Modulation of Thrombin-Mediated Activation of Factor VIII:C by Calcium Ions, Phospholipid, and Platelets

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The activation of factor VIII:C by thrombin appears to be an important prerequisite for the function of factor VIII:C as a cofactor in factor X activation in coagulation. The possible modulation of factor VIII:C activation by potential cofactors such as calcium ions, phospholipid, and platelets was studied systematically. Factor VIII:C activation could not be studied in the complete absence of Ca²⁺, since factor VIII:C activity decayed rapidly in calcium-free buffers, EDTA, or ethylene glycol tetra-acetic acid (EGTA), with only partial or no recovery of activity after readdition of Ca²⁺ or Mg²⁺. Added calcium chloride at 1.25, 2.5, 4, 10, 50, and 200 mmol/L produced progressive inhibition of factor VIII:C activation, with complete inhibition achieved by 50 mmol/L. Crude phospholipid preparations gave varying results, while purified phospholipids either had no effect or inhibited activation. This paper reports the new finding that fresh washed human platelets markedly potentiated factor VIII:C activation by a low concentration of thrombin (0.02 U/mL), even with prostaglandin E₁, (PGE₁) or dibutyryl cyclic AMP (cAMP) added to the washed platelets. However, the activity of platelets in factor VIII:C activation was inhibited by inclusion of PGE₁, or dibutyryl cAMP during platelet washing, and ionophore A23187 increased this platelet activity; these data suggest that platelet stimulation is involved in the development of this activity. When platelets were maximally stimulated by thrombin (0.5 U/mL), the external calcium concentration increased 55 to 160 μmol/L, as measured with murexide, supporting the possible modulation of factor VIII:C activation by a transient increase in Ca²⁺ at the platelet surface.

Reagents. Trizma base, murexide, rabbit brain cephalin, bovine serum albumin (BSA) (fatty acid-free), cytochalasin B, prostaglandin E₁ (PGE₁), dibutyryl cyclic AMP (cAMP), EDTA, and ethylene glycol tetra-acetic acid (EGTA) were purchased from Sigma Chemical Co, St Louis. Dimethyl sulfoxide was obtained from Baker Chemical Co, Phillipsburg, NJ; 1.0 mol/L CaCl₂ from BDH Chemicals, Poole, England; Na Chelex from Bio-Rad Laboratories, Richmond, Calif., and inosithin from Associated Concentrates, Woodside, NY. Arachidonic acid was purchased from BioData Corp., Hadboro, Pa. Purified phosphatidylglycerol (PI), phosphatidylserine (PS), phosphatidylcholine (PC) and phosphatidylethanolamine (PE) were from Supelco, Bellefonte, Pa, and Ultrol Hepes buffer from Calbiochem, La Jolla, Calif. Platelin-plus activator was purchased from General Diagnostics, Morris Plains, NJ, and human factor VIII:C-deficient plasma (<0.01 U/mL) and pooled normal plasma were purchased from George King Biomedical, Overland Park, Kan. Acid-citrate-dextrose anticoagulant, formula A, was obtained from Fenwal Labs, Deerfield, Ill. Ile-glu-γ-γ-γ-γ-glutamidetetra-amino ketone (IgG-CK) was the generous gift of Dr Charles Kettner, Brookhaven National Laboratory, Upton, NY. Proteins. Human factor VIII:C was partially purified as previously reported, with a specific activity of 20 to 25 factor VIII:C U/mg and 34 to 52 U/mg von Willebrand factor (assuming A₁ cm² = 10.0 at 280 nm). It contained no other detectable clotting factors and could be activated >20-fold with thrombin. Factor VIII:C was stored in small aliquots in plastic tubes at −70 °C in Tris-buffered saline (TBS) (0.10 mol/L NaCl, 0.05 mol/L Tris, pH 7.4) with 0.2% BSA (TBS-BSA). No contaminating enzymes could be detected in the factor VIII:C–TBS–BSA by assay with chromogenic substrates S-2222, S-2238, S-2251, and S-2160. Purified human α-thrombin (2914 NIH U/mg) was the generous gift of Dr John Fenton, New York State Department of Health, Albany. Studies with chelating agents. In order to study factor VIII:C activation in the absence of Ca²⁺, compared with the readdition of Ca²⁺, I attempted to render the factor VIII:C preparation, or the diluting buffer, virtually calcium-free without affecting the factor VIII:C activity of the preparation; I was unsuccessful. There was a gradual loss of factor VIII:C activity, apparent as early as two to five minutes after the addition of EDTA, 0.1 to 0.2 mmol/L, or EGTA, 0.2 mmol/L (but not after EDTA 1 to 5 μmol/L), or after dilution of factor VIII:C in TBS–BSA that had been dialyzed against Na Chelex. The readdition of CaCl₂ or MgCl₂ (2 to 8 mmol/L) to...
EDTA-treated factor VIII:C usually prevented further loss of factor VIII:C activity, but was not associated with recovery of factor VIII:C; readdition of MnCl₂ (2 to 8 mmol/L) was reproducibly associated with partial recovery of factor VIII:C activity, especially at the higher concentrations of MnCl₂ used (4 mmol/L and 8 mmol/L). Because of this instability of factor VIII:C in the absence of Ca²⁺, all studies assessing the effects of Ca²⁺ were performed by adding varying concentrations of CaCl₂ to factor VIII:C, which was not pretreated to remove Ca²⁺. Therefore, the true calcium concentration is actually slightly higher than the added CaCl₂ because of the contribution of the calcium in the factor VIII:C/TBS-BSA preparation (probably >5 <100 μmol/L).

