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Activation of factor VII bound to tissue factor: A key early step in the tissue factor pathway of blood coagulation

(hemostasis/factor X/factor IX)

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ABSTRACT **Whether the factor VII/tissue factor complex that forms in tissue factor-dependent blood coagulation must be activated to factor VIIa/tissue factor before it can activate its substrates, factor X and factor IX, has been a difficult question to answer because the substrates, once activated, back-activate factor VII. Our earlier studies suggested that human factor VII/tissue factor cannot activate factor IX. Studies have now been extended to the activation of factor X. Reaction mixtures were made with purified factor VII, X, and tissue factor; in some experiments antithrombin III and heparin were added to prevent back-activation of factor VII. Factor X was activated at similar rates in reaction mixtures containing either factor VII or factor VIIa after an initial 30-sec lag with factor VII. In reaction mixtures with factor VII a linear activation of factor X was established several minutes before cleavage of ^{125}I-labeled factor VII to the two-chain activated molecule was demonstrable on gel profiles. Adding antithrombin III and heparin blocked activation of factor X by factor VII/tissue factor but not by factor VIIa/tissue factor. When the antithrombin III and heparin were added 1 min after the other reagents, factor VII/tissue factor activation of factor X was not blocked. These data suggest that factor VII/tissue factor cannot activate measurable amounts of factor X over several minutes. Overall, our results support the hypothesis that a rapid preferential activation of factor VII bound to tissue factor by trace amounts of factor Xa is a key early step in tissue factor-dependent blood coagulation.**

A native factor VII/tissue factor (TF) complex is formed as the first step of TF-dependent blood coagulation. Since single-chain native factor VII is a zymogen, it has not been clear whether formation of the complex suffices to trigger the coagulation process. The question has been hard to study because the physiologic substrates of factor VII(a)/TF, factor X and factor IX, back-activate factor VII once they are activated.

Zur *et al.* (1) have provided evidence that native bovine factor VII possesses minimal enzymatic activity. They concluded that the intrinsic coagulant activity of the native molecule is adequate to initiate TF-dependent coagulation. We recently reported that native human factor VII does not possess physiologically significant enzymatic activity for a variant factor IX molecule that is unable after activation to back-activate factor VII (2). Lacking a similar variant for factor X and having at the time only a crude TF reagent, we were unable to evaluate critically the ability of native factor VII/TF to activate factor X.

We have now carried out experiments with normal factor X and purified human brain TF in which antithrombin III and heparin were used to block back-activation of factor VII by factor Xa. The data, reported herein, provide evidence that native human factor VII/TF cannot activate measurable amounts of factor X. More importantly, the data provide strong indirect evidence for the hypothesis that preferential, rapid activation of factor VII bound to TF is a key early step in TF-dependent coagulation.

MATERIALS AND METHODS

Reagents. Sodium [^{125}I]iodide and sodium boro[^{3}H]hydride were purchased from Amersham. Rabbit brain cephalin, purified bovine brain phosphatidylcholine and phosphatidylserine, and *n*-octyl β-D-glucopyranoside were from Sigma. Heparin was from Organon. Chromogenic substrate S-2222 was from Helena Laboratories (Beaumont, TX). All chemicals for analytical sodium dodecyl sulfate/polyacrylamide gel electrophoresis ($NaDodSO_4$/PAGE) were obtained from Bio-Rad. Other chemicals, reagent grade or better, were from standard sources. The buffer used to dilute reagents contained 50 mM Tris·HCl at pH 7.5, 0.15 M NaCl, and bovine serum albumin at 1 mg/ml (TBS/BSA).

Purified Human Clotting Factors. Human factors VII (3) and X (4) were purified to homogeneity by methods described earlier. Antithrombin III was purchased from Kabi Diagnostica (Stockholm). Purified factor X was activated with insolubilized Russell's viper venom and was stored and diluted for use as previously described (4).

Factor VIIa was prepared by incubating factor VII, final concentration 100 μg/ml, with factor Xa, final concentration 2 μg/ml, and cephalin, final concentration 1.2 mg/ml, in the presence of 5 mM $CaCl_2$ for 20 min at 37°C. The reaction was terminated by adding EDTA to give a final concentration of 10 mM. Factor VIIa was stored at −80°C and used within 2 months.

