa activity detected in these concentrates by a clotting assay for factor Xa was 2–4-fold greater than the results of the chromogenic assay, presumably because the clotting assay is also influenced by the presence of other factors.

Thrombin Assays

Activated and unactivated concentrates contained similar small amounts of thrombin by S-2238 assay, ranging from 12 to 76 ng/ml and 14 to 37 ng/ml, respectively (Table 1). On the basis of the demonstrated minimal activity of factors IXa and Xa on this substrate, the factor IXa and Xa content of the concentrates could account for <5% of the apparent thrombin content by this assay. The range of thrombin detected (12–76 ng/ml) represents 0.04–0.3 NIH U/ml, approximately. When the concentrates were assayed by a standard clotting technique for thrombin that detects as little as 0.2 NIH U/ml, only FEIBA contained detectable thrombin (0.2–0.5 U/ml). Although the thrombin level in Auto IX by S-2238 should also be detectable by the clotting assay, the presence of heparin in Auto IX necessitates considerable dilution prior to clotting assay, thus decreasing the sensitivity of the assay. Therefore, the results of the clotting assays may be consistent with those of the chromogenic assay; however, it is also possible that the chromogenic assay is measuring $\gamma$-thrombin as well as $\alpha$-thrombin, while only the latter would be detected by the clotting assay.

NAPTT Test

The NAPTT was positive on the activated concentrates but not the unactivated concentrates. As initially described, the NAPTT is reported as the clotting time of a 1/8 dilution of the concentrate (the shorter the clotting time, the more activated the concentrate). This reporting method is known to be inapplicable to concentrates
that contain sufficient heparin to prolong the clotting time at a \( \frac{1}{10} \) dilution. In these studies, the positive NAPTT of Auto IX, which contains heparin, shortened with increasing dilution from \( \frac{1}{10} \) to \( \frac{1}{20} \), reached a plateau at \( \frac{1}{20}-\frac{1}{40} \), then lengthened. Therefore, the activated concentrates were compared at \( \frac{1}{10} \) in order to determine their relative degree of activation. Auto IX had the shortest clotting time (44 sec; ratio of 0.22 compared to buffer), followed by FEIBA-1000 lot b > a > c. FEIBA-250 (lot d) had a negative NAPTT at \( \frac{1}{10} \), was borderline at \( \frac{1}{20} \) (ratio, 0.9), and positive at \( \frac{1}{2} \) (ratio, 0.5); whereas Konyne was negative at both dilutions (ratio >0.9). Since neither contains heparin, it is reasonable to rank FEIBA-250 as more positive than Konyne, but less than FEIBA-1000 or Auto IX. Proplex had a negative NAPTT at all dilutions, but the presence of heparin prolonged the assay on dilutions of \( \frac{1}{10} \) or less, so that it was not possible to rank Proplex in relation to Konyne or FEIBA-250. When the results of the NAPTT were plotted versus the factor IXa, Xa, or thrombin content of the concentrates, there appeared to be a positive correlation between shortening of the NAPTT and the factor IXa content (Fig. 3), which was not as evident for factor Xa (Fig. 4) or thrombin (Fig. 5). When the data were analyzed by a rank correlation method (omitting Proplex, \( n = 6 \)), there was a significant correlation of the NAPTT with factor IXa concentration (\( r = .94, p < 0.02 \)) but not with factor Xa (\( r = .77, p > 0.1 \)) or with thrombin concentration (\( r = .40 \)). Even when factor Xa levels were intentionally increased to 100–200 ng/ml by freezing and thawing the unactivated concentrates, the NAPTTs of these concentrates remained negative.
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Fig. 5. The relationship between the thrombin concentration and the NAPTT of the factor IX concentrates. The thrombin concentration (µg/ml) is plotted on the y axis versus the NAPTT ratio on the x axis for each concentrate (symbols as in Fig. 3).

Antithrombin III Content

The activated and unactivated concentrates had similar small levels of antithrombin III antigen (8-41 µg/ml and 15-42 µg/ml, respectively, Table 2). In contrast, antithrombin III biologic activity, measured as heparin cofactor, was only 2-9 µg/ml, or 8%-38% of the antigen level, in one unactivated and two activated concentrates; whereas only one unactivated concentrate (Konyne) had approximately equal levels of antithrombin III antigen and activity (Table 2). Those concentrates containing heparin (Auto IX and Proplex) had lower ratios of activity to antigen (8%-14%) than those without heparin (FEIBA and Konyne), with ratios of 21%-126%.

DISCUSSION

The identification and quantitation of both the therapeutic and thrombogenic components in factor IX concentrates had been hampered previously by the nonspecific nature of most clotting assays used to evaluate the concentrates. Several clotting assays, including the NAPTT and the thrombin generation time, have been reported to correlate with the thrombogenicity of factor IX concentrates in the animal model of stasis thrombosis.14,23 These assays may or may not detect the same components in the concentrates.24,25 The apparent thrombogenic component that is detected by the NAPTT was tentatively linked to factor IXa and Xa activity by inhibitor studies.9 In the stasis thrombus model, purified factor IXa is 7 times more thrombogenic than factor Xa and 60 times more than thrombin on a

