Detection of Factor X Activation in Humans

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A sensitive radioimmunoassay (RIA) for the fragment that is liberated from factor X when this zymogen is activated by factor VII/VIIa-tissue factor or factor IXa was developed. Antisera were raised in rabbits to a synthetic 15 amino acid peptide containing the COOH-terminal sequence of the activation fragment coupled to bovine serum albumin with glutaraldehyde. The reactivity of the antibody population obtained toward the factor X zymogen was negligible (less than 1/36,000) of that of the activation peptide on a molar basis. However, because other plasma constituents contributed to a nonspecific basal signal in the RIA, a procedure by which the peptide could be reproducibly extracted from plasma was developed. The mean level of this species in normal individuals younger than the age of 40 was 66.4 pmol/L, and elevations up to 550 pmol/L were observed in patients with evidence of disseminated intravascular coagulation. The validity of these measurements of factor X activation is supported by the fact that the RIA signal migrates on reverse-phase high pressure liquid chromatography in a manner identical to that of the native peptide and can be quantitatively recovered. The mean concentration of the activation fragment was markedly decreased to 25.7 pmol/L in patients with hereditary factor VII deficiency (P < .0001 vs normal controls), whereas the mean level in subjects with factor VIII deficiency was 61.1 pmol/L (P > .1 vs normal controls). These data indicate that the basal (ie, in the absence of thrombosis or provocative stimuli) levels of FXI under in vivo conditions result mainly from the activity of the extrinsic pathway.

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THE COAGULATION CASCADE proceeds in a series of linked proteolytic reactions. The enzymes generated by these processes are not directly available for quantitation because they are evanescent species that are rapidly neutralized by naturally occurring protease inhibitors. Faced with these obstacles, we have focused our attention on the development of a series of highly sensitive and specific radioimmunoassays (RIAs) for peptides that are liberated with the activation of hemostatic system zymogens in vivo. These by-products of zymogen transformation are stable entities with finite half-lives in the circulation. We have previously developed immunoassays for the Fx12 fragment and the protein C activation peptide, which measure the cleavage of prothrombin by factor Xa and the scission of protein C by the thrombin-thrombomodulin complex, respectively.

Human factor X is a vitamin K-dependent glycoprotein of molecular weight ~59,000 that circulates in plasma as an inactive zymogen at a concentration of ~200 nmol/L. This component consists of a heavy chain and a light chain, which are joined by a single disulfide bridge. The activation of factor X by the extrinsic (factor VII/VIIa-tissue factor) or the intrinsic (factor IXa-VIIIa complex) pathway plays a pivotal role in the hemostatic mechanism. During this reaction an Arg395-Ile396 bond is cleaved at the amino-terminal end of the heavy chain of the protein, which releases a highly glycosylated 52 amino acid activation peptide of molecular weight ~14,000.

In this communication, we describe the development of a RIA for the activation peptide, which is generated pari passu with activated factor X. This assay has been employed to quantify the extent of factor X activation in normal individuals and in patients with abnormalities of their coagulation mechanisms due to a variety of clinical disorders.

MATERIALS AND METHODS

Column chromatographic materials and reagents. Sephadex G-10, G-25, and G-100 were obtained from Pharmacia Fine Chemicals, Piscataway, NJ. Dry hydroxylapatite and Affi-Gel 15 were purchased from Bio-Rad Laboratories, Richmond, CA. Human factor X was bound to Affi-Gel 15 as described by the manufacturer and approximately 0.5 mg protein was coupled to 1 mL of "packed" gel. Disposable wide pore butylsilane (C18) extraction columns were bought from J.T. Baker Inc, Phillipsburg, NJ. Tert-butyloxy carbonyl (Boc) amino acids and Boc-Arg (Tosyl) on a Merrifield peptide resin were purchased from Vega Biochemicals, Tucson, AR and Peninsula Laboratories, Belmont, CA, respectively. Heparin was purchased from Elkins-Sinn, Inc, Cherry Hill, NJ.

Proteins. Bovine serum albumin (BSA) and ovalbumin were purchased from Sigma Chemical Co, St Louis, MO. Human factor X, human factor IX, bovine factor XIa, and the protease from Russell's viper venom that activates factor X (RVV-X) were obtained from Enzyme Research Laboratories, South Bend, IN. Recombinant human factor VIIa (specific activity 39,300 U/mg) was generously provided by Novo Alle, Bagsvaerd, Denmark. Human factor IXa was produced by activating human factor IX with bovine factor XIa (8% [wt/wt]) in the presence of 10 mmol/L calcium at 37°C for 1 hour. After the addition of EDTA and benzamidine to terminate the reaction, the mixture was dialyzed against 0.10 mol/L potassium phosphate, pH 7.0 containing 10 mmol/L benzamidine and filtered through a column of hydroxylapatite equilibrated with the same buffer. The factor IX activation peptide was present in the column effluent while the factor IXa was harvested with 0.5 mol/L potassium phosphate, pH 7.0. The various proteins were demonstrated to be homogeneous with respect to size by the sodium dodecyl sulfate (SDS) gel electrophoretic system of Laemmli.

Protein concentrations were determined from the absorbance
stance of protein solutions at 280 nm. The molar extinction coefficients for human factor Xa, human factor IXa, and RVV-X were assumed to be 11.6, 13.2, and 10.0, respectively.

