Influence of Factor VIIa and Phospholipids on Coagulation in “Acquired” Hemophilia

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Objective—This study was performed to evaluate the influences of phospholipids and recombinant factor VIIa (rFVIIa) on thrombin generation and clot formation in “acquired” hemophilia B.

Methods and Results—A synthetic mixture corresponding to hemophilia A and “acquired” hemophilia B blood (AHBB) manufactured in vitro by an anti-FIX antibody were used in this study. With 10 pmol/L tissue factor (TF), 10 nmol/L rFVIIa, and saturating phospholipid, established thrombin generation in SHA was similar to that observed in the presence of factor VIII and rFVIIa at physiological concentrations. At lower phospholipid concentrations, thrombin generation was delayed and reduced. With 5 pmol/L TF, contact pathway–inhibited AHBB clotted later than normal blood and showed reduced clot stability and thrombin generation. These parameters of effectiveness were increased by the addition of phospholipids to AHBB, which restored clot stability and increased thrombin generation. No correction of clot formation or thrombin generation was observed when rFVIIa and phospholipids were added to AHBB in the absence of TF.

Conclusions—The influence of rFVIIa is dependent on TF, and phospholipids substantially increase the hemostatic (or thrombotic) potential of rFVIIa/TF. (Arterioscler Thromb Vasc Biol. 2002;22:

Key Words: hemophilia ■ factor VIIa ■ tissue factor ■ phospholipids ■ thrombin generation

The intrinsic factor Xase, a complex enzyme composed of serine protease factor IXa and the cofactor factor VIIIa assembled on a membrane surface, is an essential component of the tissue factor (TF)-induced hemostatic mechanism. Genetic or acquired deficiencies in precursors of the intrinsic Xase, factor VIII and factor IX, lead to the most common bleeding disorders, hemophilia A and hemophilia B, respectively. One in 5000 male newborns is a carrier of a defective gene leading to factor VIII deficiency, and 1 in 30,000 male newborns has factor IX deficiency. Early treatments for hemophilies used partially purified coagulation factor concentrates. These concentrates, however, were also associated with thromboembolic complications and viral infections. During the last two decades, monoclonal antibody–purified factor VIII and IX concentrates and recombinant factors VIII and IX have become the desired therapy for hemophilia A and B. Progress has also been achieved in animal models by using gene therapy, and several clinical trials have been initiated using this approach for the treatment of human patients.

A significant fraction of hemophilia patients receiving replacement therapy develop inhibitory antibodies against the missing protein, and alternative treatments must be used. During the last two decades, factor VIIa at supraphysiological concentrations has been used for the therapy of hemophilia patients, especially for those with inhibitors against factors VIII and IX. The mechanism by which high doses of factor VIIa restore normal hemostasis in the hemophilia situation is not clear, although it has been hypothesized that elevated factor VIIa is able to trigger sufficient thrombin generation in hemophilia blood. The data acquired in our laboratory using whole blood and synthetic in vitro coagulation models suggest that factor VIIa at pharmacologically relevant concentrations is not able to restore either thrombin generation or solid clot formation and that TF is required for any procoagulant effect.

The reactions of the propagation phase of the blood coagulation cascade leading to thrombin formation in vivo occur primarily on membrane surfaces provided by platelets. In vitro studies indicate that artificial phospholipid membranes can be used for these reactions as well. Moreover, at concentrations exceeding 2 μmol/L, phospholipids per se are more efficient in supporting reactions of blood coagulation than platelets at physiological concentration.

In this study, we evaluate the ability of factor VIIa at pharmacologically relevant concentrations in combination

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with phospholipid vesicles to improve thrombin generation in synthetic model and anti-factor IX antibody–induced (“acquired”) hemophilia B blood.

