Fibrinopeptide A Cleavage and Platelet Release in Whole Blood In Vitro

EFFECTS OF STIMULI, INHIBITORS, AND AGITATION

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ABSTRACT The relationship between platelet release and fibrinopeptide A cleavage from fibrinogen to form fibrin I in vitro was examined in blood allowed to clot undisturbed or with gentle agitation. In undisturbed or agitated blood platelet release and fibrin I formation occurred simultaneously. When hirudin was added to undisturbed blood it prevented platelet release as well as fibrin I formation. In contrast, hirudin added to agitated blood had little effect on platelet release despite complete inhibition of fibrin I formation. Collagen added to either undisturbed or agitated blood increased platelet release and then fibrin I formation, and ADP added to undisturbed blood caused an initial burst of platelet release followed by slight acceleration of fibrinopeptide A cleavage. Prostaglandin E\(_1\) and theophylline prevented platelet release in both undisturbed and agitated blood, but did not affect fibrin I formation. The results with inhibitors in agitated blood suggest that fibrin I formation and platelet release can occur independently in the presence of the increased interactions induced by agitation. Addition of thrombin or tissue thromboplastin to undisturbed blood accelerated fibrin I formation with little effect on platelet release. Finally, initial thrombin formation in undisturbed blood appeared to be associated with the platelet surface. These relationships suggest that thrombin formation via the intrinsic system leads to thrombin generation on the platelet surface and simultaneous platelet release and fibrin I formation, while thrombin generated via tissue thromboplastin leads to thrombin formation in the plasma and fibrin I formation preceding platelet release. Activation by interaction of blood with collagen causes initial acceleration of platelet release and later acceleration of fibrin I formation.

INTRODUCTION

Knowledge of the interrelationship of platelet activation and fibrin formation in normal hemostasis and intravascular coagulation or thrombosis is important in understanding the mechanisms by which hemostasis and thrombosis occur. Earlier attempts to examine this interrelationship clinically used measurements of platelet and fibrinogen survival and turnover in various thrombotic disorders (1). These studies provided information about the predominance of platelet activation or fibrin formation in those disorders over periods of several days but the techniques did not allow the investigators to study the time sequence of platelet activation and fibrin formation in the initial phases of hemostasis and thrombosis. The development of sensitive and specific radioimmunoassays for \(\beta\)-thromboglobulin (\(\beta\)TG)\(^1\) (2–4) and platelet factor 4 (PF4) (3–8), two platelet-specific proteins secreted during the platelet release reaction (2, 3, 5, 9), and for fibrinopeptide A (FPA) (10), the first product of thrombin action on fibrinogen, allows a detailed study of two specific events that occur during blood clotting in vitro and also allows analysis of these molecular events during intravascular coagulation in vivo (11).

Previous studies from this laboratory have demonstrated that when thrombin is added to citrated plasma or to whole blood without anticoagulant, fibrinogen cleavage can be detected at thrombin concentrations 100 times lower than those required to induce detectable platelet release (3), suggesting that added throm-

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\(^1\)Abbreviations used in this paper: \(\beta\)TG, \(\beta\)-thromboglobulin; PF4, platelet factor 4; FPA, fibrinopeptide A; PGE\(_n\), prostaglandin \(E_n\); PPP, platelet-poor plasma; PRP, platelet-rich plasma.
bin preferentially cleaves fibrinogen. In contrast, when blood is allowed to clot at 37°C in plastic tubes, fibrinogen cleavage and platelet release are detected at the same time (12). Similarly, Shuman and Levine (13) reported that thrombin generation, as determined by radioimmunoassay, and PF4 release occurred in parallel in normal blood allowed to clot in vitro; Rybak and colleagues (14) have shown that the rate of prothrombin activation is paralleled by the rate of PF4 secretion in normal blood in vitro (14); and Prowse and colleagues (15) have found that βTG release occurs at the same time as FPA cleavage and PF4 release in normal blood in vitro. These studies suggested that thrombin generated in whole blood may actually be formed on the platelet surface and thus stimulate platelet release simultaneously with fibrinogen cleavage. Milewich et al. (16), Kane et al. (17), and Tracy et al. (32) have demonstrated that coagulation Factor Xa can bind to the surface of platelets and thereby dramatically accelerate the rate of Xa cleavage of prothrombin to generate thrombin (16, 17). It would not be unreasonable to anticipate that Xa bound to the platelet surface might convert prothrombin on the same surface.