**Purification of the factor X activation peptide (FXP).** Human factor X and RVV-X were incubated together for 2 hours at 37°C at final concentrations of 0.84 mg/mL and 16.7 μg/mL, respectively. The solvent conditions were 10 mmol/L CaCl₂ in 0.10 mol/L NaCl, 0.01 mol/L Tris-HCl, pH 7.5. The reaction was terminated by the addition of EDTA and benzamidine to final concentrations of 100 mmol/L and 10 mmol/L, respectively. After extensive dialysis against 0.10 mol/L potassium phosphate, pH 7.0 containing 10 mmol/L benzamidine, the reaction mixture containing 5.5 mg of protein in 7.5 mL was filtered through a column of hydroxypatite (0.8 x 12 cm) equilibrated with the same buffer. The chromatography was conducted at 16 mL/h at 4°C. The FXP was present in the column effluent while the remainder of the factor X molecule was adsorbed to the hydroxypatite matrix. Fractions containing significant absorbance were pooled and dialyzed overnight against 4 L of distilled water containing 10 mmol/L benzamidine. After washing the matrix with two column volumes of 0.1 mol/L potassium phosphate, pH 7.0, the factor Xa was harvested with 0.5 mol/L potassium phosphate, pH 7.0.

The dialyzed FXP preparation was then acidified to a final phosphoric acid concentration of 0.1% (vol/vol) and injected onto a Microsorb C₁₈ high pressure liquid chromatography (HPLC) column (Rainin Instrument Co, Woburn, MA) equilibrated with 0.1% (vol/vol) phosphoric acid. The column was 25 cm x 4.6 mm, and the flow rate was 1.2 mL/min. A linear gradient of acetonitrile in 0.1% (vol/vol) phosphoric acid was applied to the column and the peptide eluted at an acetonitrile concentration of ~35% (vol/vol). This material was neutralized with NaOH, evaporated to dryness in a Savant Speed Vac Concentrator (Savant Instruments Inc, Hicksville, NY), reconstituted with distilled water, and dialyzed overnight against distilled water. SDS gel electrophoresis was performed on 10% gels and demonstrated a single band with an apparent molecular weight of ~23,000. After determining the amount of FXP that had been purified, the yield of FXP was calculated to be >80% of the total amount of factor X that was activated.

A known volume of the purified FXP was hydrolyzed in 6N HCl in sealed tubes at 110°C. After removal of HCl, the amino acid content of the hydrolysate was determined utilizing a Beckman 6500 amino acid analyzer (Beckman Instruments Inc, Irvine, CA). The amount of peptide in the sample was obtained by dividing the amino acid derivatives were utilized. The peptide was synthesized sequentially from Boc-Arg (Tosyl) on a Merrifield resin with two column volumes of 0.10 mol/L potassium phosphate, pH 7.0, containing 1 mg/mL ovalbumin. The sensitivity of the assay was maximized by diluting the crude antisemur 500-fold. Under these conditions ~33% of the ¹²⁵I-labeled counts were immunoprecipitable. To act as a carrier in the second antibody separation, 4% (vol/vol) nonimmune rabbit serum was added to the solution containing the tracer. The tubes were then mixed and incubated at 4°C for 18 hours. Thereafter, radiolabeled antigen bound to the antibody was separated from free iodine by Sephadex G-25 gel filtration.

**Immunoassays.** The FXP RIA was performed using a double-antibody approach. The initial reaction mixtures were composed of 50 μL of radiolabeled tracer (~10,000 cpm), 500 μL of unlabeled FXP standards or unknown sample, as well as 100 μL of the specific antibody preparation. All of the reagents had been extensively diluted in Tris-buffered saline (0.05 mol/L Tris-HCl, 0.10 mol/L NaCl, 0.02% (wt/vol) sodium azide, pH 7.5, containing 1 mg/mL ovalbumin). The specificity of the assay was maximized by diluting the crude antisemur 500-fold. Under these conditions ~33% of the ¹²⁵I-labeled counts were immunoprecipitable. To act as a carrier in the second antibody separation, 4% (vol/vol) nonimmune rabbit serum was added to the solution containing the tracer. The tubes were then mixed and incubated at 4°C for 18 hours. Thereafter, radiolabeled antigen bound to the antibody was separated from free iodine by Sephadex G-25 gel filtration.

**Collection and processing of blood samples.** Venipunctures were performed in the morning of the day of surgery using a 19- or 21-gauge butterfly infusion sets using a two-syringe technique. Blood samples for the FXP and F₁⁺₇ RIA's were drawn into plastic syringes preloaded with the following anticoagulant: 38 mmol/L citric acid, 75 mmol/L sodium citrate, 136 mmol/L dextrose, 6 mmol/L EDTA, 6 mmol/L sodium citrate, 136 mmol/L dextrose, 6 mmol/L EDTA, 6 mmol/L sodium citrate.
adrenochrome, and 25 U/mL heparin. The ratio of anticoagulant to
blood employed was 0.2:1.0 (vol/vol). After collection of blood
samples, plasma fractions were obtained by centrifugation at 4°C for
15 minutes at 1,600 xg and stored at -80°C before use.

Normal ranges for FXP and Fx were obtained from data on
control subjects. This population consisted of healthy individuals
who gave a negative history for bleeding as well as thrombosis and
were not taking any medications at the time of sample collection.