Methods

Materials

Human coagulation factors VII, X, IX, and prothrombin were isolated from fresh frozen plasma using the general methods of Bajaj et al24 and were purged of trace contaminants and traces of active enzymes as described.25 Human factor V and antithrombin III (AT-III) were isolated from freshly frozen plasma.26,27 Recombinant factor VIII and recombinant TF (residues 1 to 242) were provided as gifts from Drs Shu Len Liu and Roger Lundblad (Hyland division, Baxter Healthcare Corp, Duarte, Calif). Recombinant human factor VIIa was purchased from NOVO Pharmaceuticals. Recombinant full-length tissue factor pathway inhibitor (TFPI) produced in Escherichia coli was provided as a gift by Dr Kirk Johnson (Chiron Corp, Emeryville, Calif). The TF/lipid reagent (2 nmol/L/10 μmol/L) and corn trypsin inhibitor (CTI) were prepared as described.28 The determination of active TF in TF/PCPS preparations was done as described.29 Phosphatidylserine (bovine brain) (PS), phosphatidylcholine (egg yolk) (PC), benzamidine hydrochloride, and EDTA were purchased from Sigma. Phospholipid vesicles (PCPS) composed of 25% PS and 75% PC were prepared as described.30 Spectrozyme TH was purchased from American Diagnostica, Inc. D-Phe-Pro-ArgCH2 Cl (FPRck) was synthesized in house. ELISA thrombin-AT-III (TAT) kit (Enzygnost TAT) was purchased from Behring. Monoclonal anti-factor IX antibody (α-FIX-91) was obtained from the Biochemistry Antibody Core Laboratory (University of Vermont). Fibrinogen, fibrinopeptides A (FPA) and B (FPB), and platelet counts were assayed immediately for thrombin activity. The hydrolysis of the substrate was monitored by the change in absorbance at 405 nm using a Molecular Devices Vmax spectrophotometer. Thrombin generation was calculated from a standard curve prepared by serial dilutions of α-thrombin.

TF-Initiated Clotting of Fresh Human Blood

The protocol used is a modification of Rand et al,31 performed at the Clinical Research Center, Fletcher Allen Health Care (Burlington, Vt). Nonanticoagulated phlebotomy blood was drawn and placed in tubes on a rocking table at 37°C preloaded with CTI.

Multiple Time Point Experiments

The collection of multiple time points was performed in 4 series of tubes (16 tubes/series) and 2 supplementary tubes. Sixty-two tubes (15 in each of 4 series and 2 supplementary) were loaded with 0.1 mg/mL CTI and 5 pmol/L repleted TF (TF/PCPS 5 pmol/L, 25 nmol/L in HBS with 2 mmol/L CaCl2). Four phlebotomy control tubes (1 tube/series) contained no TF. The specific experiments performed were as follows:

1. Sixteen tubes contained no additional reagents (normal control).
2. Sixteen tubes were loaded with 50 μg/mL of α-FIX-91 (“acquired” hemophilia B without replacement).
3. Sixteen tubes were loaded with 50 μg/mL of α-FIX-91 and 15 μmol/L PCPS (“acquired” hemophilia B with PCPS replacement).
4. Sixteen tubes were loaded with 50 μg/mL of α-FIX-91, 15 μmol/L PCPS, and 10 nmol/L factor VIIa (“acquired” hemophilia B with PCPS and factor VIIa replacement).
5. Two supplementary tubes were loaded with 10 nmol/L factor VIIa (no PCPS).

No more than 35 μL of all reagents was loaded in each tube. Normal blood was drawn by venipuncture, 1-mL aliquots were delivered into the reagent-loaded tubes, and the tubes were periodically quenched (from 0 to 20 minutes) with 1 mL of 50 mmol/L EDTA containing 20 mmol/L benzamidine hydrochloride and 10 μL of 10 nmol/L FPRck (diluted in 10 mmol/L HCl). The zero tube of each series was quenched before the addition of blood. The clotting time was determined visually by two observers. Two supplementary tubes were quenched 10 minutes after the clotting time. Tubes were centrifuged, and clots and supernatants were separated and stored for additional analyses. Thrombin generation was evaluated by ELISA for TAT, FPA, and FPB release by HPLC and fibrinogen depletion by Western blots. Solid clots were lyophilized, weighed, solubilized, and analyzed by gel electrophoresis.

Single Time Point Experiments (PCPS Titration)

In these experiments, all 41 tubes (10 tubes/series and phlebotomy control tube) were loaded with CTI and varying concentrations of PCPS in duplicates (0, 1, 3, 8, and 25 μmol/L) (no PCPS in phlebotomy control tube). In the first series, all tubes were loaded with 5 pmol/L TF/25 mmol/L PCPS alone (normal control), in the second series with 5 pmol/L TF/25 mmol/L PCPS and 50 μg/mL of α-FIX-91 (“acquired” hemophilia B control), and in the third series with 50 μg/mL of α-FIX-91 and 10 nmol/L factor VIIa (factor VIIa replacement). In the fourth series, TF was absent and 10 nmol/L factor VIIa was present. Fresh blood (1 mL) was delivered into reagent-loaded tubes. Tubes were quenched 10 minutes after the clotting time. The samples were collected and analyzed as above.
Results