This paper reports the results of studies designed to examine the relationship between platelet release and fibrinopeptide A cleavage from fibrinogen to form fibrin I under various conditions in vitro and presents evidence that initial thrombin formation occurs in association with the platelet surface during spontaneous clotting but in the plasma when tissue thromboplastin is present in the blood.

**METHODS**

Blood was collected from healthy laboratory personnel who had not taken aspirin or other platelet-inhibitory drugs for at least 10 d before venipuncture. Blood was collected through 19-gauge butterfly needles into plastic syringes that contained either saline or one of the following materials: ADP (ICN Nutritional Biochemicals, Cleveland, Ohio), prostaglandin E1; (PGE1, a gift from Upjohn Co., Kalamazoo, Mich.) and theophylline (Sigma Chemical Co., St. Louis, Mo.), collagen (Horn Chemie, Munich, West Germany), hirudin (Koch-Light Laboratories, Ltd., Bucks, England), purified human alpha thrombin 2,800 U/mg (a gift of Dr. John Fenton, II), or Diagen rabbit brain thromboplastin (Diagnostic Reagents, Ltd., Oxon, England) reconstituted with 5 ml normal saline and diluted with normal saline with 3.5 g/liter bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.).

The blood was mixed gently with the additive in the syringe and then dispersed into plastic tubes and incubated at 37°C for different time intervals. Incubation was timed from entry of blood into the syringe. In some experiments the incubation was carried out in a stationary water bath and in others the tubes were placed on a rocker which inverted them 18 times per minute (Ames aliquot mixer, Miles Laboratories Inc., Elkhart, Ind.) in an incubator. At the end of the incubation time for each tube 1/10 vol of a mixture of heparin (50 or 1,000 U/ml) 4% citrate, 0.01 M adenosine (Nutritional Biochemicals Corp.), and 0.02 M theophylline was added, the blood was mixed with this anticoagulant, and the tube was placed on ice for up to 30 min until further processing. Samples were processed as described and stored frozen for radioimmunoassay for βTG and PF4 (3, 9). An aliquot of the high speed centrifugation supernatant plasma was taken for processing and assay for FPA. 1 ml plasma was mixed with 40 mg benzentone (lab grade, Fisher Scientific Co., Pittsburgh, Pa.), and 0.05 ml Tris-buffered saline (0.1 M NaCl, 0.05 M Tris), pH 8.9, containing 0.1% ovalbumin and 0.02% sodium azide and the suspension was incubated for 10 min at 22°C on a rocker (Ames aliquot mixer) and then centrifuged at 4,900 g for 10 min at 4°C. The supernatant plasma-buffer mixture was removed and stored frozen until radioimmunoassay for FPA (10) was performed. Reagents kindly provided by Dr. H. L. Nossel. In each experiment an additional 1.8 ml blood was collected and mixed with 1/10 vol 4% citrate for determination of platelet count by phase-contrast microscopy and for determination of plasma fibrinogen concentration (18). In some experiments a further 3 ml of blood was incubated at 37°C without stirring for 1 h in a 12 × 75 mm glass clot. Clot formation and retraction occurred and this sample provided an index of total releasable βTG and PF4.

