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The Influence of Fibrinogen and Fibrin on Thrombin Generation – Evidence for Feedback Activation of the Clotting System by Clot Bound Thrombin

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Summary

In plasma the bulk of thrombin generation takes place after a clot has formed. We therefore investigated in what way the clot influences thrombin generation in plasma. The forming clot withdraws thrombin from free solution. Consequently less thrombin activity is found and less thrombin-inhibitor complexes are formed. The thrombin that is adsorbed to the clot reduces the lag time before thrombin generation in intrinsically or extrinsically triggered platelet poor plasma as well as in platelet rich plasma. We investigated the mechanism of this activation.

Clots were obtained by recalcification of plasma or by the addition of thrombin-like enzymes (Reptilase, Agihal) from snake venoms. They were thoroughly washed until the washing fluid was devoid of any detectable clotting enzyme activity. In platelet poor plasma (PPP), thrombin-induced clots shorten the factor Va-dependent lag-time of thrombin generation in the extrinsic system as well as the factor VIIIa-dependent thrombin generation in the intrinsic system. Factor V or factor VII preparations that in itself hardly influence thrombin generation patterns aquire the capacity to shorten these lag-times when incubated with clot. The last washing fluid of the clot is inactive. Snake venom induced clots are not active either. Clots that are incubated in heparinised plasma for 1 h or more are as active as clots from normal plasma are. A role of factor Xa can not be excluded but must be minor because a clot made by addition of thrombin to plasma from which the factors II, VII, IX and X have been removed is as active as a clot from normal plasma is.

When added to recalcified platelet rich plasma (PRP), in which the lag-time of thrombin formation is dependent upon activation of platelet procoagulant phospholipid activity, any type of clot shortens the lag-time before the burst of thrombin generation. Clots that are obtained by snake venom enzymes are also active in this system. This indicates that fibrin alone is capable to induce the procoagulant phospholipid activity in platelets.

We conclude that three known thrombin-dependent feedback activations in the clotting system (factor V, factor VIII and platelets) are efficiently supported by thrombin bound to the fibrin clot and that there is an additional activating effect of fibrin on the procoagulant action of platelets.

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Introduction

The clotting of fibrinogen by thrombin is caused by the enzymatic removal of fibrinopeptides A and B (1). In this process thrombin is adsorbed on the fibrin (2–6). This property of fibrin is known as Anti-thrombin I action (7). The clot bound thrombin remains enzymatically active, as has been shown by its ability to release FPA from fibrinogen (8–10), to clot fibrinogen, to induce the platelet release reaction (10) and to hydrolyse chromogenic substrate S2238 (9). It also has been reported that a washed clot will shorten the coagulation time of blood and plasma (10, 11). It is the purpose of this article to investigate the mechanism(s) by which clot bound thrombin influences thrombin generation in plasma.

The inhibition characteristics of clot bound thrombin are reportedly different from those of free thrombin in that it seems protected from inactivation by antithrombin III (AT III) even in the presence of heparin (5) but remains accessible to antithrombin III-independent inhibitors like hirudin, hirudin dodecapeptide (Hirugen), D-Phe-Pro-Arg-Chloromethyl ketone (PPACK) (8) and Dermatan Sulphate (12).

The most ready explanation for clot induced coagulation of plasma is that bound thrombin clots fibrinogen from the free solution. Another possible mechanism is that an existing clot induces thrombin generation in the surrounding plasma. Indeed the shortening of whole blood clotting time (WBCT) has been reported when clots were incubated in native blood (12, 13). Positive feedback activation, i. e. enhancement of the rate of thrombin production by the first traces of thrombin formed, is an essential part of the clotting process (14). With the initial sequential activation of clotting factors only trace amounts of thrombin are generated (15). These traces of thrombin have the ability to activate factor V, factor VIII (15–20) and platelets (21, 22). The activated co-factors V and VIII increase the catalytic capacity of their partner-enzymes (factors Xa and IXa) by several orders of magnitude (23, 24). Thrombin action on platelets fosters thrombin formation by exposing procoagulant phospholipids (22) and releasing factor V (25 and references therein). Presumably also the interstices of a platelet aggregate provide a niche in which the products of clotting factor interaction are not diluted by flow phenomena. This ensemble of positive feedback actions of thrombin on cofactors and platelets leads to explosive, nonlinear thrombin generation (26).

The possible positive feedback-function of clot-bound thrombin has not been analyzed until now. In this article we investigate whether clot bound thrombin enhances further thrombin formation by sustaining the activation of factors V and VIII as well as platelets. To this purpose we compare the influence on thrombin generation of clots produced by thrombin and non-thrombin enzymes. A preliminary report of this work has been presented in 1993, at the 14th ICTH congress (27).

Materials and Methods

Materials

Plasma

Normal plasma was prepared by collecting blood from healthy donors on trisodium citrate (9:1). Platelet rich plasma (PRP) was obtained by single centrifugation at 250 × g, 15° C, 10 min, while platelet poor plasma (PPP) was obtained by a double centrifugation at 1000 × g, 15° C, 15 min and a third centrifugation at 23,000 × g, 4° C, 1 hour. PPP thus obtained was stored at –80° C.

Factor VIII deficient plasma was obtained in the same way from a known severe haemophilia A patient who had not received substitution therapy for several weeks.

Depleted plasma is plasma depleted of the vitamin K-dependent coagulation factors by mixing it with 1/5 volume of $Al(OH)_3$ (25% in 0.15 M NaCl) and incubating at 37° C for 5 min, with constant stirring. After centrifuging at high speed in an Eppendorf centrifuge for 2 min the supernatant plasma was recovered. It was checked that no detectable amounts of the factors II, VII, IX and X remained. The factor V concentration was 60% and the factor VIII concentration 65% of the original pooled plasma.

Defibrinated plasma is plasma that was defibrinated by mixing with 1/50 volume of Reptilase, letting the clot form at 37° C for 10 min and keeping the clotted plasma for additional 10 min at 0–4° C. Fibrin thus formed was discarded by winding on a plastic spatula. For further details see ref. 28.