**Activation experiments.** The activation of human factor VIII:C (0.01 to 0.8 U/mL) by added human thrombin (0.01 to 0.2 U/mL) was studied as previously reported. Conditions were chosen to produce a fivefold to tenfold activation achievable so that the entire range of VIII:C levels in the experiment (preactivation to peak activation) could be easily assayed with the same vol of subsample placed directly into the assay mixture without requiring prior dilution. I have shown this assay method to have a coefficient of variation of 12% to 14% over the range of factor VIII:C (0.01 U/mL) assayed in these studies, when performed by the following method in my laboratory. In brief, 0.1 to 1.1 mL of factor VIII:C in TBS-BSA was placed in a polystyrene tube in a 37 °C water bath for three minutes, during which a baseline factor VIII:C assay was performed in duplicate; then 5 to 10 μL of thrombin was added (at “zero” time) and timed serial 1-μL to 100-μL aliquots were removed and placed immediately in a mixture of 0.1 mL VIII:C deficient plasma, 0.1 mL Platelet-in-plus, and 0 to 100 μL of TBS, which had been preincubated for eight minutes at 37 °C; 0.1 mL of 40 mmol/L CaCl₂ was added immediately (less than five seconds) after (or in some cases immediately before) the test sample, and the time of clotting from the final addition was measured by a mechanical timer (Fibrometer, BBL, Cockeysville, Md). Reproducible activation and assay of factor VIII:C required accurate pipetting of small aliquots (1 to 10 μL) of thrombin and of subsamples for factor VIII:C assay; I have used Drummond microdispensers (Drummond Scientific Co, Broomall, Pa), which allow the coefficient of variation (c.v.) of ≤14% found in these studies. For experiments in which the effect of CaCl₂, IGAČ, phospholipid, or platelets was assessed, the added reagent was warmed with the factor VIII:C TBS-BSA for three minutes before the addition of thrombin. In preliminary experiments, it was found that the effect of 4.0 mmol/L CaCl₂ was equivalent whether added to factor VIII:C or thrombin before the two were combined. In experiments assessing CaCl₂ added to factor VIII:C, the concentration of CaCl₂ used in the clotting assay was adjusted so that the final concentration was between 5 and 7.5 mmol/L CaCl₂, a range that does not cause variation in the factor VIII:C clotting assay. In addition, the stability of factor VIII:C was identical at 37 °C in the presence or absence of added CaCl₂ (2 to 4 mmol/L). Pooled normal human plasma (arbitrarily assigned a value of 1.0 U/mL) was the reference for the factor VIII:C clotting assays. In some experiments with platelets, factor VIII:C was also measured by the 11C-factor X activation method as previously reported.