For experiments in which thrombin antigen was measured, blood tubes were incubated at 37°C in a water bath and at the end of each incubation time, 1/10 vol of anticoagulant (4% citrate, 0.02 M theophylline, 1.04 μM PGE1) was added, the tube was placed on ice, 1/100 vol of 1.0 M diisopropyl fluorophosphate (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) in isopropanol was added, and the tube was incubated on ice for 15 min. 1/50 vol of 0.5 M benzamidene (Sigma Chemical Co.) in saline was then added. The tube of blood was centrifuged at 160 g at 4°C and the platelet-rich plasma (PRP) was removed. An aliquot of this PRP was solubilized (1% Triton X-100) for thrombin, and the remainder was centrifuged twice as for βTG and PF4 assay (3, 9) and the supernate then assayed for thrombin.

Radioimmunoassay of thrombin was performed essentially as described by Shuman and Majerus (19). The procedure for radiolabeling purified human alpha thrombin, the thrombin assay buffer, the IgG fraction of anti-human thrombin (a gift from Dr. Majerus) and the incubation times for the assay were as described (19). Goat anti-rabbit IgG was purchased from N. L. Cappel Laboratories, Inc., Cochranville, Pa. Nonimmune rabbit immunoglobulin was prepared from normal rabbit serum (Antibodies Inc., Davis, Calif.) by adsorption with 100 mg/ml BaSO4 and heating the adsorbed serum to 60°C for 30 min. The immunoglobulin was then precipitated with 15% Na2SO4 (final concentration). Thrombin-free control plasma was prepared from plasma collected as described (19) and adsorbed with 200 mg/ml bentonite for 10 min at 32°C, centrifuged at 4,900 g for 10 min, and stored frozen. Bentonite adsorption removed more than 99% of the radioactivity of 125I-thrombin added to plasma. The volumes of reagents used in the radioimmunoassay were 100 μl of thrombin-free control plasma containing dilutions of standard diisopropylthrombin or test plasma, 20 μl of anti-human thrombin, 20 μl of nonimmune rabbit immunoglobulin, 30 μl of [125I]diisopropylthrombin, and 30 μl goat anti-rabbit IgG.

Statistical analyses were performed using the t test for differences between means for comparison of ADP and PGE1-theyophylline data with saline data and the paired t test for comparison of the thrombin content of platelet-poor plasma (PPP) and PRP. To compare the two sets of data for collagen incubation with blood with the mean data for saline incubation with blood, a modification of the test for comparison of two means was used and the P values for the two sets of collagen data were then combined to give a P value for the difference between collagen and saline data (20).
RESULTS

Contribution of thrombin formation to platelet release during incubation without agitation. The time-courses of FPA generation and release of βTG and PF4 for control incubations and incubation with hirudin are shown in Fig. 1. FPA, βTG, and PF4 levels in the plasma in the control incubations changed minimally during the first 3 min of incubation, increased slightly between 3 and 6 min, and then increased more rapidly. The level of FPA at 30 min in the controls represents ~0.3% of the total releasable FPA calculated from the plasma fibrinogen concentration and the levels of βTG and PF4 represent ~1.7 and 1.5%, respectively of total releasable βTG and PF4 based on the values obtained by radioimmunoassay of blood clotted in glass tubes. Hirudin at 20 U/ml plasma (Fig. 1) prevented cleavage of FPA from fibrinogen during the first 15 min of incubation and markedly inhibited cleavage between 15 and 60 min. βTG release was essentially blocked for 60 min. There was a small amount of PF4 release in the first 6 min in the presence of hirudin, but no increase from 6 to 60 min. Hirudin was also tested at 1 and 5 U/ml with little inhibition of FPA cleavage or α-granule release with 1 U/ml and moderate inhibition of FPA cleavage and α-granule release with 5 U/ml.