Reagents

Buffer A: 50 mM Tris HCl, 100 mM NaCl, 0.5 g/l Bovine Serum Albumin (BSA, Sigma), pH = 7.35. Buffer B: same as Buffer A with 20 mM EDTA, pH = 7.9. Buffer C: 50 mM Tris HCl, 175 mM NaCl, 0.5 g/l BSA, pH = 7.9. The phospholipids vesicles were 20 mole% phosphatidyl serine (PS) and 80 mole% phosphatidyl choline and were prepared as previously described (23). Chromogenic substrate used for thrombin was S2238: H-D-Phe-Pip-Arg-PNA.2 HCl. Reptilase was supplied by Laboratories Stago (Asnières, France) and Recombinant Tissue Factor by Baxter-Dade (Düdingen, Switzerland). The 4th International standard heparin (ISH) was obtained from the National Bureau of Standards and Control (London). Recombinant Hirudin (Knoll AG) was a kind gift of Dr. W. Hornberger. Agihal, a purified fraction of Agkistrodon Halys Halys snake venom, that splits fibrinopeptide A from fibrinogen and does not activate factors V, VII or platelets, was obtained from Prof. L. Yukelson (Tashkent, Uzbekistan). Bovine purified factors V, VIII, IX were kindly provided by Dr. R. Wagenvoord.

Methods

Preparation of Clots

Three different types of clot were produced by: recalcification of normal plasma, adding 100 nM of thrombin to depleted plasma and adding Reptilase or Agihal to normal plasma.

Normal plasma clots: Platelet poor plasma (250 µl for one clot) was coagulated by recalcification to a final conc. of 23 mM $CaCl_2$ in a glass tube by adding 12 µl of 0.5 M $CaCl_2$ solution and incubation at 37° C for ½ h. The clot was wound on a plastic spatula, dried by blotting and put in human serum for 10 min. In certain experiments heparin was added to this serum to a final concentration of 0.1 U/ml. Subsequently, the clot was washed at least 4 times in 2 ml aliquots of Buffer A over the course of several hours to eliminate any soluble material trapped in the clots. That no unbound thrombin remained with the clot was checked by the absence of detectable amidolytic activity of the last washing buffer on S2238. This means that less than 0.2 picomole of thrombin could have leaked from a clot into the surrounding fluid during 30 min. The last washing fluid was also tested for its effect in thrombin generation curves.

Thrombin clots in depleted plasma were prepared by adding 10 µl of human α-thrombin (2.5 µM) to 250 µl of plasma depleted of the vitamin K-dependent clotting factors by $Al(OH)_3$ adsorption at the same time as 12 µl of 0.5 M $CaCl_2$.

Snake venom clots were prepared from the same volume of plasma by adding 1/50 volume of Reptilase or Agihal. The defibrinated plasma remaining after coagulation by the snake venoms was tested for the activity of the clotting factors and compared to the original plasma. No activation of clotting factors was found.

Prolongation of Clotting Time by Recombinant Hirudin

In 120 µl of plasma at 37° C supplied with PS/PC (1.1 µl final conc.) and varying conc. of hirudin (0.05 µM–0.2 µM) coagulation was triggered with recombinant tissue factor (final dilution 1/240) in 100 mM $CaCl_2$ and clotting times were recorded. The final concentration of 0.15 µM hirudin, extending the clotting time from 31 s (in absence of hirudin) to 2 min 45 s, was used in our experiments.

Measurement of Thrombin Generation in Plasma

Thrombin generation curves were obtained as described in detail in references 28–30. In short, to 240 µl of normal or defibrinated plasma was added 40 µl of Buffer A (which may contain any other required substance, e. g. hirudin) and 20 µl PS/PC (20 µM). The mixture was incubated at 37° C for 5 min and coagulation was triggered with 60 µl of triggering solution. Triggering solution A (extrinsic pathway) is recombinant tissue factor (final dilution 1/240) in 100 mM $CaCl_2$ (16.7 mM final conc.). Under our conditions this dilution of thromboplastin yields a clotting time of around 31 s in non-defibrinated plasma in the absence of hirudin. This trigger is used to study factor V activation. Hirudin (150 nM final concentration) has to be present, as will be discussed in the experimental section. The activation of factor VIII was studied using trigger B (intrinsic pathway), consisting of factor IXa (12 nM), and PS/PC (12 µM) and Ca^{2+} (1.5 mM). Of this trigger 38 µl was taken in the same pipette as 22 µl Ca^{2+} (250 mM) to obtain 60 µl of recalcifying trigger. In the case of PRP, 240 µl of plasma was diluted with 60 µl Buffer A and coagulation was triggered with 60 µl $CaCl_2$ to obtain 16.7 mM final concentration.

After addition of the trigger, 10 µl of reaction mixture was subsampled at equally spaced intervals (5, 10 or 15 s) in prewarmed (37° C) cuvettes with 490 µl of Buffer B containing 200 µM of S2238. As soon as the mixture coagulated, the clot was wound on a small spatula and removed. The reaction was stopped by the addition of 300 µl of 1 M citric acid after about two minutes. The optical density was read at 405 nm. The spectrophotometer as well as the subsampling and the stopping pipette were connected to a personal computer that calculated the increase of OD in time from the OD and the moment of subsampling and stopping. From the OD/min values the amidolytic activity was calculated by comparing with a standard calibration curve of active site titrated human α-thrombin. The lag time of thrombin formation is defined as the moment at which the thrombin concentration rises from a 0–5 nM level to a 10–20 nM level, because we observed that in fibrin containing plasma at the moment of clotting the thrombin concentration arrived at that level.

Clots or other substances of which the influence on thrombin generation was tested, were added to the reaction mixture 90 s after triggering coagulation. Unless otherwise indicated, three clots have been used.

For ease of presentation, in cases where the only essential difference between the curves was a shift in time, i. e. a shortening of the lag-time, we have also rendered the results as that shortening only (Tables 2 and 3).

Measurement of the Thrombin Potential

The thrombin potential is defined as the area under the thrombin generation curve (29). It is a measure of the amount of any substrate that the thrombin generated can potentially convert during its existence in a free form. It has been determined by calculation of the area under the thrombin generation curves that we determined.

Measurement of Prothrombin and α_2-Macroglobulin-thrombin Complex

Prothrombin was determined as described in ref. 28. Briefly, a 10 µl sample was incubated for 5 min in cuvettes containing 415 µl Buffer B and 50 µl staphylocoagulase, giving a final concentration of 300 nM, i. e. a molar excess of staphylocoagulase over the highest prothrombin value to be expected. The amidolytic activity that generates is due to the stoichiometric staphylocoagulase-thrombin complex formed (31). This complex has the same specific activity on chromogenic substrate as thrombin has (28). The total amidolytic activity was measured after addition of S2238 to a final concentration of 200 µM. In samples in which thrombin has been present the thrombin is partitioned between its different inhibitors, antithrombin III, α_2-macroglobulin and others. The α_2-macroglobulin-thrombin complex retains thrombin-like amidolytic activity. It has 0.556 times the specific activity of thrombin under our experimental conditions (28). The amount of total prothrombin present in plasma sample was calculated from the total amidolytic activity minus the activity of thrombin complexed with α_2-macroglobulin as measured in sample to which no staphylocoagulase had been added.