Processing of plasma for FXP RIA. To extract the FXP from
plasma, the larger proteins were precipitated by adding 0.1 vol of 7
mole/L perchloric acid to 0.9 vol of plasma, and were then removed
by centrifugation at 48,000 xg for 20 minutes at room temperature.
The supernatant fluid was decanted, 5N NaOH was added to raise
the pH to greater than 7, and 20% (vol/vol) trifluoroacetic acid
(TFA) was added to lower the pH to less than 3. A butylsilane C4
(6 mL) extraction column was prepared with 6 mL of absolute
methanol containing 0.5% (vol/vol) TFA followed by 6 mL of 0.5%
TFA. The sample was applied to this column, which was then
washed with 12 mL of 0.5% TFA. The peptide was subsequently
eluted with 4 mL of 75% methanol containing 0.5% TFA into 12 x
75 mm test tubes. The contents of the tubes were evaporated to
dryness overnight in a Savant Speed Vac Concentrator. Samples
were individually reconstituted with 1.5 mL of 0.10 mol/L NaCl in
0.05 mol/L Tris-HCI, pH 7.5, containing 0.02% (wt/vol) sodium
azide and 1 mg/mL ovalbumin. The specimens were then assayed by
RIA for FXP immunoreactivity.

Coagulation studies. Routine coagulation studies including pro-
thrombin time, activated partial thromboplastin time, thrombin
time, and fibrinogen were performed by standard laboratory meth-
ods. FDPs were determined using the Thrombo-WetcoTest (Well-
come Reagents, Research Triangle Park, NC). An amidolytic assay
was utilized for the measurement of plasma antithrombin-heparin
cofactor activity.

Antithrombin concentrates. Antithrombin purified from pooled
human plasma of normal donors (heat-treated) was supplied by
Cutter Biological (Berkeley, CA). One unit is defined as the amount
of antithrombin in 1 mL of pooled normal human plasma.

Informed consent. Approval was obtained from the Institutional
Review Board of the Beth Israel Hospital for these studies. Patients
were informed that blood samples were obtained for research
purposes, and that their privacy would be protected.

Analysis of data. Estimation of relative immunoreactivity, com-
putation of the slopes of the dose-response curves, and determina-
tions of the various associated indices were obtained by a least-
squares fit of the RIA results to a “four parameter” model as
described by Rodbard. Statistical analyses of data were con-
ducted by standard techniques. In most instances the means are
provided with associated standard deviations.

RESULTS

Human factor X was activated by RVV-X at an enzyme-
to-substrate ratio of 1:50 (wt/wt) at 37°C in the presence 10
mmol/L CaCl2. The reaction was terminated at 2 hours by the
addition of EDTA and SDS gel electrophoresis documenting the
complete conversion of factor X to factor Xa. The activation peptide
was purified from this mixture by hydroxylapitate chromatography and reverse-phase HPLC.

Amino acid analysis demonstrated the same relative propor-
tions of the relevant amino acids as that calculated from the
known amino acid composition of FXP. We initially immu-
nized rabbits with the native activation peptide coupled to
BSA with glutaraldehyde. The antibody populations
obtained from these animals demonstrated high avidity for
the FXP but were not significantly more specific for the
activation peptide than the parent zymogen, and filtration
of the various IgG fractions through factor X-Affi-Gel 15 did not
substantially increase their specificity.

We therefore decided to raise antibodies to a synthetic
peptide containing a portion of the sequence of the native
activation fragment. Based upon an analysis of hydrophobicity of the amino acids coding for FXP, a relatively hydrophilic
region was identified at the COOH-terminal end of the
molecule. A peptide containing the COOH-terminal 15 residues of the FXP, NH2-Phe-Asp-Gln-Thr-Gln-Pro-Glu-
Arg-Gly-Asp-Asn-Asp-Leu-Thr-Arg-COOH, was then syn-
thetized using the solid-phase method of Merrifield. After
isolation by gel filtration and reverse-phase HPLC, the
composition of the peptide was determined by amino acid
analysis. A single mole of peptide was found to contain 4.00,
1.74, 3.29, 1.03, 0.96, 0.90, 0.97, and 2.02 mol of aspartic
acid, threonine, glutamic acid, proline, glycine, leucine,
phenylalanine, and arginine, respectively. The synthetic
peptide was also analyzed by mass spectrometry, which demon-
strated an intense protonated ion peak at a molecular weight
of 1,790 (data not shown) identical to that calculated from
the amino acid composition. Furthermore, a series of spectra

![Graph](https://www.bloodjournal.org)
exhibited fragment ions, which confirmed the primary sequence of the polypeptide for the first nine NH₂-terminal residues.

The synthetic peptide was covalently linked to BSA with glutaraldehyde, and the conjugate was employed to raise antisera in rabbits. The immunized animals produced high titer antibody populations as judged by binding assays utilizing ¹²⁵I-FXP. However, a single rabbit provided an antibody population with optimal sensitivity and negligible reactivity toward human factor X. The FXP RIA was constructed utilizing antisera from this animal, which had been immunized for 5 months. Figure 1 shows a typical FXP titration curve conducted with this antibody population. The ¹²⁵I-labeled ligand bound to the specific antibody in the presence of a given amount of competing antigen divided by the ¹²⁵I-labeled ligand bound to the specific antibody in the absence of competing antigen (B/B₀) is plotted against the log B₀ of the molar amount of competing antigen. The solid lines represent a computer-generated fit of the data to the four-parameter model of Rodbard et al.¹⁷⁻¹⁸ The mean slope and midpoint of the log-log dose-response curves for 37 individual FXP assays were 1.02 ± 0.07 and 0.268 ± 0.03, respectively. Results obtained with duplicate assays at varying concentrations of FXP demonstrate that the intraassay coefficient of variation of this method within the linear portion of the titration curve is less than 4%. The detection limit of the technique is 0.02 nmoL/L of peptide.