Contribution of platelet release to thrombin formation during incubation without agitation. Fig. 2 shows the time-courses of generation of FPA and release of βTG and PF4 in blood mixed with collagen in the syringe to stimulate platelet release, in blood mixed with saline in the syringe (control), or with PGE1 and theophylline in the syringe to inhibit platelet release. Addition of collagen to the blood increased platelet release over the 12-min period. FPA formation was increased at 6, 9, and 12 min compared with the saline control, but was not elevated at 2 min. The differences between FPA cleavage with collagen and with saline were significant with P = 5.46 x 10⁻² at 6 min, 9.97 x 10⁻³ at 9 min and 2.0 x 10⁻⁴ at 12 min. The combination of PGE1 and theophylline blocked release of βTG and PF4 but did not affect cleavage of FPA from fibrinogen during the 12 min incubation.

The effects of incubating blood with 5 μM ADP are shown in Table I. By 2 min there was a 10- to 20-fold increase in βTG and PF4 levels compared with the levels in blood incubated with saline, but the subsequent increases in βTG and PF4 levels with ADP were small (1½- to 2-fold). FPA levels were higher with ADP than with saline at 6, 9, and 12 min incubation but the differences were not significant at the 5% level. When the experiment was repeated with 50 μM ADP, βTG, and PF4 levels showed a further increase (15.3±2.0 and 18.2±2.6 pmol/ml, respectively, at 2 min and 27.1±4.2 and 34.4±5.1 pmol/ml at 12 min, n = 4). Mean FPA levels with 50 μM ADP were similar to the control values at 2 min and approximately three times the control values at 6, 9, and 12 min, but again the differences were not statistically significant.
Platelet release and thrombin action on fibrinogen during incubation with agitation. In Fig. 3 the time-course of thrombin cleavage of fibrinogen is compared with the time-course of release of βTG and PF4 in tubes agitated during incubation. Agitation markedly accelerated both fibrinogen cleavage and platelet release compared with the controls shown in Figs. 1, 2, and 4. To try to define whether the mechanism of this acceleration was increased activation of plasma coagulation by surface contact or increased platelet release mediated by the agitation, incubations with agitation were performed with the addition of hirudin or with the addition of the combination of PGE1 and theophylline. Hirudin blocked fibrinogen cleavage by thrombin but only slowed platelet release somewhat. The combination of PGE1 and theophylline blocked platelet release for ~9 min but did not affect the time or rate of FPA increase. Addition of collagen to agitated blood accelerated both platelet release and fibrinogen cleavage and as with the stationary incubation, acceleration of platelet release was evident before acceleration of fibrinogen cleavage.

**Effect of addition of thrombin or tissue thromboplastin to undisturbed blood.** In the experiments described thus far, in both undisturbed and agitated blood, thrombin-induced cleavage of fibrinogen and platelet release began simultaneously in the absence of stimulators or inhibitors of either reaction. It is possible that thrombin was generated through the intrinsic pathway in these studies. Previous studies from this laboratory suggested that thrombin added exogenously might behave differently from that generated through the intrinsic pathway, so the time-courses of FPA cleavage and platelet release were studied after the addition of a low concentration of thrombin to blood without anticoagulant. The effect of thrombin generated through the extrinsic pathway by addition of dilute tissue thromboplastin was also studied and the results are shown in Fig. 4. FPA cleavage was accelerated by both added thrombin and added tissue thromboplastin (Fig. 4, left), whereas βTG and PF4 release showed little difference from the control (Fig. 4 middle and right). By 15 min incubation the increases in FPA cleavage with thrombin and tissue thromboplastin were small.

**Association of thrombin with platelets in spontaneously clotting blood.** The distinct difference between the patterns of FPA generation and platelet release seen with added thrombin or tissue thromboplastin and those seen with thrombin generated spontaneously suggested that in the latter case thrombin was actually generated on the platelet surface so that it would have equal access to plasma fibrinogen

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**Table I**

<table>
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<th>Incubation</th>
<th>FPA</th>
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<td>ADP</td>
<td>Saline</td>
</tr>
<tr>
<td>min</td>
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<td>pmol/ml</td>
<td>pmol/ml</td>
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<td>6.32±1.72</td>
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FIGURE 3  Time-course of increase in plasma level of FPA, βTG, and PF4 in blood incubated on an Ames aliquot mixer at 37°C. At the indicated time 1/10 vol of anticoagulant solution containing 1,000 U/ml heparin, 4% citrate, 0.01 M adenosine, and 0.02 M theophylline was mixed with the blood and the tube was placed in an ice bath. ○, Control, no addition to syringe; ○, PGE₂ and theophylline, at 260 nM and 5 mM in plasma, respectively; ▲, hirudin, 50 U/ml in plasma; Δ, collagen, μg/ml in plasma.