Determination of Thrombin/Antithrombin III Complex (TAT)

TAT levels were determined using an enzyme immunoassay (Enzygnost TAT, Behring, Germany) working on the sandwich principle; 100 µl of 1/4000 diluted samples of serum were incubated for 30 min in tubes coated with rabbit antibodies to human TAT. The tubes were washed and peroxidase-conjugated antibodies to human ATIII were added. After a 30 min incubation, tubes were washed again and peroxidase substrate o-phenylenediamine 2 HCl was introduced in the tubes. They were stored in the dark for 30 min after which the substrate conversion was stopped by adding 0.5 N sulphuric acid and absorbency measured at 492 nm. The TAT concentrations in the samples were obtained via the reference curve provided by the manufacturer.

Results

Thrombin Adsorption on the Forming Clot

The amount of prothrombin consumed during the coagulation of plasma was not significantly influenced by the presence or absence of fibrinogen (Table 1). Nevertheless, in absence of fibrinogen, the thrombin generation curve is significantly higher than in its presence (Fig. 1, upper frame). The area under the thrombin-time curve is 31 ± 3% (mean ± SEM, n = 10) lower in the presence than in the absence of the forming clot. Also the amount of thrombin found in the form of TAT complexes or bound to α_2-macroglobulin is lower after coagulation of normal plasma than after thrombin generation in defibrinated plasma (Table 1).

These results show that prothrombin is converted that does not appear as amidolytic activity during thrombin generation and that is not accounted for by the amount of TAT complexes formed or by the amount of α_2-macroglobulin-thrombin complexes formed. This suggests that during coagulation a significant amount of thrombin is bound to the forming fibrin clot. The phenomenon is also observed in the presence of heparin (Fig. 2). Heparin catalyses the ATIII-thrombin interaction. The free thrombin observed is therefore lower than without heparin, both in the presence and the absence of fibrin(ogen). The difference between the two curves is smaller in the presence of heparin than in its absence. This suggests that in the presence of heparin less thrombin is adsorbed on the clot. Additional evidence for binding of thrombin to the clot is found in the fact that a thrombin generation curve of fibrin containing plasma shows a dip at the moment that the clot is forming in situ, which is not seen in a curve from defibrinated plasma (Fig. 1). This can be seen clearly from the details of the first minutes of the graph (Fig. 1, lower frame).

Table 1 Thrombin related variables after thrombin generation in normal and defibrinated plasma

	Normal	Defibrinated
Prothrombin consumed (%)	93.9 ± 1.8	93.8 ± 0.6
α_2 Macroglobulin-thrombin (%)	4.3 ± 0.3	11.0 ± 0.4*
TAT complexes (%)	61.5 ± 0.4	63.9 ± 0.3*
Thrombin potential (nM · min)	356 ± 8.2	511 ± 10.1*

Results are expressed ± SDM; n = 10. The molar concentration of the original prothrombin in the plasma, is the 100% value.
* Indicates statistical significance at a $p < 0.05$ level

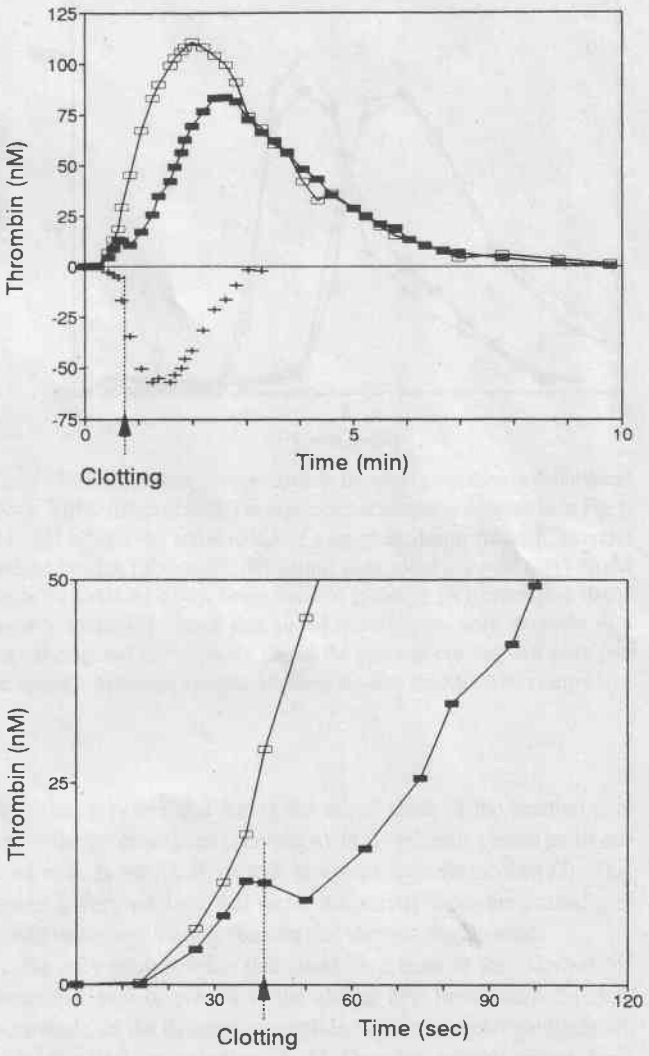


Fig. 1 The influence of fibrin formation on extrinsic thrombin generation. Thrombin generation was triggered in normal pooled PPP with recombinant tissue factor (1/240), Ca^{2+} (16.7 mM) and phospholipids (PS/PC, 1.1 µM). Amidolytic activity was determined in subsamples taken at 15 s intervals. From the amidolytic activity the thrombin activity was calculated (28). The experiment was performed in normal plasma (■) and in the same plasma defibrinated with reptilase (□). The difference of the two curves is represented below the time axis (+). Lower frame: Details of the first minutes