The RIA described above was used to study the reactivity of purified human factor X, which contains the FXP amino acid sequence at the NH₂-terminus of its heavy chain. As shown in Fig 1, this species reacts minimally with our specific antibody population, and we estimate that the immunoreactivity of the factor X zymogen is less than 1/36,000 that of the FXP on a molar basis. Similar data were obtained with three other preparations of human factor X.

To demonstrate that our RIA procedure is capable of detecting FXP antigenic regions hidden within the factor X molecule, we completely converted a known amount of the zymogen to factor Xa and the activation fragment with RVV-X. The environmental conditions were identical to those used to purify the FXP and the reaction was terminated by the addition of EDTA. Examination of this activation mixture with our RIA procedure confirmed that the theoretical amount of FXP had been released (Fig 1).

After activating factor X with RVV-X, several investigators have observed that the separation of factor Xa from the FXP by gel filtration requires the use of denaturing buffers such as 10% formic acid or 8 mol/L urea-1% SDS. These observations have led to the conclusion that there exists a noncovalent association between the enzyme and the activation peptide. Although our ability to separate factor Xa from the FXP by hydroxylapatite chromatography suggests that such an interaction does not occur, we have analyzed the behavior of these components on Sephadex G-100 (200 cm x 0.6 cm) in a solvent system containing 0.05 mol/L Tris-HCl, 0.10 mol/L NaCl, pH 7.5, and 0.1 mg/mL ovalbumin. To this end, we individually radiolabeled factor Xa harvested from the hydroxylapatite columns used in the purification of the activation peptide as well as the FXP. These species were then subjected to gel filtration at a flow rate of 3.6 mL/h, and the peak fraction of ¹²⁵I-factor Xa eluted within 1 mL of the ¹²⁵I-FXP peak, which had an elution volume similar to that of ovalbumin (molecular weight ~43,000). It should be noted that factor Xa and the FXP have molecular weights of ~46,000 and ~14,000, respectively. Based upon the above data, we conclude that factor Xa and the FXP appear to coelute in gel filtration systems under non-denaturing conditions due to the anomalous migration of the highly glycosylated FXP.

Despite the specificity of our RIA, several independent lines of evidence strongly suggested that plasma constituents other than the FXP contributed to a nonspecific basal signal in the assay, and we concluded that it was impossible to accurately quantitate FXP levels in human plasma directly. We therefore developed a routine method by which the peptide could be extracted from 6 mL of plasma from individual subjects. In the final step of this procedure, the specimens are reconstituted with Tris-buffered saline to a final volume of 1.5 mL before RIA. Thus the extraction process enables us to concentrate the FXP signal fourfold and lowers the detection limit of the assay technique to 5 pmol/L. Along with each set of samples to be extracted, we have also employed a normal plasma control that had been “spiked” with 0.5 pmol of native peptide. The results obtained on 10 occasions with a plasma pool obtained from normal donors utilizing an appropriate anticoagulant for blood collection indicate that the uncorrected basal level of FXP is 72.5 ± 8.6 pmol/L. Furthermore, we were able to recover 85.9% ± 8.5% of the added peptide. The extent of recovery is independent of the amount of FXP added to the plasma, or the volume of plasma utilized in the extraction process (data not shown). Because we have observed that the average recovery of added FXP in the extraction procedure is extremely constant, we have elected to not divide the values obtained by the fractional recovery. Whereas the intraassay coefficient of variation for the FXP RIA was less than 4%, the within-day and between-day coefficients of variations for a plasma sample subjected to our extraction procedure were 8% and 12%, respectively.

To demonstrate that the RIA signal obtained after the extraction and concentration procedure represents FXP, 6 mL of plasma from a normal individual as well as a smaller volume of the same plasma “spiked” with native peptide were processed in the customary fashion. The samples were then reconstituted in 1 mL of 0.5% (vol/vol) TFA and injected onto an Alltech C₈ HPLC column. The flow rate was 1.2 mL/min. The mobile phase consisted of 0.5% (vol/vol) TFA and a linear gradient of acetonitrile in 0.5% (vol/vol) TFA was applied to the column. The column effluents were monitored with the FXP RIA, which revealed that the peptide in both samples eluted at an acetonitrile concentration of approximately 60% (vol/vol) (Fig 2). Greater than 80% of the applied immunoreactivity could be recovered in the peak fractions. These results indicate that the FXP RIA, used in conjunction with appropriate sample processing techniques, is able to accurately measure plasma concentrations of the activation fragment.

During the quantitation of plasma FXP levels, we have insured that in vitro factor X activation is completely suppressed after venipuncture. The solution utilized for this
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The elution from a C4 HPLC column of FXP immunoreactivity from normal plasma (●), plasma of a patient with DIC associated with metastatic prostatic carcinoma (□), and native FXP (□). The column was 25 cm × 4.6 mm and was equilibrated with 0.5% (vol/vol) TFA. Peptide immunoreactivity was eluted with an acetonitrile gradient in 0.5% (vol/vol) TFA and fractions of 1.2 ml were collected. The contents of the tubes were evaporated to dryness, redissolved in 1.2 ml of Tris-buffered saline with 1 mg/ml ovalbumin to give a final pH of 7.5, and assayed for FXP content.

purification contains ACD (citric acid, sodium citrate, dextrose), EDTA, adenosine (platelet inhibitor), and heparin. The efficacy of this anticoagulant was demonstrated by immediately mixing blood samples from three normal donors with the above anticoagulant mixture. Purified human factor VIIa (final concentration, 0.4 μg/mL) or buffer was added to aliquots of each sample, and FXP immunoreactivity was quantitated. The levels of this component in aliquots to which enzyme had been added were not significantly different from those in which buffer had been admixed (55.8 ± 17 pmol/L vs 56.8 ± 14 pmol/L). Experiments in which human factor IXa (final concentration, 0.5 μg/mL) was the added enzyme gave similar results (49.9 ± 9.7 pmol/L vs 54.3 ± 16 pmol/L).