Purified Human TF. Human brain TF apoprotein was purified to homogeneity as described earlier (5). The apoprotein was incorporated into phospholipid vesicles by using octyl glucoside (6) and a molar ratio of protein to phospholipid of 1:100,000. When the apoprotein was incorporated into 100% phosphatidylcholine vesicles, TF activity was minimal. When vesicles containing different ratios of phosphatidylserine to phosphatidylcholine were used, a 40:60 molar ratio yielded maximal activity, and this ratio was therefore used for the present experiments.

Radiolabeling of Proteins. Sialyl[^{3}H]factor IX and sialyl-[^{3}H]factor X were prepared by the general technique of van Lenten and Ashwell (7) with slight modifications as described earlier (8). Specific radioactivity for [^{3}H]factor IX was 1.5×10^8 cpm/mg of protein and that for [^{3}H]factor X was 2.1×10^8 cpm/mg. Factor VII was labeled with ^{125}I as described earlier (8), and its specific radioactivity was 4×10^9 cpm/mg of protein.

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Abbreviation: TF, tissue factor.

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Measurement of Rate of Activation of Factor X and Factor IX. An activation peptide release assay was used to obtain progress curves of factor X activation (9) and factor IX activation (10). One hundred percent activation of factor X yielded 30–35% of the labeled factor soluble in trichloroacetic acid. In some experiments, activation of factor X was monitored by measuring factor Xa amidolytic activity with the chromogenic substrate S-2222. The test sample, 0.7 ml, was added to a cuvette containing 0.1 ml of S-2222 at 1 mg/ml and the resultant rate of change in absorbance at 405 nm was measured. This was converted to factor Xa concentration from a reference curve prepared with factor Xa at known concentrations.

Measurement of Activation of Factor VII. Factor VII activation was measured by subjecting serial aliquots from reaction mixtures to $NaDodSO_4$/PAGE under reducing conditions and analyzing the resulting radioactivity profiles. The procedure is described in detail in a previous publication (8). This technique permits detection of as little as 5% factor VIIa in a reaction mixture.

Electrophoresis. $NaDodSO_4$/PAGE was performed according to the method of Laemmli (11).

RESULTS

The data presented below are representative examples obtained in experiments repeated under the same conditions on at least three different occasions.

Factor VIIa/TF and Factor VII/TF Activation of Factor X. Factor VIIa/TF and factor VII/TF activated factor X at similar rates, with no lag phase for factor VIIa/TF and with a 30-sec lag for factor VII/TF (Fig. 1*A*). Results differed strikingly when antithrombin III and heparin were added to reaction mixtures before recalcification (Fig. 1*B*). In reaction mixtures containing factor VIIa/TF, the antithrombin III/heparin did not delay activation. In reaction mixtures containing factor VII/TF, the antithrombin III/heparin suppressed activation. Factor Xa was not measurable after 5 min of incubation and was measurable in only minimal amounts (0.5–2% trichloroacetic acid-soluble ^{3}H-labeled material) after 15 min. Raising the concentration of factor VII/TF complex in the reaction mixture 100-fold (final concentrations of TF of 200 ng/ml and of factor VII of 5 μg/ml) did not enhance the rate of factor VII/TF activation of factor X in the presence of antithrombin III and heparin.

In further experiments the order of addition of antithrombin III/heparin to the reaction mixture was varied. In one reaction mixture native factor VII, TF, calcium, antithrombin III, and heparin were incubated together for 1 min before the factor X was added. In the other reaction mixture native factor VII, TF, calcium, and factor X were incubated together for 1 min before the antithrombin III and heparin were added. Activation of factor X differed strikingly in the two mixtures (Fig. 2). If the antithrombin III and heparin were already present when the [^{3}H]factor X was added, then factor X was not activated. If native factor VII, TF, calcium, and [^{3}H]factor X were incubated together for 1 min before antithrombin III and heparin were added, factor X was activated. In a supplemental experiment, native factor VII at 0.5 μg/ml, TF at 2.0 ng/ml, and 5 mM $CaCl_2$ were incubated with factor Xa at 0.25 μg/ml for 1 min before antithrombin III, heparin, and [^{3}H]factor X were added in final concentrations of 150 μg/ml, 5 units/ml, and 5 μg/ml. Allowing this concentration of factor Xa to react with native factor VII, TF, and calcium for 1 min also bypassed the suppressive effect of antithrombin III/heparin upon the activation of [^{3}H]factor X (data not shown).