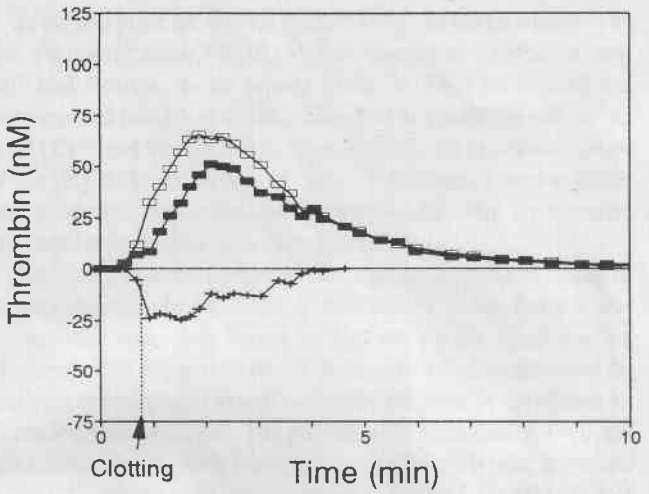


Fig. 2 The influence of fibrin formation on thrombin generation in the presence of heparin. The experiment of Fig. 1 was repeated in the presence of 0.04 IU/ml of unfractionated heparin

Effect of the Clot on the Coagulation of Fibrinogen

That fibrin bound thrombin directly attacks fibrinogen has been proven by the demonstration of clot-dependent generation of fibrinopeptides (8–10). This is not likely to be a sufficient explanation for the procoagulant effect observed, because newly formed fibrin would soon build a considerable diffusion barrier that will prevent the access of further fibrinogen to the bound thrombin. We have tried to induce coagulation of non-recalcified normal plasma by the addition of normal clots. If the clots were well washed and incubated in serum, such as was our standard procedure for making them, no coagulation was ever observed in 12 out of 12 experiments even after 1 h. So indeed the bulk of fibrinogen remains unconverted.

Effect of the Clot on Factor V Activation

To investigate the possibility that clot bound thrombin can mediate feedback activation of factor V in clotting plasma, we needed a system in which factor VIII was guaranteed to play no role. Therefore we used extrinsic thrombin generation measurements carried out in factor VIII deficient plasma.

Reinforcement of feedback activation reactions in the generation of thrombin show as shortening of the lag-time before the burst of thrombin occurs, inhibition of the feedback activation causes prolongation of the lag-time (14, 32, 33). However, no perceptible lag-phase is seen in extrinsic coagulation (34) not even at small time resolutions and low (up to 1/4000 diluted) thromboplastin concentrations. A delay of thrombin generation can however be induced by hirudin. It has been shown to be caused by retardation of the feedback activation of factor V by thrombin (35).

We observed that addition of clots to the system shortens this hirudin induced delay (Fig. 3). This must be attributed to the clot causing prothrombinase activity. In principle any of the three components of prothrombinase, i.e. factor Xa, factor Va or procoagulant phospholipids may be provided by the clot. Factor Xa activity indeed has been demonstrated to be contained in the clot (36). We observed that clots, produced by addition of thrombin (100 nM) to plasma devoid of factors II, VII, IX and X, did cause shortening of the delay (Table 2). This shows that factor Xa is not required for the observed shortening. It has

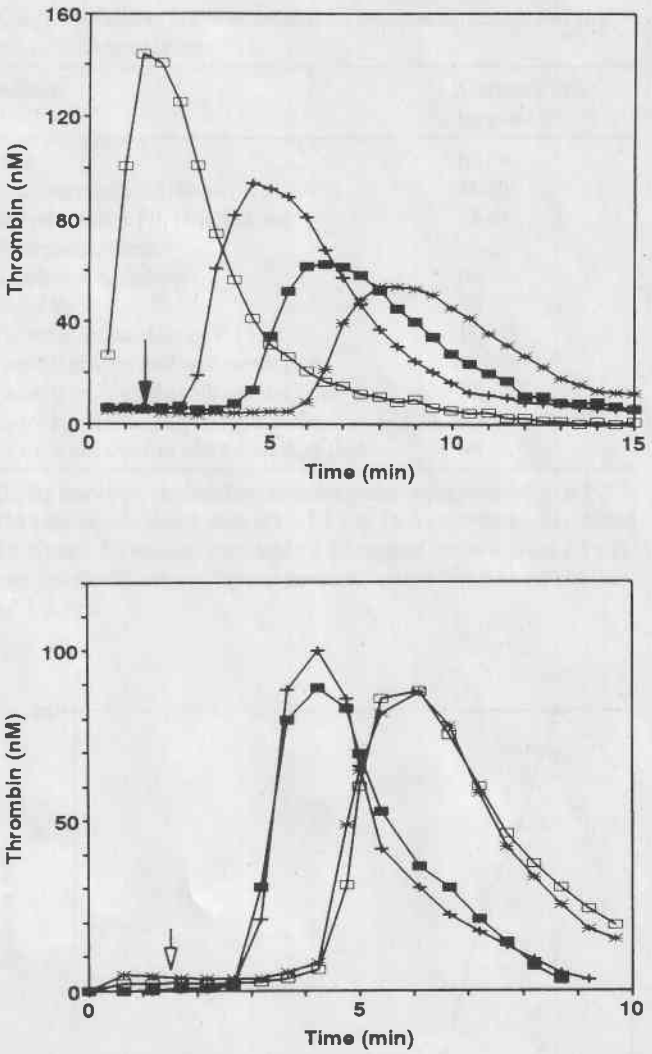


Fig. 3 Effect of plasma clots on extrinsic thrombin generation in defibrinated factor VIII deficient plasma. The experimental setup was the same as in Fig. 1, 0.15 μM hirudin was added to induce a lag-phase. Upper frame: (□) control without hirudin; (×) control; (■) normal clots added at t = 90 s; (+) 10 nM factor Va added at t = 90 s. Lower frame: (□) control; (×) control plus 10 nM factor V added; (+) control plus 10 nM factor V previously incubated with normal clots and Ca^{2+} (5 mM); (■) as the previous one but with clots preincubated in heparinised serum. All additions were done at t = 90 s (arrow)

been shown before that during the initial phase of the reaction (i.e. before the thrombin burst takes place), in the extrinsic system under our conditions factor Xa is present in excess over factor Va (37). This makes it very unlikely that factor Xa activity from the normal clot would be the rate limiting reactant that shortens the lag time.