In two other series of experiments, we have established that the FXP immunoreactivity measured by our antibody population is stable for several hours within the plasma environment and that the peptide does not distribute preferentially into the cellular element fraction of blood (data not shown).

To initiate an assessment of this assay in various clinical conditions, studies have been conducted in which the extent of factor X activation has been analyzed in normal individuals as well as in patients with alterations in hemostatic system activity. FXP levels in 23 normals below the age of 40 ranged between 39.7 and 112 pmol/L with a mean of 66.4 ± 20 pmol/L. We have evaluated seven individuals with disseminated intravascular coagulation (DIC) in association with either metastatic carcinoma, acute promyelocytic leukemia, or pregnancy following a cesarian section (Table 1). The DIC syndrome was identified by characteristic abnormalities of routine clinical tests, such as prolonged thrombin time, diminished fibrinogen concentration, elevated fibrinogen (fibrin) split products, and reduced platelet count.22 None of the subjects had received heparin prior to blood collection, nor did any exhibit clinical or laboratory evidence of renal or hepatic dysfunction at the time of sampling. The levels of F1.2, an index of the in vivo enzymatic activity of factor Xa on prothrombin, were also measured by RIA12 and found to be markedly elevated. These patients exhibited substantial elevations in FXP levels compared with normal individuals with a mean concentration of 343 ± 84 pmol/L.

Figure 2 depicts the HPLC elution profile of peptide extracted from 3 mL of plasma obtained from patient 1 with DIC in association with prostatic carcinoma. The FXP immunoreactivity eluted at the same acetonitrile concentration as the native peptide and 86% of the applied signal was recovered in the peak fractions of the gradient profile. These results confirm our supposition that the RIA signal accurately reflects the levels of the activation fragment. The data also suggest that other NH2-terminal fragments of the heavy chain of factor X, which could be produced during DIC by the action of proteases other than factor VIIa or factor IXa, do not contribute to the signal measured with our assay.

Plasma FXP levels have also been determined in nine stably anticoagulated patients receiving sodium warfarin with prothrombin times greater than 1.4 times control (Table

Table 1. Levels of FXP in Patients With Disseminated Intravascular Coagulation

<table>
<thead>
<tr>
<th>Patient No./Age (yr)</th>
<th>Disorder</th>
<th>Fibrinogen (mg/dL)</th>
<th>FDPs (μg/mL)</th>
<th>Platelets (x 10^5/μL)</th>
<th>F1.2 (nmol/L)</th>
<th>FXP (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/69</td>
<td>Prostatic carcinoma</td>
<td>50</td>
<td>640-1,280</td>
<td>62</td>
<td>73.8</td>
<td>550</td>
</tr>
<tr>
<td>2/61</td>
<td>Prostatic carcinoma</td>
<td>50</td>
<td>80-160</td>
<td>79</td>
<td>11.0</td>
<td>348</td>
</tr>
<tr>
<td>3/56</td>
<td>Pancreatic carcinoma</td>
<td>115</td>
<td>40-80</td>
<td>70</td>
<td>6.92</td>
<td>233</td>
</tr>
<tr>
<td>4/39</td>
<td>Esophageal carcinoma</td>
<td>52</td>
<td>160-320</td>
<td>52</td>
<td>50.2</td>
<td>311</td>
</tr>
<tr>
<td>5/59</td>
<td>Promyelocytic leukemia</td>
<td>82</td>
<td>80-160</td>
<td>20</td>
<td>32.0</td>
<td>375</td>
</tr>
<tr>
<td>6/37</td>
<td>Promyelocytic leukemia</td>
<td>126</td>
<td>80-160</td>
<td>56</td>
<td>24.0</td>
<td>443</td>
</tr>
<tr>
<td>7/34</td>
<td>Pregnancy, post C-section</td>
<td>82</td>
<td>160-320</td>
<td>63</td>
<td>37.2</td>
<td>250</td>
</tr>
<tr>
<td>Normal range</td>
<td>150-325</td>
<td>&lt;10</td>
<td>150-440</td>
<td></td>
<td>1.51 ± 0.66</td>
<td>66.4 ± 20</td>
</tr>
<tr>
<td>Normal mean ± SD</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td></td>
<td>—</td>
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</tr>
</tbody>
</table>
and had a mean \( F_{1+2} \) concentration that was significantly reduced compared with normal controls (\( P < .0001 \)).

We have measured the levels of FXP in several patient populations with inherited deficiencies of factor VII, factor VIII, or factor IX (Table 3). None of the subjects had received clotting factor concentrates or plasma infusions within the previous 7 days. The mean plasma concentration of this species was reduced to 25.7 ± 11 pmol/L in five individuals with plasma factor VII activity levels of ≤7% of normal (\( P = .0001 \) vs normal controls), and the mean FXP level in 10 subjects with factor VIII levels of less than 1% was 61.1 ± 19 pmol/L (\( P > .1 \)). The mean \( F_{1+2} \) values were similarly noted to be significantly reduced in the former population and normal in the latter group, 0.31 ± 0.17 nmol/L (\( P < .0001 \)) and 1.30 ± 0.51 nmol/L (\( P > .1 \)), respectively (Table 3). Four patients with severe factor IX deficiency (factor IX levels of less than 1%) have also been examined, and the mean levels of FXP and \( F_{1+2} \) were not substantially different from those of normal controls or patients with factor VIII deficiency, 69.5 ± 31 pmol/L and 1.26 ± 0.45 nmol/L, respectively (Table 3).