PCPS Titration in the Synthetic Coagulation Model

In all experiments of this series, the concentration of relipidated TF was 10 pmol/L (in 50 nmol/L PCPS). Figure 1 depicts thrombin generation profiles over time at varying PCPS concentrations. In the control experiment (all proteins were present at their mean physiological concentrations) with 200 μmol/L PCPS, thrombin was generated at a maximum rate of 320 nmol/L/min after an initiation phase of ~4 minutes (●). The highest level of thrombin observed was 340 nmol/L. In the absence of factor VIII (approximating hemophilia A) with 200 μmol/L PCPS, the initiation phase was prolonged to 15 minutes and the maximum rate of thrombin generation was decreased to 6.2 nmol/L per min (□). Only 18 nmol/L thrombin was detected after 20 minutes. The addition of 10 nmol/L factor VIIa in the absence of factor VIII at 200 μmol/L PCPS decreased the initiation phase to 1 minute and increased the maximum thrombin generation rate to 140 nmol/L per min (■), a result similar to that reported by van 't Veer et al. A decrease in PCPS to 100 μmol/L had little effect on the maximum rate of thrombin generation during the propagation phase (110 nmol/L per min) and maximum thrombin levels (~250 nmol/L at both PCPS concentrations), while the initiation phase was prolonged to 2 minutes (●). Additional reductions in PCPS concentration caused prolongation of the initiation phase and decreased rates of prothrombin activation during the propagation phase. At 50 μmol/L PCPS (○), the initiation phase duration was similar to that of the normal control, whereas the maximum rate of thrombin generation was only 60 nmol/L per min with the maximum thrombin level of 160 nmol/L. At 14 μmol/L PCPS, the initiation phase was extended to 9 minutes, thrombin generation rate decreased to 13 nmol/L/min (▲), and the maximum thrombin level decreased to 70 nmol/L. No thrombin generation was observed in the absence of PCPS.

PCPS and Factor VIIa in “Acquired” Hemophilia B Blood

PCPS Titration

CTI inhibited normal blood activated with 5 pmol/L TF/25 nmol/L PCPS clotted in 6.9 minutes (Figure 2A, ●). The addition of 1 μmol/L PCPS decreased the clotting time to 5.5 minutes. Additional increases in PCPS concentration (to 25 μmol/L) had no additional effect on the clotting time. “Acquired” hemophilia B blood in the presence of 5 pmol/L TF/25 nmol/L PCPS clotted in 11.8 minutes (□). At 3 μmol/L PCPS, the clotting time of this blood was decreased to 10.1 minutes. An increase in PCPS to 8 μmol/L decreased clotting time to 3.8 minutes. An additional increase in PCPS to 25 μmol/L had no additional effect on clotting time. The addition of 10 nmol/L factor VIIa at 5 pmol/L TF/25 nmol/L PCPS decreased the visually observed clotting time of “acquired” hemophilia B blood from 11.8 minutes (without exogenous factor VIIa, □) to 4.3 minutes (▲). Increases in PCPS concentration decreased the clotting time to 2.5 minutes at 25 μmol/L phospholipid. When TF was omitted from the reaction, the “acquired” hemophilia B blood clotted in 35 minutes in the presence of 10 nmol/L factor VIIa (●). In the absence of TF, the additions of PCPS (1 to 25 μmol/L) had no procoagulant effect on the clotting time of the “acquired” hemophilia B blood (clotting time varied from 33 to 41 minutes).

The maximum thrombin concentration (evaluated as the plateau level of the TAT generation) achieved in normal blood treated with 5 pmol/L TF/25 nmol/L PCPS was ~500 nmol/L. An increase in PCPS to 3 μmol/L increased the maximum thrombin observed to ~700 nmol/L. Additional increases in the PCPS concentration (up to 25 μmol/L) had no additional effect on the ultimate thrombin concentration. The maximum thrombin concentration observed in “acquired” hemophilia B blood at the same initiator concentration (no additional PCPS) was ~240 nmol/L. The addition of PCPS (25 μmol/L) increased the thrombin level to 570
nmol/L. When 10 nmol/L factor VIIa was added to “acquired” hemophilia B blood at 5 pmol/L TF and 3 μmol/L PCPS, an equivalent thrombin level (570 nmol/L) was achieved. No additional increase in thrombin was observed when PCPS was increased to 8 and 25 μmol/L. In the absence of TF, the addition of 10 nmol/L factor VIIa to “acquired” hemophilia B blood produced a maximum thrombin level of 56 nmol/L after 45 minutes. In the absence of TF, the addition of PCPS (1 to 25 μmol/L) with 10 nmol/L factor VIIa to “acquired” hemophilia B blood did not increase the final thrombin concentration.