The only phospholipids that could be present in the clots are the traces that may be present in the normal PPP from which the clots were made. In the thrombin generation experiments phospholipids are added to a final concentration >1 μM. Therefore possible phospholipid concentrations in the clots are several orders of magnitude lower and cannot influence thrombin generation any further than the added phospholipid does. It therefore is also highly unlikely that the clots contribute to the prothrombinase action by bringing phospholipids to the reaction mixture. This left factor V activation as the most likely cause of the advancement of the thrombin burst. We therefore attempted to demonstrate factor V activation by the clots.

In the first place we saw that the effect of a clot can be mimicked by the addition of factor Va (10 nM final concentration) whereas non-activated factor V has no activity (Table 2). Then we showed that non-activated factor V in which 3 clots were incubated (10 min, 37° C, 5 mM Ca^{2+}) and then discarded, has a similar effect as activated factor V has (Fig. 3, lower frame). The factor V preparation used or buffer into which clots had been incubated, as well as the factor V preparation incubated with reptilase clots were all inactive.

Incubating clots for several hours in serum to which 0.1 U/ml of heparin is added did not diminish their activity. When factor V was preincubated with clots formed by reptilase the lag phase was not shortened. Clot induced factor Va generation was also measured directly, in normal plasma as well as in plasma depleted of the vitamin K-dependent clotting factors. The plasmas were recalcified at t = 0 and clots were added at t = 90. Every minute 10 µl aliquots were drawn and diluted 200-fold in tubes on ice, containing Buffer C with 0.1 U/ml of heparin and 5 mM $CaCl_2$. From these tubes 10 µl aliquots were transferred to prewarmed cuvettes containing 105 µl of buffer C and an excess of constituents of prothrombinase [human factor Xa, 20 pM, PS/PC 10 µM, and $CaCl_2$ 5 mM (final conc.)]. After 1 min, 10 µl of human prothrombin (200 nM final conc.) was added. At 3 min, 350 µl of Buffer B was added to stop thrombin generation. Then thrombin was measured via its amidolytic activity on S2238 (see Methods). The amount of Va generated was calculated by reading on a standard curve generated by titration of purified factor Va with active site titrated factor Xa in the presence of phospholipid. The results show that in normal plasma a tremendous increase in factor Va occurs (Fig. 4, top), this coincides with the burst of thrombin. This is to be expected because factor Va causes thrombin formation, but freshly generated thrombin activates factor V. It was impossible to analyse this type of curve in terms of the precise temporal relationships between the rise of both activities. In order to demonstrate that clots directly activate factor V in a plasma medium, we repeated the experiment in plasma from which the vitamin K-dependent factors had been removed. It was seen (Fig. 4, lower frame) that normal clots are capable to activate traces of factor V. The effect in itself is modest. To demonstrate factor V activation more convincingly the depleted plasma was supplied with additional factor V (70 nM) and indeed an enhancement in Va concentration could be seen (Fig. 4, lower frame). The bulk of factor Va generation in normal plasma must be considered to be secondary to thrombin formation. The importance of the small activation of factor V by the clot is that it starts a positive feedback loop by causing the generation of traces of free thrombin that in their turn cause further factor V activation. The effect of clots that had been incubated in heparinised serum (0.1 U/ml) for over 1 h was identical to that of clots from normal plasma.

Effect of the Clot on Factor VIII

When thrombin generation is initiated with incomplete tenase (containing IXa, PS/PC and Ca^{2+}) instead of tissue factor, clotting occurs via the intrinsic pathway but the contact phase is short circuited (32). A lag phase is always observed, because initial traces of thrombin have to activate factor VIII in order to induce the thrombin burst (32, 37, 38). The effect of various types of clot on these factor VIII dependent lag times are completely comparable to those obtained in a factor V dependent system (Fig. 5, Table 3). We interpreted that, analogously to factor V, factor VIII is also activated by thrombin adsorbed on a clot. Indeed, preincubation of a factor VIII preparation (15 nM in 1 mM $CaCl_2$) with 3 clots for 1 min at 37° C under gentle stirring, induces the capaci-

Table 2 Shortening of hirudin induced lag time in extrinsic thrombin generation on different additions

Addition	Shortening of the lag phase (s)
None	NS
Clot from recalcified plasma	78–89
Thrombin-clot in FII, FVII, FIX and FX depleted plasma	77–93
Reptilase or Agihal clot	NS
Factor V	NS
Thrombin activated factor V	174–197
Factor V preincubated with normal clots	96–118
Factor V preincubated with reptilase clots	NS
Factor V preincubated with PPACK clots	NS
Factor V preincubated with last washing fluid	NS

The lag times were obtained from thrombin generation experiments as in Fig. 3. The addition was always done at t = 1.5 min. Each experiment was repeated 4 to 6 times. No statistics were done but the range of results is given. NS indicates that the difference of lag time measured was less than the sampling interval, i.e. 30 s.