Activation of Factor VII in Reaction Mixtures Containing Factor X. Experiments were next carried out with ^{125}I-labeled factor VII (^{125}I-factor VII) and unlabeled factor X to measure in the same reaction mixture activation of factor VII, by its conversion to a two-chain molecule, and generation of factor Xa, by its amidolytic activity. Reaction mixtures contained ^{125}I-factor VII at the plasma concentration of factor VII, 0.5 μg/ml, factor X at 10 μg/ml, TF at 2 ng/ml, and $CaCl_2$ at 5 mM. Within 1 min after the calcium was added, a linear rate of activation of factor X was established (Fig. 3), but no cleavage of ^{125}I-factor VII was demonstrable on a gel radioactivity profile (Fig. 4). After 5 min, when about 20% of factor X had been converted to factor Xa, about 90% of the ^{125}I-factor VII was still present in its native one-chain form (Fig. 3). In an additional experiment serial subsamples of reaction mixtures were subjected to $NaDodSO_4$/PAGE under reducing conditions followed by autoradiography. The generation of sufficient two-chain factor VIIa to be demonstrable by autoradiography (Fig. 5) also lagged several minutes behind the onset of a linear rate of activation of factor X.

Factor VIIa/TF and Factor VII/TF Activation of Factor IX. Experiments similar to the above were also carried out with

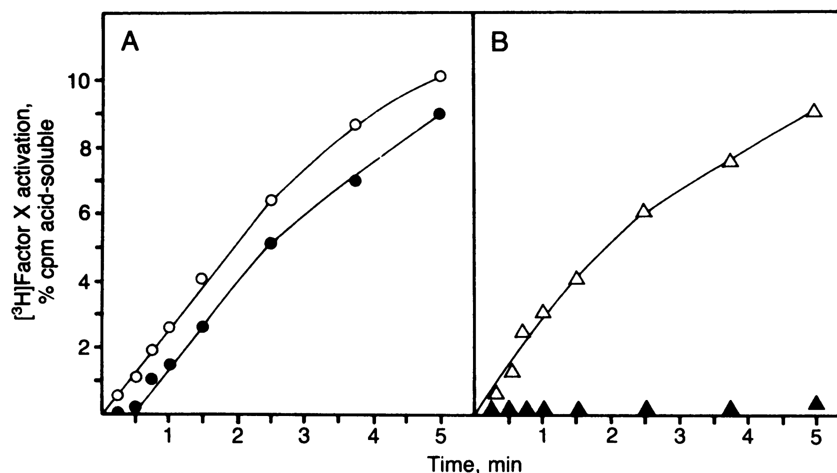

FIG. 1. Activation of factor X by factor VIIa/TF and by factor VII/TF in the absence (*A*) and in the presence (*B*) of antithrombin III and heparin. Final concentrations are [^{3}H]factor X, 5.0 μg/ml; factor VII or factor VIIa, 0.5 μg/ml; TF, 2.0 ng/ml; $CaCl_2$, 5 mM. The reaction mixtures of *B* also contained final concentrations of antithrombin III of 150 μg/ml and heparin of 5 units/ml. In *A*, ○, factor VIIa/TF; and ●, factor VII/TF; in *B*, △, factor VIIa/TF; and ▲, factor VII/TF. Factor VII or factor VIIa, TF, and [^{3}H]factor X were incubated with antithrombin III and heparin for 3 min at 37°C before the calcium was added.

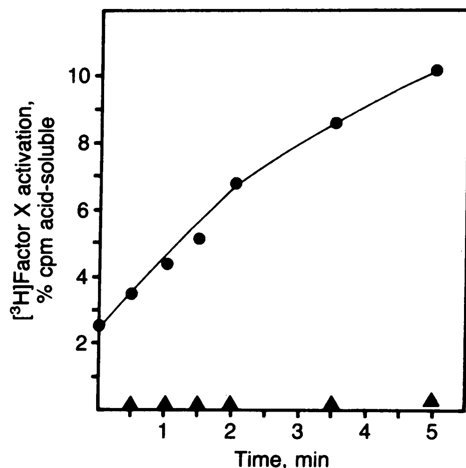

FIG. 2. Exposure of factor VII/TF/Ca^{2+} to factor X for 1 min prevents antithrombin III/heparin from blocking factor X activation. Antithrombin III and heparin were added to reaction mixtures containing factor VII, TF, and $CaCl_2$ 1 min before (▲) or 1 min after (●) [^{3}H]factor X was added. Final concentrations are as listed in the legend to Fig. 1. For the reaction mixture in which the antithrombin III and heparin were added before the factor X, the time shown on the abscissa is time after addition of the factor X; for the reaction mixture in which the antithrombin III and heparin were added after the factor X, the time shown on the abscissa is time after addition of the antithrombin III and heparin.