FIGURE 4  Time-course of increase in plasma level of FPA, βTG, and PF4 in blood incubated undisturbed at 37°C and the effect of added thrombin or tissue thromboplastin. At the indicated time 1/10 vol of an anticoagulant solution containing 1,000 U/ml heparin, 4% citrate, 0.01 M adenosine, and 0.02 M theophylline was mixed with the blood and the tube was placed in an ice bath. ○, Tissue thromboplastin (mean of two experiments), 1/1,500,000 dilution; ×, thrombin 0.001 U/ml (mean of three experiments).
and to the platelet thrombin receptors for induction of the release reaction in contrast to generation in the plasma with added thrombin or tissue thromboplastin. This hypothesis was tested by assaying both PRP and platelet-free plasma for thrombin content after a 2-min incubation of whole blood with saline. The results for eight experiments are shown in Table II. At 2 min there was slightly, but significantly, more thrombin assayed in PRP than in PPP. In two experiments in which blood was incubated for 6 min, there was also a significant difference between PPP and PRP, whereas at later times (15 and 30 min), no differences were found. When dilute tissue thromboplastin (1/1,500,000 dilution) was added to the blood there was no difference in the thrombin content of PRP as compared with PPP at 2, 6, 15, or 30 min.

**DISCUSSION**

Thrombin generation on the platelet surface is first suggested by the initial experiments, shown in Fig. 1, in which it was demonstrated that FPA formation and platelet α-granule protein release followed parallel time-courses. Similar parallel time-courses have been reported by other workers who measured thrombin antigen and PF4 secretion (13), prothrombin activation fragment and PF4 secretion (14), or FPA formation and PF4 and βTG secretion (15). All of the above studies in which thrombin was generated in the blood are in contrast to previous studies from this laboratory in which thrombin was added to whole blood and which showed FPA cleavage at thrombin concentrations at least 100-fold lower than those which caused βTG and PF4 release (3). The present results suggest that thrombin generated in the blood has equal access to plasma fibrinogen for fibrin formation and to platelets for induction of the release reaction in contrast to added thrombin which preferentially cleaves fibrinogen. Platelet release in this undisturbed system is entirely dependent on thrombin action since blocking thrombin action with hirudin completely prevents platelet release. Shuman and Levine (21) have reported that PF4 release in whole blood is delayed in the presence of hirudin.

Platelet release and fibrin formation were not parallel when ADP or collagen was added to stimulate release. Collagen markedly increased release of βTG and PF4 at the earliest time tested (2 min) as compared with release in the presence of saline while FPA acceleration occurred later (at 6, 9, and 12 min). Two potential mechanisms by which the acceleration of FPA cleavage might occur are activation of the contact activation system by collagen (22) and release of platelet Factor V by collagen (23, 24). Although release of Factor V from platelets was not examined in the present study, it is likely that it occurred in parallel with release of βTG and PF4 (23, 24). Released platelet Factor V could be activated by low levels of thrombin formed in the first few minutes of incubation and the Va could then provide a binding site for Xa (17) and accelerate further thrombin formation. This would be consistent with the finding of acceleration of thrombin formation at 6–12 min but not at 2 min.