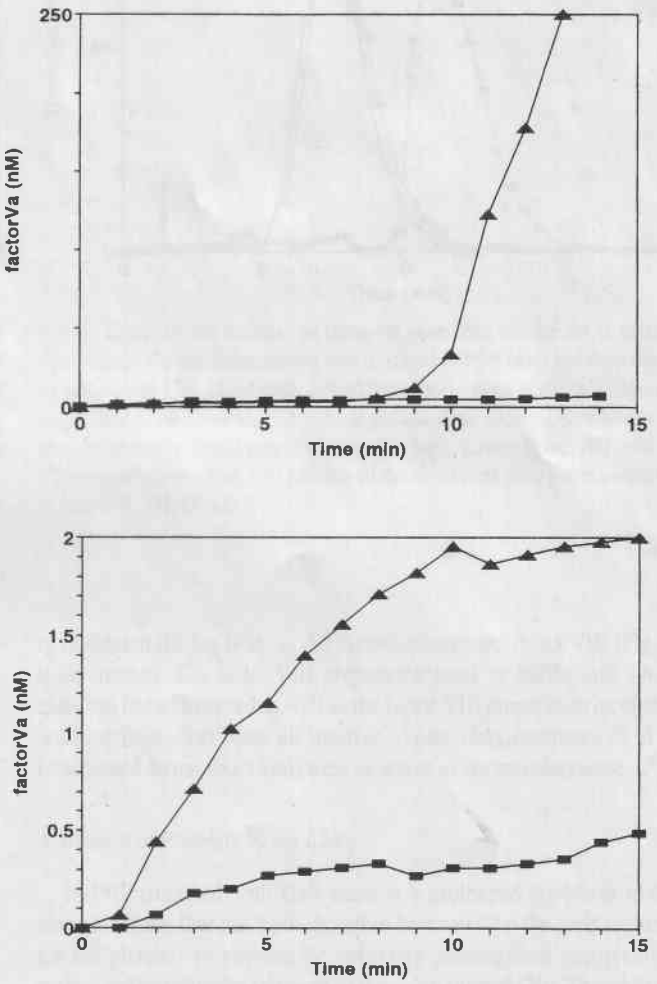
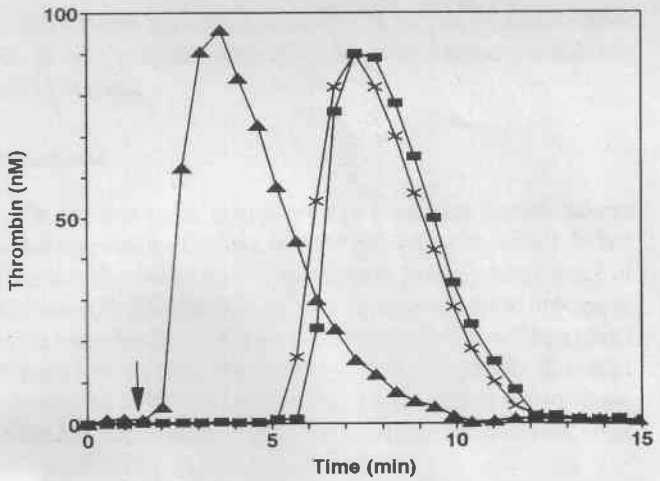


Fig. 4 The influence of normal plasma clots on factor Va generation in plasma. The development of activated factor V was measured in defibrinated normal pooled plasma, in subsamples taken at 1 min intervals after recalcification and simultaneous addition of three normal clots. Upper frame: (■) control without clots; (▲) clots added at zero time. Lower frame: (■) clots added at t = 0 to plasma depleted of factors II, VII, IX and X; (▲) idem with additional factor V (70 nM). See text for experimental details



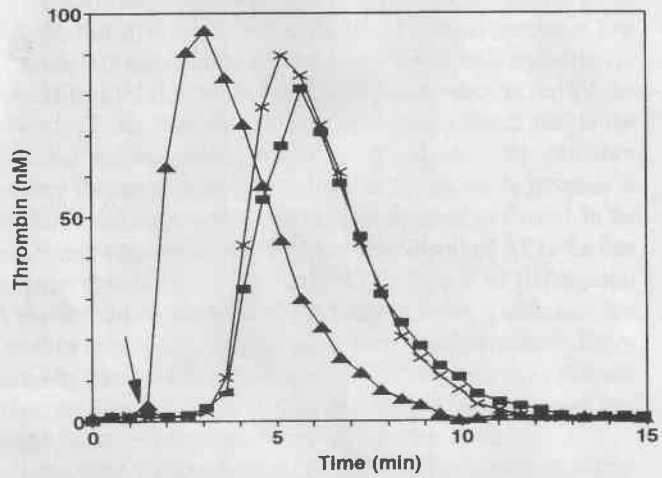
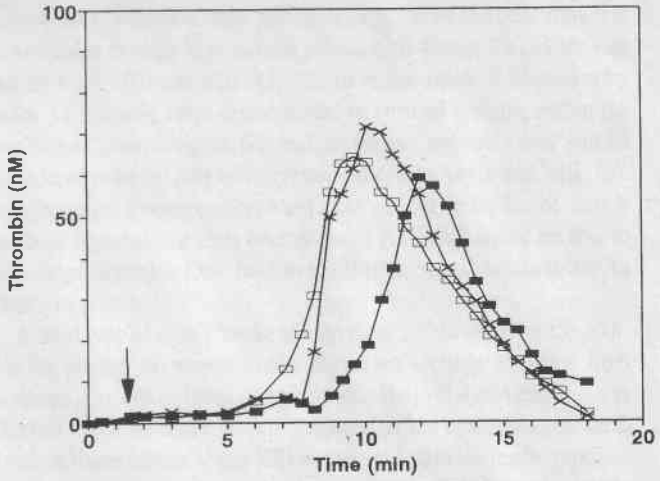


Fig. 5 Effect of plasma clots on intrinsic thrombin generation. Thrombin generation was measured in defibrinated normal pool plasma. It was triggered with Ca^{2+}, factor IXa and phospholipids (incomplete tenase) as described in the methods section. All additions were done at t = 90 s. Upper frame: (■) control; (▲) normal plasma clots added; (×) reptilase clots added. Lower frame: (■) control; (▲) addition of factor VIII, 10 µl of a 15 nM solution in 1 mM $CaCl_2$ in which 3 normal clots were incubated at 37° C for 1 min; (×) the same but factor VIII not incubated with clots



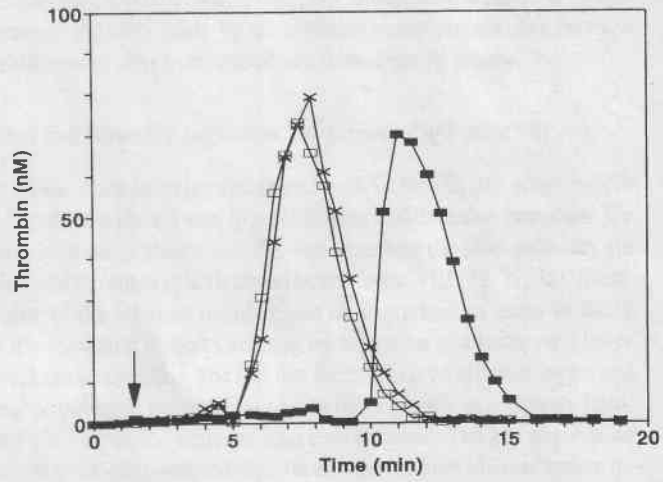


Fig. 6 Effect of clot addition on thrombin generation carried out in platelet rich plasma. Thrombin generation was triggered in PRP from a normal donor by addition of Ca^{2+} (16.7 mM). All additions were done at t = 90 s (arrow). Upper frame: (■) control; (□) normal plasma clots added; (×) addition of clots incubated in serum plus 0.1 U/ml of heparin. Lower frame: (■) control; (□) reptilase clots added; (×) addition of thrombin clots from plasma depleted in factors II, VII, IX and X