normal factor IX to confirm earlier observations made with the variant factor IX molecule, factor IX_{BmLE} (2). Factor VIIa/TF promptly activated [^{3}H]factor IX. In contrast, factor VII/TF failed to activate measurable amounts of factor IX during the first 10 min of incubation. Then, a linear activation began that approached the rate obtained with factor VIIa/TF. Adding a low final concentration of factor Xa, 0.02 μg/ml, to a reaction mixture made with factor VII abolished the 10-min lag. Adding antithrombin III and heparin prevented activation of factor IX in reaction mixtures made with factor VII but not in reaction mixtures made with factor VIIa (data not shown). In additional experiments, unlabeled factor IX was activated with ^{125}I-factor VII. Only about 5% of the total factor VII of the reaction mixture was in the two-chain activated form after 10 min of incubation—i.e., at the incubation time of observed beginning linear activation of factor IX in reaction mixtures containing [^{3}H]factor IX.

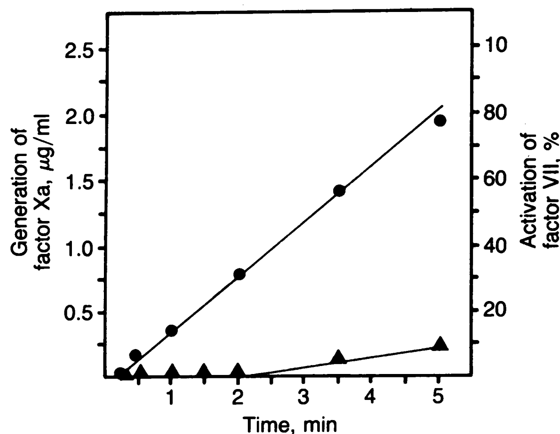

FIG. 3. Relationship between activation of factor VII and activation of factor X in a reaction mixture made with native factor VII. Final concentrations are unlabeled factor X, 10 μg/ml; ^{125}I-factor VII, 0.5 μg/ml; TF, 2.0 ng/ml; and $CaCl_2$, 5 mM. The progress curve of activation of factor VII (▲) was determined from radioactivity profiles; the progress curve of activation of factor X (●) was determined by assay of amidolytic activity.