The levels of FXP have also been examined in the blood of nonanticoagulated individuals with hereditary antithrombin deficiency (Table 4), a disorder known to be correlated with the subsequent development of thrombosis. We have previously shown that most of these subjects have significant increments in factor Xa activity (ie, elevated \( F_{1+2} \) levels) but not thrombin activity (ie, normal FPA levels) in their blood.23 Our studies indicate that the mean concentration of FXP was 78.5 ± 22 pmol/L in eight patients with this disorder, which was not significantly different from that in normal controls of similar age (\( P > .1 \)). We also examined the effects of antithrombin concentrate infusions in six of the above patients with a deficiency of the protease inhibitor. After obtaining baseline blood studies, each subject received an infusion of the purified protein via a peripheral vein at a dosage of 75 U/kg over ~20 minutes. Blood samples were obtained by separate venipuncture prior to and 24 hours after the administration of the medication. Our results indicate that the plasma antithrombin concentration increased from a mean baseline value of 43.3% ± 10.4% in patients with this disorder, which was not significantly different from that in normal controls of similar age (\( P > .1 \)). We also examined the effects of antithrombin concentrate infusions in six of the above patients with a deficiency of the protease inhibitor. After obtaining baseline blood studies, each subject received an infusion of the purified protein via a peripheral vein at a dosage of 75 U/kg over ~20 minutes. Blood samples were obtained by separate venipuncture prior to and 24 hours after the administration of the medication. Our results indicate that the plasma antithrombin concentration increased from a mean baseline value of 43.3% ± 10.4% in patients with this disorder, which was not significantly different from that in normal controls of similar age (\( P > .1 \)). We also examined the effects of antithrombin concentrate infusions in six of the above patients with a deficiency of the protease inhibitor. After obtaining baseline blood studies, each subject received an infusion of the purified protein via a peripheral vein at a dosage of 75 U/kg over ~20 minutes. Blood samples were obtained by separate venipuncture prior to and 24 hours after the administration of the medication. Our results indicate that the plasma antithrombin concentration increased from a mean baseline value of 43.3% ± 10.4% in patients with this disorder, which was not significantly different from that in normal controls of similar age (\( P > .1 \)).

### Table 2. Levels of FXP in Patients on Oral Anticoagulant Therapy

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Prothrombin Time (sec)</th>
<th>FXP (nmol/L)</th>
<th>F1+2 (pmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.0/10.7</td>
<td>19.6</td>
<td>27.5</td>
</tr>
<tr>
<td>2</td>
<td>28.3/10.7</td>
<td>0.07</td>
<td>17.5</td>
</tr>
<tr>
<td>3</td>
<td>19.2/10.7</td>
<td>0.11</td>
<td>37.5</td>
</tr>
<tr>
<td>4</td>
<td>26.8/10.7</td>
<td>0.11</td>
<td>21.6</td>
</tr>
<tr>
<td>5</td>
<td>16.7/10.7</td>
<td>0.07</td>
<td>14.9</td>
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<tr>
<td>6</td>
<td>23.0/11.1</td>
<td>0.07</td>
<td>20.9</td>
</tr>
<tr>
<td>7</td>
<td>20.8/10.5</td>
<td>0.03</td>
<td>3.6</td>
</tr>
<tr>
<td>8</td>
<td>26.6/10.5</td>
<td>0.04</td>
<td>25.4</td>
</tr>
<tr>
<td>9</td>
<td>15.0/10.7</td>
<td>0.37</td>
<td>27.2</td>
</tr>
</tbody>
</table>

Results for the prothrombin time represent the value for the individual patient plasma over that for the control plasma in seconds.

### Table 3. Levels of FXP and \( F_{1+2} \) in Patients With Hereditary Deficiencies of Factor VII, Factor VIII, or Factor IX

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Factor Deficiency</th>
<th>FXP (pmol/L)</th>
<th>( F_{1+2} ) (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>14</td>
<td>VII</td>
<td>16.4</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>22</td>
<td>VII</td>
<td>28.1</td>
<td>0.29</td>
</tr>
<tr>
<td>3</td>
<td>71</td>
<td>VII</td>
<td>17.5</td>
<td>0.47</td>
</tr>
<tr>
<td>4</td>
<td>48</td>
<td>VII</td>
<td>22.5</td>
<td>0.19</td>
</tr>
<tr>
<td>5</td>
<td>27</td>
<td>VII</td>
<td>43.8</td>
<td>0.50</td>
</tr>
<tr>
<td>6</td>
<td>26</td>
<td>VIII</td>
<td>39.6</td>
<td>0.69</td>
</tr>
<tr>
<td>7</td>
<td>28</td>
<td>VIII</td>
<td>73.8</td>
<td>1.15</td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>VIII</td>
<td>69.2</td>
<td>1.87</td>
</tr>
<tr>
<td>9</td>
<td>49</td>
<td>VIII</td>
<td>37.5</td>
<td>1.58</td>
</tr>
<tr>
<td>10</td>
<td>19</td>
<td>VIII</td>
<td>71.1</td>
<td>1.24</td>
</tr>
<tr>
<td>11</td>
<td>10</td>
<td>VIII</td>
<td>47.1</td>
<td>1.05</td>
</tr>
<tr>
<td>12</td>
<td>17</td>
<td>VIII</td>
<td>36.4</td>
<td>0.39</td>
</tr>
<tr>
<td>13</td>
<td>9</td>
<td>VIII</td>
<td>64.6</td>
<td>1.96</td>
</tr>
<tr>
<td>14</td>
<td>36</td>
<td>VIII</td>
<td>90.0</td>
<td>1.30</td>
</tr>
<tr>
<td>15</td>
<td>47</td>
<td>VIII</td>
<td>81.6</td>
<td>1.76</td>
</tr>
<tr>
<td>16</td>
<td>18</td>
<td>IX</td>
<td>60.8</td>
<td>1.91</td>
</tr>
<tr>
<td>17</td>
<td>63</td>
<td>IX</td>
<td>114</td>
<td>1.14</td>
</tr>
<tr>
<td>18</td>
<td>13</td>
<td>IX</td>
<td>58.4</td>
<td>0.92</td>
</tr>
<tr>
<td>19</td>
<td>35</td>
<td>IX</td>
<td>44.7</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Normal range = 39.7-112 pmol/L, 0.35-3.51