Thrombin generation rates (based on TAT formation) are presented in Figure 2B. In normal blood activated with 5 pmol/L relipidated TF at 25 nmol/L PCPS (no exogenous factor VIIa added), thrombin generation rates varied from 60 nmol/L per min without PCPS addition to 135 nmol/L per min at 25 μmol/L TF (Figure 2B, ◆). In “acquired” hemophilia B blood activated with the same TF concentration in the absence of additional PCPS, the thrombin generation rate was approximately one third that of normal blood (21 nmol/L per min) ( []). With increasing PCPS this rate increased, reaching 97 nmol/L per min at 25 μmol/L phospholipid. The addition of 10 nmol/L factor VIIa to “acquired” hemophilia B blood induced to clot with 5 pmol/L TF increased the thrombin generation rate at all PCPS concentrations tested (●). At 3 μmol/L PCPS, the thrombin generation rate in “acquired” hemophilia B blood with 10 nmol/L factor VIIa was similar to that observed in normal blood at the same PCPS concentration in the absence of exogenous factor VIIa (134 and 132 nmol/L per min, respectively). In the absence of TF and PCPS, the addition of 10 nmol/L factor VIIa to “acquired” hemophilia B blood produced low levels of thrombin (56 nmol/L at a low (1.6 nmol/L per min) rate (▲). Additions of PCPS (1 to 25 μmol/L) did not increase thrombin generation rate.

These data indicate that for normal blood in the presence of TF, saturation with PCPS was achieved at 3 μmol/L phospholipid. In “acquired” hemophilia B blood in both the absence and presence of 10 nmol/L exogenous factor VIIa, the maximum thrombin generation rates were observed at 25 μmol/L PCPS. In the absence of TF and in the presence of 10 nmol/L factor VIIa, thrombin generation in “acquired” hemophilia B blood was negligible and almost independent of PCPS.

Time Course of Thrombin Generation and Clot Formation

In all experiments of this series, blood was activated with 5 pmol/L TF/25 nmol/L PCPS. Normal CTI-inhibited blood clotted 5.5 minutes after the addition of TF (Table; Figure 3A, ◆). Thrombin generation occurred at a maximum rate of 68 nmol/L per min, and the reaction was complete ~15 minutes after TF addition. The clotting time of “acquired” hemophilia B blood at the same TF concentration was extended to 7.8 minutes ( []) with a maximum thrombin generation rate of 5.7 nmol/L per min and the maximum level of 36 nmol/L, values >10% those observed in normal blood. The addition of 15 μmol/L PCPS to “acquired” hemophilia B blood (●) decreased the visually observed clotting time to 3.9 minutes, ie, a clot was observed almost 2 minutes sooner than in normal blood; however, the inception of the propagation phase of thrombin generation occurred at the same time as in normal blood (6 minutes). The maximum rate of thrombin generation was enhanced to 48 nmol/L per min, and the
maximum level increased to 70 nmol/L. The addition of 10 nmol/L factor VIIa to “acquired” hemophilia B blood activated with 5 pmol/L TF in the presence of 15 μmol/L PCPS (★) had almost no effect on the visually observed clotting time (3.7 minutes); ie, it was similar to that observed without added factor VIIa. The initiation phase of thrombin generation, however, was shortened to 3 minutes, and maximum thrombin levels increased to 200 nmol/L. The addition of 10 nmol/L factor VIIa alone, without PCPS, to “acquired” hemophilia B blood activated with 5 pmol/L TF produced 60 nmol/L thrombin over 15 minutes of the reaction (★; a single-point experiment).