Fresh washed human platelets were prepared as previously described with minor modifications (described below) from whole blood obtained by venipuncture from normal volunteers on the same day of the experiment and used as quickly as possible (generally <4 hours). The entire procedure was performed at room temperature. Written informed consent was obtained from each donor under a protocol approved by the Northport Veterans Administration Human Studies Committee and the SUNY-Stony Brook Committee on Research in Human Subjects. In brief, whole blood was anticoagulated with 0.1 vol of 40% Na citrate, or in later experiments with 0.15 vol of acid–citrate–dextrose. Platelet-rich plasma (PRP) was prepared by centrifugation, the platelets were pelleted and washed three times with 10 mL of TBS, 0.1 mol/L NaCl, and resuspended in TBS, and in later experiments with 0.15 mol/L NaCl, 10 mmol/L Hepes, pH 6.5, and were resuspended in the washing buffer for a final count of 2 to 5 x 10⁵/μL in the fVIII:C activation experiments. In preliminary experiments, 11C-serotonin release from platelets prepared in this manner was assessed as previously reported and found to remain stable for at least three hours when the platelet suspension was kept in a plastic tube at room temperature. Platelets washed in this manner (in Hepes–NaCl buffer) in this laboratory do not support prothrombin activation in the absence of added factor Xa, and the prothrombin activation observed when factor Xa is added can be abolished by the presence of oligopeptide factor Xa inhibitor (Sidone Morrison, SUNY-Stony Brook, unpublished observations, May 1984). The effect of PGE₁ (11 μmol/L) or dibutyryl cAMP (5 mmol/L) was tested either by addition to the platelets after washing or by addition to whole blood and inclusion in the washing buffer. The effect of cytochalasin B was tested by including cytochalasin B in DMSO (7.5 μg/mL final concentration [f.c.]) in the whole blood and washing buffer and was compared with platelets washed without cytochalasin but with an equivalent volume of DMSO added. The effect of agonists—ionophore A23187 (5 μmol/L) or arachidonic acid (50 μmol/L)—was assessed by incubating the agonist or control buffer with the washed platelets and factor VIII:C at 37 °C for three to five minutes and then adding thrombin (0.03 U/mL) and following the activation by VIII:C clotting assay. In experiments with arachidonic acid, purified human fibrinogen, 1 mg/mL (a gift of Dr Dennis Galanakis, SUNY-Stony Brook) was added to the washed platelets and release of ATP and aggregation were monitored in a dual-channel Lumi aggregometer. Purified phospholipids (PL, PE, PS, PC) were prepared as described. One vial of cephalin (from 20 mg of rabbit brain acetone powder) was resuspended in 1.0 mL of TBS (f.c. 4.0 mmol/L phospholipid). A single stock suspension of inositol (20 mg/mL) was further diluted such that a final concentration of 60 μg/mL was achieved in all experiments. All experiments assessing the effect of Ca²⁺ concentration were performed with dilutions made from the same 1.0 mol/L CaCl₂ stock from BDH Chemicals.

**Studies with murexide.** Fresh washed human platelets were resuspended after the final wash in 10 mmol/L Hepes, 80 mmol/L NaCl, 0.1 mmol/L EDTA (Hepes–NaCl–EDTA) and exposed to 0.5 U/mL thrombin at 37 °C for one minute. Control platelets were incubated simultaneously without added thrombin. The platelets were pelleted by centrifugation at 10,000 g for four minutes and the supernatant was assayed for total calcium concentration by the calcium-sensitive indicator dye, murexide, in the following manner: 100 μL of supernatant and 200 μL of murexide (final concentration 1.0 mmol/L), in Hepes–NaCl–EDTA buffer (as described above) were placed in a cuvette, and the OD at 460 nm was recorded. This wavelength setting was empirically determined to give the maximum change in absorption with increasing CaCl₂ in a single-beam spectrophotometer. The OD of the supernatant from the control platelets was subtracted from the OD of the supernatant from the thrombin-stimulated platelets, and this difference was assumed to reflect calcium released from the platelets. The concentration of calcium was calculated from a reference curve obtained by measuring the OD of 100 μL of varying CaCl₂ concentrations (50 to 200 μmol/L), made from the same 1.0 mol/L stock (BDH Chemicals).