2). These individuals were receiving this medication for the prophylaxis of venous thrombosis or intracardiac thrombosis and had a mean \( F_{1+2} \) concentration that was significantly reduced compared with normal individuals (0.113 ± 0.11 pmol/L vs 1.51 ± 0.68 pmol/L, \( P < .0001 \)). The mean FXP level in these patients was 21.8 ± 9.5 pmol/L, which was significantly decreased compared with the control group of healthy individuals (\( P < .0001 \)).

### Table 4. Response of FXP and \( F_{1+2} \) Levels in Antithrombin-Deficient Patients to Antithrombin Concentrate Infusions

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>Age (yr)</th>
<th>Antithrombin Activity (% of normal)</th>
<th>FXP (pmol/L)</th>
<th>( F_{1+2} ) (nmol/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preinfusion</td>
<td>Postinfusion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>-------------</td>
<td>------------</td>
<td>------------------------------------</td>
<td>--------------</td>
<td>---------------------</td>
</tr>
<tr>
<td>1</td>
<td>46</td>
<td>43</td>
<td>105</td>
<td>5.09</td>
</tr>
<tr>
<td>2</td>
<td>31</td>
<td>46</td>
<td>84.9</td>
<td>4.84</td>
</tr>
<tr>
<td>3</td>
<td>21</td>
<td>37</td>
<td>110</td>
<td>3.45</td>
</tr>
<tr>
<td>4</td>
<td>33</td>
<td>43</td>
<td>82.6</td>
<td>1.41</td>
</tr>
<tr>
<td>5</td>
<td>41</td>
<td>50</td>
<td>82.7</td>
<td>2.66</td>
</tr>
<tr>
<td>6</td>
<td>19</td>
<td>41</td>
<td>63.4</td>
<td>2.45</td>
</tr>
<tr>
<td>7</td>
<td>17</td>
<td>47</td>
<td>42.9</td>
<td>3.09</td>
</tr>
<tr>
<td>8</td>
<td>31</td>
<td>31</td>
<td>77.3</td>
<td>3.60</td>
</tr>
</tbody>
</table>

Our studies indicate that the mean concentration of FXP was 47.8 ± 22 pmol/L in eight patients with this disorder, which was not significantly different from that in normal controls of similar age (\( P > .1 \)). We also examined the effects of antithrombin concentrate infusions in six of the above patients with a deficiency of the protease inhibitor. After obtaining baseline blood studies, each subject received an infusion of the purified protein via a peripheral vein at a dosage of 75 U/kg over ~20 minutes. Blood samples were obtained by separate venipuncture prior to and 24 hours after the administration of the medication. Our results indicate that the plasma antithrombin concentration increased from a mean baseline value of 43.3% ± 4.4% of normal to a mean level of 106% ± 12% at the 24-hour time point. The mean plasma \( F_{1+2} \) measurement decreased from 3.32 ± 1.4 nmol/L to 1.52 ± 0.52 nmol/L (\( P = .006 \)), whereas the mean level of FXP went from 84.7 ± 20 pmol/L to 75.6 ± 22 pmol/L in the six patients (\( P < .02 \)).
To use the FXP RIA to estimate the amount of factor X that is activated in the circulation, it is necessary to know the rate of clearance of the peptide. We have therefore undertaken studies of the metabolic behavior of FXP in an animal model. The dog was chosen for these experiments as we have previously obtained clearance rates for activation peptides, such as human F1,2 in dogs that are similar to those in humans.23 To this end, purified FXP was radiolabeled with $^{125}$I. The radiolabeled FXP was infused as a bolus into a peripheral leg vein of the dog, and blood samples were drawn through a catheter placed in the jugular vein into preloaded syringes containing the "FXP anticoagulant." Within 5 days of this initial experiment, a second study was carried out employing 1.87 nmol of unlabeled protein to raise the plasma level to ~1,000 pmol/L. Samples were obtained at various time points and assayed for FXP immunoreactivity. The plasma radioactivity and immunoreactivity data were plotted against time (Fig 3). $^{125}$I-FXP plasma radioactivity and FXP plasma immunoreactivity can each be described by a two-exponential curve, $C_1e^{-r_1t} + C_2e^{-r_2t}$. The fractional breakdown rate, $k_B$ (h$^{-1}$), was calculated from $[C_1/r_1 + C_2/r_2]^{-1}$. The values for $C_1$, $r_1$, $C_2$, $r_2$, and $k_B$ in the radioactive and immunologic studies were 0.432, 0.689, 0.820, 4.31, 1.22, and 0.145, 0.327, 0.867, 2.50, 1.26, respectively. Similar results were obtained in a second animal.