Table 2. Antithrombin III (AT III) in Factor IX Concentrates

<table>
<thead>
<tr>
<th>Concentrate</th>
<th>AT III Antigen (µg/ml)</th>
<th>AT III Activity (µg/ml)</th>
<th>Activity/Antigen (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activated concentrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Auto IX*</td>
<td>24</td>
<td>1.8</td>
<td>8</td>
</tr>
<tr>
<td>2a. FEIBA-1000†</td>
<td>41</td>
<td>8.8</td>
<td>21</td>
</tr>
<tr>
<td>b. FEIBA-1000</td>
<td>26</td>
<td>6.1</td>
<td>24</td>
</tr>
<tr>
<td>c. FEIBA-1000</td>
<td>19</td>
<td>7.3</td>
<td>38</td>
</tr>
<tr>
<td>d. FEIBA-250</td>
<td>8</td>
<td>2.8</td>
<td>35</td>
</tr>
<tr>
<td>Unactivated concentrates</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3a. Proplex*</td>
<td>40</td>
<td>5.5</td>
<td>14</td>
</tr>
<tr>
<td>b. Proplex*</td>
<td>42</td>
<td>4.6</td>
<td>11</td>
</tr>
<tr>
<td>4. Konyne</td>
<td>15</td>
<td>19</td>
<td>126</td>
</tr>
</tbody>
</table>

*Contain heparin, 1-1.5 U/ml Lots a and b of Proplex are the first and second lot numbers given under Materials and Methods.
†Lots a–d of FEIBA correspond to the lot numbers of FEIBA listed in order under Materials and Methods.
molar basis; the minimal thrombogenic dose of factor IXa was 200–400 ng in a 2-kg rabbit. Similar studies of the dose-response relationship of factor IX concentrates would be feasible if specific assays for each activated clotting factor could be performed on the concentrates.

This study has demonstrated that a newly developed assay for factor IXa, which measures 3H-factor-X activation, can be used to assay factor IXa in factor IX concentrates without loss of specificity and only minor loss of sensitivity. The relative specificity of chromogenic substrate assays for factor Xa and thrombin has been confirmed and their feasibility for the assay of the concentrates demonstrated. These studies showed a marked difference in factor IXa content between the activated and unactivated concentrates, but not in factor Xa or thrombin content. The negative factor IXa assay on one lot of activated concentrate, FEIBA-250, is consistent with its lower protein content and lesser degree of activation (250 FEIBA U according to the manufacturer) compared to the three lots of FEIBA-1000. The factor IXa content of the concentrates correlated significantly with the NAPTT results; whereas the factor Xa and thrombin content did not. Although thrombogenic lots of unactivated concentrates were not available for assay in this study, it is probable that those lots with short NAPTTs would contain measurable factor IXa by the tritiated factor X assay. The amount of factor IXa in the activated concentrates (20–46 µg/vial) was 100–200 times the minimal thrombogenic dose in normal 2-kg rabbits and thus might be thrombogenic in humans. In contrast, the factor Xa and thrombin content per vial of all the concentrates tested were well below the minimal thrombogenic dose for a rabbit and thus probably for humans as well. However, synergistic effects of factor Xa and thrombin might occur in the presence of factor IXa and contribute to thrombogenic potential. Furthermore, the action of factor IXa is markedly dependent on the presence of its cofactor, factor VIII. Since the activated concentrates are used solely in factor-VIII-deficient patients, the risk of thrombosis from factor IXa may be small in this setting. It is interesting that no thrombotic complications have been reported for Auto-IX and only two cases for FEIBA, one of which occurred when cryoprecipitate was given simultaneously.

The persistence of active enzymes—factors IXa, Xa, and thrombin—despite measurable antithrombin III activity in the concentrates might at first seem inconsistent. However, recent studies have shown that the inhibition of factor Xa and thrombin by antithrombin III in molar excess does not proceed to completion, and the apparent equilibrium concentration of free enzyme depends on the antithrombin III concentration. Therefore, it is plausible that most, but not all, of the activated factors generated during preparation of the concentrates would be inactivated by complex formation with antithrombin III. The apparent excess of antithrombin III antigen compared to activity in the two activated concentrates, and in one unactivated concentrate containing heparin, is consistent with the hypothesis that most of the antithrombin III is complexed with activated factors. Since heparin appears to act by increasing the rate of inactivation by antithrombin III without affecting the final equilibrium, it is understandable that the presence of antithrombin III, with or without heparin, during the purification of the concentrates appears to have much more effect on thrombogenicity than the addition of heparin to the reconstituted concentrate.
Another activated factor that might have therapeutic or thrombogenic potential in some settings is factor VIIa, particularly as it is not inhibited by antithrombin III. This study did not include a factor VIIa assay on the concentrates, since neither clotting assays nor a \(^3\mathbf{H}\)-factor-X activation assay in the presence of tissue factor was sufficiently specific for factor VIIa versus factor VII. Other investigators, using clotting and amidolytic assays, have recently reported that both activated and unactivated factor IX concentrates contain factor VIIa. Factor VIIa was measured in plasma after transfusion of concentrate and found to have a half-disappearance time of 2.5 hr. No accurate estimate of the in vivo survival of factor IXa is available, but the slow inhibition of factor IXa by antithrombin III makes it very possible that factor IXa has a measurable in vivo survival. Whether factor IXa, VIIa, or some other factors are responsible for the apparent therapeutic benefit of some factor IX concentrates in factor VIII inhibitor patients is unclear. This study, along with the new factor VIIa assay, demonstrates that specific assays are feasible for each of the activated vitamin-K-dependent factors in the concentrates. Therefore, future studies using these assays should provide quantitative data on the relationship of these activated factors, in particular factors IXa and VIIa, to the therapeutic and thrombotic effects of the concentrates.

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REFERENCES


Activated clotting factors in factor IX concentrates

MB Hultin