**RESULTS**

When the activation of factor VIII:C (0.01 U/mL) by thrombin (0.02 U/mL) was studied in the absence of added CaCl₂ and compared with the presence of added CaCl₂ at
varying concentrations (1.25, 2.5, 4.0 mmol/L) in four independent experiments, a progressive, increasing inhibition of activation and inactivation was found as CaCl₂ concentration was raised (Fig 1). When the data were normalized to the three-minute time point and the means of the independent determinations at each time point were compared, a significant difference was found between the activation in 4.0 mmol/L CaCl₂ and the control activation at all time points \( P < .05 \) except 1.5 minutes, and between the activation in 1.25 mmol/L CaCl₂ and 4.0 mmol/L CaCl₂ at 3, 12, and 20 minutes \( (P < .05) \). In order to assess the possible effects of physiologic levels of Ca²⁺ (1 to 2 mmol/L) at a physiologic level of factor VIII:C, the activation of factor VIII:C (0.6 to 0.8 U/mL) by thrombin (0.02 U/mL) with 1.25, 2.0, and 4.0 mmol/L or no added CaCl₂ was studied in six independent, identical experiments (Fig 2). When the data for each time point were pooled and the means were determined either for the raw data (Fig 2) or for data normalized as in Fig 1, the addition of 1.25 or 2.0 mmol/L CaCl₂ gave significant inhibition of inactivation of factor VIII:C at later time points (12 to 21 minutes, \( P < .05 \) at 12 to 16 minutes and \( P < .02 \) at 21 minutes) (Fig 2). In addition, the difference in activation between no added CaCl₂ and 4.0 mmol/L CaCl₂ was significant \( (P < .02) \) at the 1.5-minute time point for the normalized data. Factor VIII:C activation by thrombin was also studied at higher added calcium concentrations (4, 10, 50, and 200 mmol/L), and again a progressive inhibition of activation and inactivation was found with increasing calcium concentration (Fig 3). The degree of inhibition by 4 mmol/L CaCl₂ was very similar to that found under the somewhat different experimental conditions of Figs 1 and 2. Complete inhibition of factor VIII:C activation was found at 50 and 200 mmol/L CaCl₂ (Fig 3). If a contaminating protease such as activated protein C were present below the detection limit of the chromogenic substrate assays \( \leq 0.2 \) mmol/L for activated protein C on S-2160 at 0.4 mmol/L \( ^3 \), the possible slow inactivation of factor VIII:C by such a protease in the presence of Ca²⁺ would be expected to decrease factor VIII:C levels below the control activation (without added Ca²⁺) at later time points, rather than the higher levels observed with added Ca²⁺. Thus, the pattern of lesser activation and lesser inactivation seen in the presence
of added Ca\textsuperscript{2+} is consistent with a direct inhibition of the thrombin–factor VIII:C interaction and inconsistent with a potential of factor VIII:C degradation by a contaminating protease such as activated protein C.

Factor VIII:C activation by thrombin has been evaluated previously by different laboratories under varying conditions, that is, with no added phospholipid or calcium, or with added phospholipid or calcium.\textsuperscript{5,7,8} I studied the effect on the crude commercial phospholipid preparations, cephalin and inosithin, with and without added CaCl\textsubscript{2}. Cephalin (f.c.70 \textmu mol/ L phospholipid) inhibited the peak of factor VIII:C activation by thrombin reproducibly, and this inhibition was consistently exaggerated by the presence of added CaCl\textsubscript{2}. Inosithin at 60 \textmu g/mL potentiated factor VIII:C activation on one occasion, whereas another batch of inosithin consistently inhibited activation. I then evaluated the effect of purified phospholipid vesicles, singly and in combination, on factor VIII:C activation. PI at 50 and 100 \textmu g/mL inhibited the peak of factor VIII:C activation by 30\% and 38\%, respectively, whereas 10 and 20 \textmu g/mL produced neither inhibition nor potentiation of activation. PE at 10, 20, and 50 \textmu g/mL inhibited the peak of factor VIII:C activation by 26\%, 39\% and 48\%, respectively. An equimolar mixture of PS–PC at two concentrations, 20 and 100 \textmu g/mL, inhibited the peak of factor VIII:C activation by 67\% and 71\%, respectively. It is pertinent to note that cephalin, inosithin, and PS–PC at these concentrations all potentiate the activation of \textsuperscript{3}H-factor X by purified factors IXa and thrombin-activated factor VIII.\textsuperscript{2,3}