**DISCUSSION**

We have developed an RIA for the measurement of the peptide, that is liberated from factor X when this zymogen is activated by factor VII/VIIa-tissue factor or factor IXa. This was accomplished by immunizing rabbits with a 15 amino acid peptide containing the COOH-terminal sequence of the FXP coupled to BSA with glutaraldehyde. The antibody populations obtained were used together with $^{125}$I-FXP and various concentrations of unlabeled FXP to construct a sensitive double antibody RIA. The reactivity of the factor X zymogen within our assay is less than 1/36,000 that of the activation peptide on a molar basis.

Despite the specificity of our antiserum for the FXP, we observed that other plasma components were able to interfere in a nonspecific fashion with the reliable determination of the concentration of the peptide. Thus, it was necessary to devise a procedure by which the FXP could be extracted from plasma prior to measurement of the levels of the peptide. This was accomplished by precipitating plasma proteins with perchloric acid and concentrating the activation fragment within the supernatant fraction with butylsiline ($C_4$) extraction columns. This methodology allows us to reproducibly recover more than 80% of the FXP signal from plasma and to demonstrate that normal values of this peptide average ~70 pmol/L. The levels of FXP were found to be significantly elevated in patients with DIC ranging from 3.5- to 8.3-fold above the mean normal concentration. The validity of these measurements of factor X activation in normal individuals as well as those with excessive hemostatic system activity is supported by our demonstration that in both of these situations nearly 80% of the plasma RIA signal migrates on reverse-phase HPLC in a manner similar to that of the native peptide. We also found that stably anticoagu-
intrinsic cascades in generating procoagulant activity within the vasculature, we have determined the plasma concentrations of FXP and F₁₋₂ in patients with isolated clotting factor deficiencies. Individuals with a severe hereditary deficiency of factor VII exhibited substantial decrements in the measurements of these two activation fragments. These observations are in marked contrast to a group of hemophilia A patients, a severe bleeding disorder characterized by marked reductions in factor VIII activity, who had a mean level of FXP and F₁₋₂ that were not significantly different from those of normal subjects. The mean concentrations of these two peptides in a smaller number of individuals with factor IX deficiency were similar to those in patients with factor VIII deficiency. These data indicate that the basal (ie, in the absence of thrombosis or provocative stimuli) levels of FXP result mainly from the activity of the extrinsic pathway under in vivo conditions. It also suggests that the intrinsic cascade makes little if any contribution to factor X activation and prothrombin conversion in this situation.

However, it is likely that the hemostatic mechanism will respond in a different manner to thrombotic stimuli. In patients with DIC or acute thrombosis, we speculate that the intrinsic cascade is mobilized to amplify the generation of factor Xa and thrombin with the subsequent development of a fibrin-platelet meshwork. This could result from an increase in the rate of production of factor IXa and/or factor VIIIa and/or the provision of a critical surface for assembly of the factor IXa-factor VIIIa-factor X complex. To examine a disease model in which the intrinsic cascade might be activated to a less extreme degree than in DIC, we have studied a cohort of asymptomatic individuals with hereditary antithrombin deficiency. A previous report from our laboratory has demonstrated that many of these subjects exhibit increased prothrombin to thrombin conversion in their blood as measured by the F₁₋₂ assay due to a severe defect in the capacity of the endogenous heparan sulfate-antithrombin mechanism to inhibit the enzymatic activity of factor Xa.¹² To determine whether factor Xa generation is also increased in such individuals, the levels of FXP were determined in eight antithrombin-deficient patients. We observed that the mean concentration of this activation peptide was not significantly elevated in this group compared with normal controls, but did decrease slightly upon infusion of purified antithrombin concentrates into six patients. However, this decline was a consistent finding and suggests that the intrinsic cascade may make a small contribution to factor X activation in this group of patients. These findings may be explained by a slight augmentation in intrinsic cascade function mediated by the actions of factor Xa and thrombin, or by a reduced ability of the anticoagulantly active heparan sulfate-antithrombin system to inhibit the enzymatic activity of factor IXa.

In future studies it will be important to develop tools that are able to monitor the activity of the intrinsic pathway in thrombotic disorders. These include assays for the peptide liberated from factor IX by factor VIIa-tissue factor or factor Xla, as well as techniques for measuring the thrombin-dependent activation of factor VIII and the activated protein C-dependent destruction of factor VIIa. These indices in conjunction with the FXP assay could provide us with new approaches for elucidating the roles of the extrinsic and intrinsic pathways and assessing the ability of the intrinsic cascade to be activated under in vivo conditions.

ACKNOWLEDGMENT

The authors gratefully acknowledge the assistance of Dr Marek Kloczewiak who prepared the hydrophapy and antigenicity plots of the amino acid sequence encompassing the FXP. We also thank Drs Bruce Ewenstein (Boston, MA), Leo R. Zacharski (White River Junction, VT), and Harvey Weiss (New York, NY) for their help in the study of patients with coagulation factor deficiencies.

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DETECTION OF FACTOR X ACTIVATION IN HUMANS


Detection of factor X activation in humans

KA Bauer, BL Kass, H ten Cate, MA Bednarek, JJ Hawiger and RD Rosenberg