Platelet activation (based on α-granule osteonectin release) in normal blood induced to clot with 5 pmol/L TF/25 nmol/L PCPS (Figure 3B, ●) was detected between 1 and 2 minutes after the initiation of the reaction, and release occurred at a rate of 1.3 nmol/L per min, ▲10% the maximum rate (12.9 nmol/L per min). In “acquired” hemophilia B blood (□), detectable platelet activation occurred after a similar interval with initial rates as in normal blood. However, the maximum rate of this process (6.9 nmol/L per min) was decreased by half and delayed, occurring between 7 and 9 minutes of the reaction. The addition of 15 μmol/L PCPS to “acquired” hemophilia B blood had little effect on platelet activation (△). When both 10 nmol/L factor VIIa and 15 μmol/L PCPS were added to this blood, early and rapid platelet activation occurred at a rate of 9.6 nmol/L per min (★).

### Parameters of Clot Formation in Normal Blood and AHBB Activated With 5 pmol/L TF/25 nmol/L PCPS

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Clot Time (CT), min</th>
<th>Analyte in Solution at CT, % of Total</th>
<th>Final Clot Weights, mg/mL Blood</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal blood</td>
<td>5.5</td>
<td>10 Fibrinogen</td>
<td>70</td>
</tr>
<tr>
<td>AHBB</td>
<td>7.8</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>AHBB + 10 nmol/L factor VIIa</td>
<td>5.7</td>
<td>NE</td>
<td>NE</td>
</tr>
<tr>
<td>AHBB + 15 μmol/L PCPS</td>
<td>4.0</td>
<td>65</td>
<td>50</td>
</tr>
<tr>
<td>AHBB + 10 nmol/L factor VIIa + 15 μmol/L PCPS</td>
<td>3.7</td>
<td>10</td>
<td>85</td>
</tr>
</tbody>
</table>

NE indicates not evaluated.
In normal blood activated with 5 pmol/L TF/25 nmol/L PCPS, ~70% of total FPA was released at the clotting time at a maximum rate of 4.2 μmol/L per min (Table). In “acquired” hemophilia B blood, detectable FPA release was delayed by >1 minute with ~60% of this peptide released at the clotting time and the maximum rate decreased to 1.2 μmol/L per min. The addition of 15 μmol/L PCPS to “acquired” hemophilia B blood substantially shifted the inception of the FPA release to the left, although maximum rate of release (1.3 μmol/L per min) was almost not affected. Less than 50% of FPA was released at clotting time. When 10 nmol/L factor VIIa together with 15 μmol/L PCPS was added to “acquired” hemophilia B blood, the maximum rate of the FPA release was increased to 2.9 μmol/L per min. At the visually observed clotting time, 85% of total available FPA was in solution.

Soluble fibrinogen depletion and insoluble clot formation in normal and induced hemophilia B blood are presented in Figure 4 and the Table. In normal blood induced to clot with 5 pmol/L TF/25 nmol/L PCPS (panel A), soluble fibrinogen was almost completely (~90%) removed from the solution at the visually observed clotting time. In “acquired” hemophilia B blood, fibrinogen depletion and solid clot formation were delayed and ~40% of total fibrinogen was in solution at the

Figure 4. Depletion of fibrinogen (Fgn) and solid clot formation during TF-induced clotting of whole blood. Samples for analyses were taken from experiments of Figure 3. Upper lanes in each panel represent soluble fibrinogen, lower parts represent solubilized solid clots. A, Normal blood; B, “Acquired” hemophilia B blood; C, “Acquired” hemophilia B blood with 15 μmol/L PCPS; D, “Acquired” hemophilia B with 15 μmol/L PCPS and 10 nmol/L factor VIIa. The numbers above the lanes represent quenching times (min). Arrows indicate clotting times.
clotting time (panel B). Only 35% of total fibrinogen was depleted from the solution at the clotting time when 15 μmol/L PCPS was added to “acquired” hemophilia B blood (panel C). When both 10 nmol/L factor VIIa and 15 μmol/L PCPS were added to “acquired” hemophilia B blood (panel D), the onset of the fibrinogen depletion started substantially earlier than in normal blood (compare panels A and D). Only 10% of fibrinogen was in solution at the visually observed clotting time.

The final clot weights in normal blood were 1.0 mg/mL blood (Table). In “acquired” hemophilia B blood, final clot weights were decreased to 0.7 mg and not affected by the addition of 10 nmol/L factor VIIa. When 15 μmol/L PCPS was added to “acquired” hemophilia B blood, final clot weights increased to an average of 0.9 mg, whereas addition of both 10 nmol/L factor VIIa and 15 μmol/L PCPS increased clot weights to 1.1 mg.