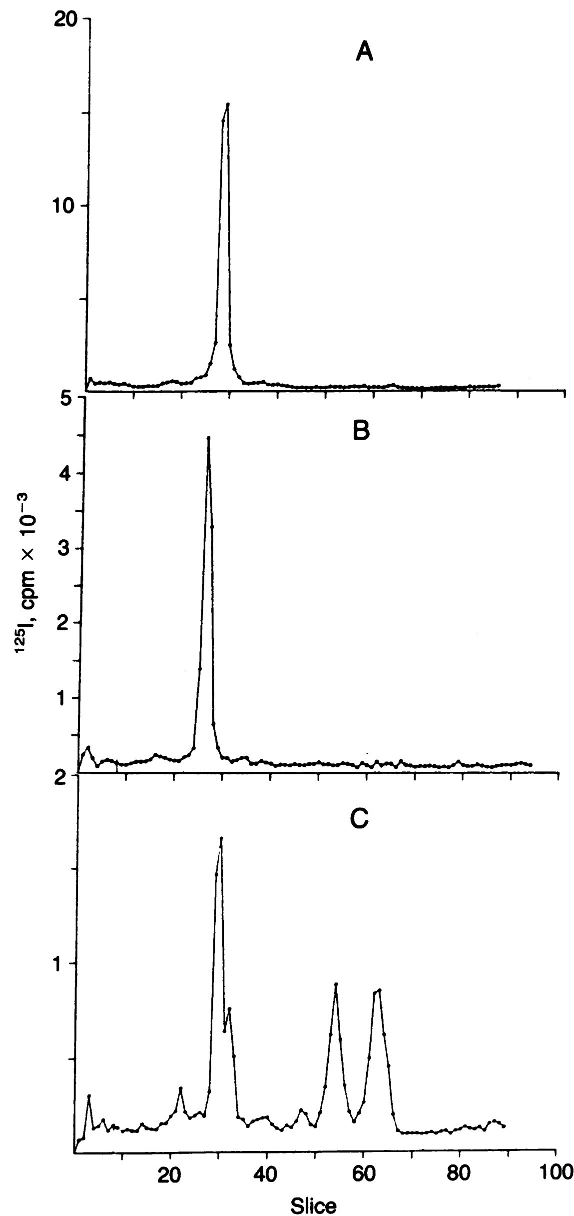

FIG. 4. Radioactivity profiles obtained on $NaDodSO_4$/PAGE under reducing conditions of ^{125}I-factor VII in a reaction mixture made with radiolabeled factor VII and unlabeled factor X. The reaction mixture is that described in the legend to Fig. 3. (*A*) Profile of a zero-time subsample removed just before the addition of calcium. (*B*) Profile of a subsample removed 1 min after the addition of the calcium, at which time a linear activation of factor X was evident. (*C*) Profile of a sample of ^{125}I-factor VIIa prepared by incubating ^{125}I-factor VII (0.5 μg/ml) with factor Xa (0.02 μg/ml) and phosphatidylcholine/phosphatidylserine vesicles (0.2 mM phospholipid) in the presence of Ca^{2+} (5 mM) for 20 min at 37°C.

DISCUSSION

Activated two-chain factor VII can activate its substrates, factor IX and factor X, only when bound to TF as a factor VIIa/TF enzyme/cofactor complex. Vessel wall injury exposes TF sites on cells in the vessel wall and surrounding tissues to native single-chain factor VII in blood. A native factor VII/TF complex forms, which should not initiate TF-dependent coagulation unless one or more of the follow-

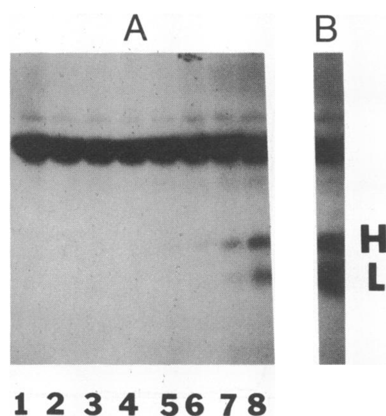

FIG. 5. Autoradiograph obtained on $NaDodSO_4$/PAGE under reducing conditions of serial subsamples from a reaction mixture containing ^{125}I-factor VII and unlabeled factor X. The gel was dried and exposed to x-ray film for 72 hr at −80°C. (*A*) Lanes 1–8 represent subsamples removed at the following intervals: zero time, 1 min, 1.5 min, 2 min, 3.5 min, 5 min, 7.5 min, and 10 min. (*B*) Autoradiograph of the factor VIIa preparation described in the legend of Fig. 4. H and L denote the heavy and light chains, respectively, of factor VIIa. The film was overexposed purposefully to show that activation of factor VII is not measurable by this technique until at least 3.5 min after reactions have been initiated with calcium.

ing hypotheses are true. The first is that although factor VII is a zymogen, a factor VII/TF complex possesses minimal enzymatic activity sufficient to initiate activation of factor X, factor IX, or both. The second hypothesis is that a low concentration of factor VIIa is normally present in blood and so trace amounts of a factor VIIa/TF complex also form at the site of vessel wall injury and this triggers the coagulation process. The third is that traces of factor Xa, factor IXa, or both, however formed, rapidly and preferentially back-activate factor VII when it is part of a factor VII/TF complex.

The data of Fig. 1*A* show that reaction mixtures made with either native factor VII or factor VIIa activated factor X at similar rates after less than a 1-min lag for the reaction mixture made with factor VII. This means to us that after 1 min both reaction mixtures contained the same concentration of factor VIIa/TF complex. The data of Fig. 1*B* show that antithrombin III/heparin did not prevent factor VIIa/TF from activating factor X. This fits with earlier observations (12, 13) that antithrombin III inactivates factor VIIa at a very slow rate, even in the presence of high concentrations of heparin. The data of Fig. 1*B* also show that antithrombin III/heparin blocked factor VII/TF from activating measurable amounts of factor X. This could not have resulted from a direct inactivation of native factor VII; antithrombin III/heparin inactivates native factor VII even slower than it inactivates factor VIIa (12). Prevention of factor X activation had to have stemmed from neutralization of traces of factor Xa in the reaction mixtures.