In contrast to purified phospholipids, fresh washed human platelets markedly potentiated factor VIII:C activation at a concentration of thrombin (0.02 to 0.03 U/mL) that produces little platelet release, and the addition of a potent inhibitor of platelet release (PGE\textsubscript{1}, 11 \textmu mol/L) to the washed platelets had no effect on the ability of the platelets to potentiate factor VIII:C activation (Fig 4). I have previously reported that this concentration of PGE\textsubscript{1} completely blocked the release of \textsuperscript{3}C-serotonin from washed platelets at similar concentrations of thrombin.\textsuperscript{3} These data support the hypothesis that the platelet effect on factor VIII:C activation measured under these experimental conditions does not require the simultaneous occurrence of the release reaction. However, when PGE\textsubscript{1}, (11 \textmu mol/L) or dibutyryl cAMP (5 mmol/L) was added to the freshly drawn whole blood and included in the washing buffer, the washed platelets did not enhance the activation of factor VIII:C by thrombin. These data indicate that some type of platelet stimulation occurring during preparation of the washed platelets (in the absence of PGE\textsubscript{1}, on dibutyryl cAMP) was responsible for the ability of washed platelets to enhance factor VIII:C activation by thrombin. I therefore investigated the ability of various platelet agonists to exaggerate the activity of platelets in factor VIII:C activation. The calcium ionophore A23187 (5 \textmu mol/L) markedly increased the platelet activity in factor VIII:C activation; whereas arachidonic acid had no effect at concentrations (50 to 100 \textmu mol/L) that were simultaneously shown to cause release and aggregation of the same batch of washed platelets. I also investigated the effect of cytochalasin B at a concentration (7.5 \textmu g/mL) that inhibited shape change of the washed platelets and found no inhibition of the ability of washed platelets to enhance factor VIII:C activation.

In order to assess the possibility that factor Xa bound to the platelets is responsible for the enhanced factor VIII:C activation, I incubated washed platelets at 37 °C with and without a potent specific inhibitor of factor Xa, IGGA-CK (0.22 \textmu mol/L). The platelets with IGGA-CK supported factor VIII:C activation by thrombin as much as the platelets without IGGA-CK (Fig 5). Furthermore, platelets washed in this manner without added IGGA-CK do not support prothrombin activation in the absence of added factor Xa.
Additional evidence for the specificity of the factor VIII:C clotting assays in these experiments with platelets was obtained by comparing the peak of factor VIII:C activation measured by simultaneous clotting assays and ³H-factor X activation assays (Table 1). The 2.56-fold increase observed in the clotting assays is entirely consistent with the 2.81-fold increase in VIII:C activity measured by the ³H-factor X activation method.

Although release of platelet granule contents may not be an essential prerequisite for the platelet effect on factor VIII:C activation, calcium released from platelets by thrombin may nevertheless be involved in modulating factor VIII:C activation. I measured the amount of calcium released from platelets (washed identically to the preceding experiments) maximally stimulated with thrombin (0.5 U/mL), using the indicator dye, murexide, and calibrated this release by measuring known CaCl₂ concentrations in murexide. At 0.24 to 1.0 x 10⁶ platelets per microliter, a mean increase of 55 to 160 nmol/L Ca²⁺ was measured after thrombin stimulation. These limited data agree closely with the original extensive studies with murexide by Detwiler and Feinman. It is therefore conceivable that in vivo Ca²⁺ release from platelets is involved in modulating factor VIII:C activation at the platelet surface.

**DISCUSSION**

These studies show that added CaCl₂ (1.25 to 200 mmol/L) exerts a significant inhibitory effect on thrombin-mediated factor VIII:C activation and inactivation. Although the actual ionized Ca²⁺ concentration in the experiments is not known with certainty, the range of CaCl₂ concentration added to factor VIII:C spans the physiologic range of total (1 to 2 mmol/L) and ionized Ca²⁺, and therefore the data support the possible physiologic significance of Ca²⁺ effects on factor VIII:C activation. Previous studies support the concept that calcium is an inhibitor of thrombin,¹¹ and this may explain the present observations. On the other hand, Ca²⁺ is clearly important for the functional stability of factor VIII:C, as shown by the effects of EDTA or EGTA in this and other studies,¹²,¹³ and partial recovery of activity after MnCl₂ addition is similar to that reported for factor V.¹⁴ Thus, the calcium effect on factor VIII:C activation may result from a conformational change in factor VIII:C itself, possibly making the thrombin-susceptible site less accessible.