The dynamics of clot growth over time revealed a pronounced dependence on the conditions of the reaction. In normal blood, initial clot weights were ~60% of those final (on average 0.6 versus 1.0 mg). In “acquired” hemophilia B blood, initial weights were ~50% of final (0.33 versus 0.7 mg). In “acquired” hemophilia B blood with 15 μmol/L PCPS, although clots were formed relatively early (4 minutes after the initiation), they were initially small (0.1 to 0.3 mg) and stayed such for ~5 minutes. In the presence of 10 nmol/L exogenous factor VIIa and 15 μmol/L PCPS in “acquired” hemophilia B blood, clots of a normal weight (0.9 to 1.2 mg) were formed early in the reaction (3 minutes after TF addition).

**Discussion**

Recombinant factor VIIa has been extensively used for the treatment of hemophilia A and B patients with inhibitors, although the exact mechanism by which this enzyme, used at supraphysiological concentrations, restores normal hemostasis is not clear. Additionally, the lack of correlation between the in vivo factor VIIa levels during the treatment and the efficacy makes the outcome somewhat unpredictable. As a consequence, similar regimens of treatment in separate studies lead either to reports of a high level of success or failure to provide reliable hemostasis.

The data of a previous study from our laboratory indicate that a single (supra)pharmacological dose of factor VIIa alone is not able to restore normal thrombin generation, clot formation, and clot stability in hemophilia A and B blood. The data of the present study indicate that anionic phospholipids remarkably improve the hemostatic potential of factor VIIa in the tissue factor–induced coagulation of “acquired” hemophilia B blood in vitro. In the presence of factor VIIa at a pharmacological concentration (10 nmol/L) and phospholipids at ~8 μmol/L, all parameters of thrombin generation and clot formation in “acquired” hemophilia B blood are similar to those observed in normal blood. Factor VIIa and PCPS alone are each able to correct the visually observed clotting time. This time, however, does not correlate either with thrombin generation or with solid clot formation as is seen in a normal blood.

In the absence of TF, only negligible amounts of thrombin are generated at a low rate when 10 nmol/L factor VIIa is added to the hemophilia B blood, and they are not increased by the addition of phospholipids. These data are consistent with our previous conclusion that TF is essential to achieve some thrombin generation in hemophilia blood in the presence of factor VIIa at pharmacological concentrations.

All reactions leading to thrombin formation in vivo are presumed to occur on membrane surfaces provided primarily by platelets. In many in vitro experiments, which explore processes of blood coagulation, artificial phospholipid vesicles are used. It was established that similar thrombin generation profiles are observed for the reactions accomplished either in the presence of platelets present at physiological concentrations or in the presence of 1 to 2 μmol/L phospholipids. These concentrations of phospholipids, however, are much lower than those required to saturate the complex enzymes of blood coagulation. An addition of phospholipids to blood provides an extra surface for the reactions of blood coagulation to occur and, as a consequence, increases the potency of enzymatic complexes of blood coagulation. The proteolytic activity of the factor VIIa/TF complex is substantially affected because of the high saturating concentrations of phospholipids (>100 μmol/L) required for the maximum efficiency of this enzymatic complex. On phospholipid addition, factor VIIa/TF is able to generate factor Xa at a high rate. An addition of 10 nmol/L factor VIIa to blood saturates TF present and, as a consequence, increases the concentration of the factor VIIa/TF complex. However, neither phospholipids alone nor factor VIIa alone are able to increase factor Xa concentration to the levels required for an efficient propagation phase of thrombin generation to occur. Only a combined effect of both increased concentration and increased efficiency of the factor VIIa/TF complex produces factor Xa levels substantial enough to drive thrombin generation to near normal levels.

In summary, phospholipids substantially improve the hemostatic potential of factor VIIa in the TF-induced coagulation of “acquired” hemophilia B blood. Based on our initial data, platelets present at supraphysiological concentrations also substantially increase thrombin generation during the TF-initiated coagulation of “acquired” hemophilia B blood in the presence of 10 nmol/L factor VIIa. These results provide a new insight into the factor VIIa-dependent hemostasis, implicating that the in vivo efficacy of factor VIIa replacement therapy is probably related to a simultaneous presence of TF and excess membrane surface as a consequence of platelet accumulation at the site of a vascular lesion. In this mechanism, factor VIIa at the pharmacological levels used could be expected to initiate thrombin production only in the event of a vascular lesion and restricted to that site.

**Acknowledgments**

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