Table 3 Shortening of the lag time in intrinsic thrombin generation on different additions

Addition	Shortening of the lag phase (s)
None	NS
Clot from recalcified plasma	111–134
Clot by thrombin in depleted plasma	97–121
Snake venom clot	NS
Factor VIII	NS
Thrombin activated factor VIII	128–147
Factor VIII preincubated with normal clots	98–113
Factor VIII preincubated with reptilase clots	NS
Factor VIII preincubated with PPACK clots	NS
Factor VIII preincubated with last washing fluid	NS

The lagtimes were obtained from thrombin generation experiments as in Fig. 4. Each experiment was repeated 4 to 6 times. No statistics were done but the range of results is given. All additions were done at t = 1.5 min, NS indicates that the difference of lag time measured was less than the sampling interval, i. e. 30 s.

ty to shorten the lag time, as did thrombin activated factor VIII (Fig. 5, lower frame). The factor VIII preparation itself or buffer into which clots had been incubated as well as the factor VIII preparation incubated with reptilase clots were all inactive. Again clots incubated (1 h) in heparinised serum (0.1 U/ml) were as active as normal clots were.

Activation of Platelets by the Clot

In PRP triggered with Ca^{2+} there is a prolonged lag-phase to the thrombin burst that has been shown to be caused by the time required for the platelets to provide the necessary procoagulant phospholipid surface under influence of traces of thrombin formed (30). The addition of a clot again shortens this lag time of the thrombin burst, also when the clot has been incubated for 1 h in heparinised serum (Fig. 6, top). The interesting and unexpected finding however was that any clot, also clots formed by reptilase and Agihal, that are inactive in platelet free systems, shorten the lag phase in PRP like a thrombin induced clot does (Fig. 6, bottom). It thus seems that the presence of thrombin adsorbed

on the clot is not an essential requirement in the case of platelet activation, in other words that fibrin alone is capable to induce procoagulant activity in platelets.

Discussion

The fact that a clot, even hours after it has been formed, induces further coagulation of plasma, is known since the 19th century. In fact it was this observation that led Buchanan to postulate the existence of thrombin (13). The phenomenon is presumably not without importance for the pathophysiology of thrombosis, because it may well be a model of one of the mechanisms of in vivo thrombus growth. Thrombus growth is one of the major problems in the management of thrombosis, so clot induced coagulation and its inhibition by antithrombotic drugs deserves detailed attention.

That thrombin adsorbs onto fibrin during coagulation is known from the literature (see Introduction) and it also appears from Fig. 1, in which it is seen that, from the moment on that a clot forms the amount of free thrombin in triggered defibrinated plasma is significantly higher than in non-defibrinated (i.e. normal) plasma. In the presence of heparin the amounts of free thrombin detected are generally lower, due to the increased scavenging of thrombin by AT III. Also the difference between the curves in the presence and in the absence of thrombin is smaller, which means that less thrombin is adsorbed on the clot. In the presence of heparin the competition between fibrin and AT III for free thrombin evidently shifts in favour of AT III. Even if AT III (-heparin) is unable to inhibit thrombin that is bound to fibrin, it will cause less thrombin to be bound to fibrin during the coagulation process. Hypothetically this could be a beneficial effect of heparin prophylaxis and treatment. Our experiments confirm that heparin does not in any way inhibit the activity of thrombin once it is adsorbed to the clot.

From Table 1 it can be seen that the difference between thrombin generation in the presence and in the absence of fibrin(ogen) cannot be explained by a lack of prothrombin conversion. It is also seen in this table that the level of the main thrombin-inhibitor complexes (TAT and α_2M-thrombin) that form in the presence of fibrin are lower than in non-defibrinated plasma. Evidently clotting fibrinogen competes for free thrombin with the other physiological inhibitors in an irreversible process. This is also seen when heparin is present (Fig. 2). The question of how fibrin competes with antithrombin III in absence and in presence of heparin is at present under further investigation in our laboratory.

In order to investigate the action of a clot on the clotting system in more detail we prepared clots by recalcification of normal PPP in a glass tube ("normal clots"). By using the intrinsic pathway without adding an other trigger than recalcification we prevented the presence of exogenous procoagulant substances such as kaolin, phospholipids or tissue factor. In order to inhibit free thrombin, all clots were incubated for 10 to 30 min in serum, in some experiments spiked with heparin (0.1 U/ml). We observed that well washed clots that were left in their serum for 10 minutes or more never induced coagulation in plasma that was not recalcified. This in our opinion excludes the possibility that significant free thrombin is contained in our clots and it also shows that bound thrombin does not act on the great mass of fibrinogen in a solution. Probably the bound thrombin causes some fibrin formation at the clot-solute interface, otherwise the fibrinopeptide generation by washed clots (8–10) is difficult to explain.

We also made clots by addition of 100 nM of thrombin to plasma depleted of the vitamin K-dependent clotting factors by $Al(OH)_3$ adsorption. These clots ("depleted plasma clots") have been introduced because they will not contain factor Xa. This factor has been shown to be contained in clots from normal plasma (36). Factor Xa can activate factors V (39, 40) and VIII (41, 42), be it that factor V activation by factor Xa has only been demonstrated in purified systems and in the presence of phospholipids, and that, in plasma, thrombin may still be considered to be the only effective activator of factors V and VIII (19). It nevertheless was necessary to test clots without factor Xa, because it has been reported that clots contain factor Xa which would be able to activate prothrombin (36). In clots made of depleted plasma factor Xa cannot be present.

A third type of clots ("snake venom clots") were made by the addition to plasma of non-thrombin fibrinogen clotting enzymes from Bothrops Atrox (Reptilase) or Agkistrodon Halys Halys(Agihal). It was checked that both snake venom fractions, at the concentrations used, do not activate factors V and VIII or platelets. Also we made "reptilase clots" from plasma depleted of the vitamin K-dependent clotting factors by $Al(OH)_3$ adsorption. No differences were ever found between the clots made by the different snake venoms, nor between snake venom clots from normal and from depleted plasma.