The observations that some concentrations of crude and purified phospholipid preparations inhibited factor VIII:C activation by thrombin may not be relevant to normal hemostasis but emphasize the importance that varying conditions and reagents may have in affecting the results obtained by different laboratories in studies of factor VIII:C activation. It is likely that factor VIII:C binds to phospholipid¹⁵ and that this binding partially protects factor VIII:C from inactivation by specific factor VIII:C antibody in vitro,¹⁶ a finding that may explain the factor VIII inhibitor bypassing activity of some therapeutic concentrates. The inhibition by phospholipids of factor VIII:C activation and inactivation by thrombin may be an analogous protective effect of phospholipid.

All of the crude and purified phospholipids that I have previously studied (cephalin, inosithin, and equimolar PS:PC mixture) potentiated factor X activation by factors IXa and activated VIII:C,²,³ in contrast to the present findings for factor VIII activation by thrombin. The present studies suggest that the platelet activity in factor VIII:C activation is a separate, previously undescribed function of platelets that may be mediated by a different receptor and/or pathway from the platelet activity in factor X activation.

All of the preceding studies in semipurified systems are far removed from the physiologic setting of circulating whole blood flowing over vascular surfaces. The studies with fresh washed human platelets are one step closer to the normal physiologic setting and support the role of platelets in factor VIII:C activation, perhaps involving a localization of the reactants at the platelet surface. The inhibiting effects of PGE₁ and dibutylryl cAMP on the development of this platelet activity in factor VIII:C activation, and the ability of A23187 to increase this platelet activity, support the interpretation that stimulation of the platelets occurs during the washing procedure and is necessary to elicit this platelet activity. In contrast, the studies with cytochalasin B and arachidonic acid suggest that neither platelet shape change, nor release or aggregation inducible by arachidonic acid, is a necessary prerequisite to development of this platelet activity in factor VIII:C activation. One explanation of these data is the possibility that the stimulus involved in the development of this platelet activity causes a platelet membrane change, which might be associated with stimulation of intracellular pathways but does not necessarily lead to release or aggregation. However, the release of intracellular contents might also modulate factor VIII:C activation in vivo, since the measurable total calcium release is sufficient to support the hypothesis that a transient increase in Ca²⁺ concentration at the platelet surface may occur and be sufficiently high (eg, several hundred micromolar) to influence factor VIII:C activation.

Both thrombin and von Willebrand factor bind to specific receptors on platelets.¹⁷,¹⁸ Thrombin causes a number of changes in the platelet membrane, including the stimulation of PI turnover by the PI-specific phosphodiesterase,¹⁹ and a trace of thrombin might be responsible for the stimulation of platelets during the washing procedure. Because the semipurified human factor VIII:C used in these studies may be at least partly complexed with the von Willebrand factor also

**Table 1. Quantitation of Factor VIII:C Activation by Clotting Assay and Simultaneous ³H-Factor X Activation Assay**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Factor VIII:C Peak* by Clotting Assay (U/mL) (G ± SE)</th>
<th>Factor VIII:C Peak* by ³H-FX Activation (cpm/45 s (G ± SE))</th>
</tr>
</thead>
<tbody>
<tr>
<td>With platelets (n = 6)</td>
<td>14.6 ± 2.4</td>
<td>410 ± 74 P &lt; .01</td>
</tr>
<tr>
<td>Without platelets (n = 6)</td>
<td>5.7 ± 0.3</td>
<td>146 ± 47</td>
</tr>
<tr>
<td>Ratio of A/B</td>
<td>2.56</td>
<td>2.81</td>
</tr>
</tbody>
</table>

*The peak of fVIII:C activation by thrombin was measured at three minutes after thrombin addition by means of the standard factor VIII:C clotting assay and by simultaneous ³H-factor X activation assay (Materials and Methods).
present in the preparation, it is possible that the potentiation of factor VIII:C activation by platelets is dependent in part on the presence of von Willebrand factor. Recent reports have appeared showing that a high purity of human factor VIII:C without residual von Willebrand factor contamination can be achieved.\(^{5,20}\) Because it should be possible to define better the role of platelets in factor VIII:C activation with the use of high-purity unactivated human factor VIII:C, further studies in this laboratory are directed toward this goal.

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REFERENCES

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