Therefore, one may conclude the following from the combined data of Fig. 1. First, measurable amounts of factor Xa cannot be generated in a reaction mixture made with factor VII until some factor VIIa/TF complex is formed. Second, our reaction mixtures made with native factor VII, TF, and [^{3}H]factor X must have been contaminated with traces of factor Xa, traces of factor VIIa, or both, to allow a linear rate of activation to begin within 1 min in the absence of antithrombin III/heparin. Third, a low concentration of factor Xa in a reaction mixture can fully convert a native factor VII/TF complex into a factor VIIa/TF complex within 1 min. The data of Fig. 2 provide further evidence for this last point. When native factor VII, TF, factor X, and calcium were allowed to incubate for 1 min before addition of antithrombin III and heparin, activation of factor X was not suppressed.

In our reaction mixtures, which contained a final concentration of 10 nM (0.5 μg/ml) factor VII or factor VIIa and 42.5 pM (2 ng/ml) TF, limited TF sites were saturated initially by whichever form of factor VII we had used to make the reaction mixture. However, 1 min after recalcification, mixtures made with either factor VII or factor VIIa must have contained essentially the same concentration of factor VIIa/TF to yield similar linear rates of factor X activation. Bach *et al.* (14) reported that one-chain native bovine factor VII binds to TF with only slightly less affinity than does two-chain factor VIIa, which means that when TF sites are limiting factor VII will compete with factor VIIa for these sites. Indeed, such competition has been demonstrated in both bovine (1) and human (2) systems. Therefore, for full conversion of the native factor VII/TF complex present initially in our reaction mixtures into factor VIIa/TF one of two events had to happen. Within 1 min either all of the factor VII of a reaction mixture was activated to factor VIIa or the factor VII bound to TF was preferentially activated to factor VIIa.

The data of Figs. 3–5 establish that the latter event must have occurred. Activation of total factor VII was not measurable in reaction mixtures made with factor VII at the time that a linear activation of factor X had been established. This would be expected with a rapid preferential activation of factor VII bound to TF, since the low concentration of TF in the reaction mixture could bind less than 0.5% of the total factor VII of the mixture. These data bring home the important point that the activation state of unbound factor VII need not mirror the activation state of factor VII bound to TF in a reaction mixture. The results fit with the concept that a rapid preferential activation of factor VII bound to TF is an essential early step in the initiation of TF-dependent coagulation.

Nemerson and Repke (15) have shown that factor Xa catalyzes activation of bovine factor VII much more rapidly in reaction mixtures containing relipidated bovine TF than in reaction mixtures containing only the phospholipid used for the relipidation. They also found that the rate of factor VII activation increased with increasing TF concentration of their reaction mixtures. Our data with human TF and factor VII extend their observations and establish that factor VII bound to a low concentration of TF can be fully activated within seconds by a low concentration of factor Xa. Thus, even though only trace amounts of factor VIIa are thought to be formed during normal hemostasis (8) only trace amounts should be needed because the factor VIIa will be positioned just where it can function—bound to TF sites in the vessel wall and surrounding tissues.

Our earlier data on the activation of factor IX (2) and the present data on the activation of factor X suggest to us that human zymogen factor VII/TF complex does not possess a minimal enzymatic activity essential for triggering TF-dependent coagulation. The demonstration by Bauer, Rosenberg, and associates (16, 17) of a low concentration of prothrombin fragment F_{1+2} in normal blood represents strong presumptive evidence that normal individuals continuously generate small amounts of factor Xa. This factor Xa, in the presence of a phospholipid cofactor on the luminal surface of vascular endothelium (18) or on the surface of activated platelets (19), could generate traces of factor VIIa. Moreover, unlike other activated clotting factors, any factor VIIa generated would circulate with a long intravascular half life of about 2.5 hr (20). Indeed, Miller *et al.* (21) have reported that normal blood, in contrast to hemophilic blood, contains demonstrable factor VIIa activity.

Therefore, our present concept of the sequence of events initiating TF-dependent coagulation in hemostasis may be summarized as follows:

(*i*) Vessel wall injury allows circulating factor VII to saturate TF sites on cells in the vessel wall and surrounding tissue. Almost all of the circulating factor VII will be native factor VII, but a trace of factor VIIa will also be present.

(*ii*) Consequently, the vast majority of enzyme/cofactor complexes that are formed will be factor VII/TF, but a small number will be factor VIIa/TF.

(*iii*) A trace concentration of factor Xa will be generated by the small number of factor VIIa/TF complexes and possibly also by reactions of the intrinsic pathway of coagulation.

(*iv*) The factor Xa so formed will preferentially and very rapidly activate only the factor VII of factor VII/TF complexes in a key amplifying step of the initiation sequence.

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