Clots and Intrinsic Coagulation, the Activation of Factor VIII

In the intrinsic system triggered with factor IXa and phospholipid a lag-phase is always seen to precede a burst of thrombin formation. We have previously shown that this lag represents the time necessary for traces of thrombin to activate sufficient factor VIII (32, 37, 38). Shortening of the lag-time therefore can be interpreted in terms of factor VIIIa formation if short circuiting by activation of a factor on a lower level can be excluded. The fact that factor IXa is an efficient trigger and that no thrombin generation is seen before 12 min if only phospholipids and Ca^{2+} are added excludes such a short circuit. The key observation was that thrombin induced clots shorten the lag time whereas venom induced clots do not (Fig. 5, Table 2). The fact that clots from depleted plasma act like normal clots do, shows in addition that factor Xa is not likely to play a role in the shortening mechanism. As a positive control we added thrombin activated factor VIII, which shortened the lag-time whereas the same factor VIII preparation when not activated by thrombin did not. Thrombin clots, also when incubated in heparin containing serum or when produced by adding thrombin to depleted plasma, induced activity in the factor VIII preparation that was comparable to that caused by free thrombin.

Clots and Extrinsic Coagulation, the Activation of Factor V

It is an as yet unresolved problem why factor V activation does not cause a similar lag phase in extrinsic coagulation as factor VIII activation does in the intrinsic pathway. Diminished thrombin production, such as seen in orally anticoagulated plasma does induce such a lag-phase. Enhanced thrombin inactivation, such as induced by heparin does not. This is reflected by the well known fact that oral anticoagulation prolongs the thromboplastin time whereas heparin administration does not. This suggests that, in extrinsic thrombin generation, there exists a form of thrombin that is capable of activating factor V whereas it is not inhibited by ATIII-heparin. Meizothrombin has been shown to be insensitive to the action of heparin (43). It will remain bound to the phospholipid surface because it still contains the C-terminal part of prothrombin through which the molecule binds to negatively charged phospholipids. The feedback activation therefore could take place at the phospholipid surface. This problem is at present under investigation in our laboratory. The natural explanation of the existence of a lag time in

the intrinsic system then would be that factor VIII, unlike factor V, remains in the fluid phase, i.e. cannot enter the "compartment" because it is bound to von Willebrand factor.

To investigate the clot induced activation of factor V we had to induce a lag time in the extrinsic system. The addition of hirudin does cause such a lag-time in the extrinsic system, probably because hirudin does inhibit meizothrombin. It has been shown before (35) that this lag-phase is caused by retardation of thrombin generation and not by immediate inactivation of normally formed thrombin, we checked this previous result by incubating samples taken at the end of the lag-time with staphylocoagulase, they invariably developed an amidolytic activity of >85% of the mixture at t = 0, in accordance with the earlier results. We used factor VIII deficient plasma in order to rule out any side effects via the intrinsic pathway.

We found essentially the same results as with factor VIII and the intrinsic system: again thrombin induced clots shorten the lag time whereas venom induced clots do not. This in itself shows nothing more than enhancement of prothrombinase activity at an earlier moment than in the absence of a clot, which can in theory be due to any component of the prothrombinase complex. It is unlikely however that it should be due to factor X activation because it has been shown that under our experimental conditions, during the lag phase, factor Xa is present in large excess over factor Va (34). Also it is not likely that phospholipids are supplied by the clot (see above). Anyhow, in that case there would be no reason for the difference between thrombin induced and venom induced clots.

Elevating the concentration of factor V in plasma did not change the lag-time. However when the factor V preparation was incubated with the clots and then added to the plasma without the clots, marked shortening could be again observed.

The generation of factor Va by addition of a thrombin induced clot could be demonstrated in normal plasma and in plasma depleted of clotting factors II, VII, IX and X. The burst of factor Va production in normal plasma is in all probability secondary to thrombin generation, but factor Va generation in depleted plasma can only be explained by a direct action of the clot on factor V.

We conclude that the thrombin adsorbed on a clot during its formation but not the fibrin itself enhances prothrombinase activity via the activation of plasmatic factor V. Heparin is neither able to inhibit thrombin present on a clot nor to prevent thrombin to adsorb on a forming clot.

The Activation of Platelets

We have previously shown that thrombin generation in PRP is critically dependent upon the phospholipids exposed by thrombin activation of the platelets (30), in other words that the lag-time in clotting PRP is the time necessary for traces of thrombin to make the platelets expose procoagulant phospholipids. The negatively charged phospholipids, in practice mostly phosphatidyl serine, are normally practically exclusively present in the inner leaflet of the platelet plasma membrane and therefore not exposed to plasma. Bevers et al. have demonstrated that platelets dispose of a mechanism, thus far unique to these cells, that causes a transbilayer movement of the PS to the outside of the activated platelet, the "flip-flop" mechanism (22). Also is PS exposed in microvesicles shed by activated platelets (44, 45). It has been shown that in isolated platelets thrombin and collagen are each capable to induce the flip-flop reaction (in the presence of Ca^{2+}) but that the combination of the two is much more potent than each of the two alone (22). In PRP however we found that thrombin alone is capable to cause a maximal procoagulant effect and that the addition of collagen does not significantly increase the procoagulant response (30).

Rather surprisingly, all clots exhibited an activating influence on the platelets during thrombin generation in PRP, not only the thrombin induced clots but also those clots obtained by reptilase and Agihal that do not contain thrombin. This indicates that the flip-flop reaction of platelets, contrary to the activation of factors V and VIII is not obligatorily restricted to the presence of thrombin on the clot. One can surmise that fibrin has a similar type of action on platelets as collagen has. If this is true then fibrinogen would be a kind of cofactor to the flip-flop inducing action of thrombin in plasma. This hypothesis has to be substantiated by the demonstration of the effect of thrombin and/or fibrinogen and/or fibrin on the transbilayer movement in isolated platelets. Such experiments are on their way now in our laboratory.

In summary: our findings demonstrate that thrombin induced clots initiate coagulation in plasma by activation of plasma cofactor proteins. This may cause generation of more thrombin at the site of a thrombus and hence thrombus growth. The fact that heparin does not inhibit this process may limit the usefulness of heparin as an antithrombotic. In general, potential antithrombotics should be characterised as to their ability to inhibit these positive feedback reactions. Even more intriguing that the role of clot bound thrombin in the feedback activation of clotting factors is the activation of platelets by fibrin to which no thrombin can be adsorbed, i.e. the collagen like action of fibrin. This once more indicates how intricately interwoven clotting- and platelet reactions are. On a practical level it underlines the rationale of anticoagulation as antithrombotic therapy and suggests that inhibitors of fibrin-platelet interactions would make interesting antithrombotics.

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