When blood was drawn into syringes that contained ADP there was an initial rapid release of βTG and PF4 in the first 2 min compared with the controls but over the next 10 min the βTG and PF4 concentrations approximately doubled in comparison to five- to sevenfold increases with collagen, perhaps due to destruction of the ADP in the blood. Several workers have reported that ADP does not induce platelet release at physiologic Ca2+ concentrations (25–27) as were present in the blood in these experiments. However the initial contact of blood with local very high ADP concentrations in the blood collection syringe does appear to lead to platelet alpha granule release. The exact mechanism of this release remains to be determined. Despite the dramatic increases in platelet release seen with ADP, the increases in FPA cleavage were small and not statistically significant although they were consistently present. These data are consistent with the hypothesis that increased platelet release contributes to increased thrombin formation although the increment contributed by platelet release is clearly small.

With PGE1 and theophylline, initial levels of βTG and PF4 were lower than those in the saline controls and there was little change over the 12-min incubation period. FPA levels in the presence of PGE1 and theophylline were not different from the controls. This is similar to the report by Shuman and Levine who did not find a difference in thrombin formation in clotting blood in the presence of prostacyclin although PF4 secretion was delayed (21). The failure of PGE1 and theophylline to influence cleavage of FPA from fibrinogen suggests that the relatively small amount of

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**TABLE II**

<table>
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<th>Experiment</th>
<th>PPP</th>
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<tr>
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Incubation time = 2 min. *P < 0.05, paired t test.*
release (1–2 pmol/ml) that occurs in the control state, i.e., with saline, is unimportant in determining the rate of thrombin formation.

Agitation of the blood increased both fibrin I formation and platelet release immediately so that both were complete by 12 min. Mixing must increase the interaction of Factor XII and the polystyrene tube surface, the interaction of platelets with the tube surface, and the interaction of platelets with other cells and any or all of these increased interactions might be responsible for the dramatic acceleration in rates observed. Additionally, mixing decreases regional differences in concentration of secreted platelet proteins and FPA and thus may permit better sampling. When hirudin was present during agitation, the FPA curve was essentially flat from 2 to 15 min although the 2-min level was elevated compared with undisturbed blood. Despite the flat FPA curve βTG and PF4 release occurred at a rate close to the control rate although the actual levels were lower at all times. Thus the increased interactions between platelets and surface and platelets and cells in agitated blood can induce the release reaction in the absence of thrombin action. Addition of PGE1 and theophylline to agitated blood caused only brief inhibition of platelet release, apparently because the rapid generation of thrombin overcame the inhibitory effect of the PGE1 and theophylline, consistent with previous observations that high concentrations of thrombin can induce release even in the presence of elevated platelet cyclic AMP levels (28, 29).

During the period when platelet release was inhibited, fibrin formation occurred at a normal rate reflecting lack of inhibition of thrombin generation. With the rapid thrombin formation that occurs in the agitated system, the increment in thrombin formation rate that can be provided by platelets appears to be insignificant.

When thrombin was added directly to blood or when it was generated by the extrinsic pathway by addition of tissue thromboplastin there was a marked disparity on its effects on fibrin formation and platelet release, with immediate acceleration of fibrin formation but little effect on platelet release. This is compatible with the previously reported findings from this laboratory that a much lower concentration of exogenous thrombin was needed in whole blood to cause FPA cleavage than to cause platelet release (3). These results suggest that thrombin generated by the extrinsic system is not formed on the platelets, but in the plasma, perhaps on the surface of the lipid contained in the thromboplastin preparation. Like exogenous thrombin, the thrombin generated by the extrinsic system acts more readily on plasma fibrinogen than on platelets.

Finally, experiments were undertaken in an attempt to confirm the hypothesis that thrombin generated by the intrinsic system is formed on the platelet surface. It was found that the thrombin concentration in PRP was slightly greater than that in high-spun PPP at the early incubation times, suggesting association of thrombin with the platelets. The differences between PPP and PRP in these experiments are at the limits of detection of differences by radioimmunoassay and thus, although suggestive, the data do not prove the hypothesis. The increment seen is consistent, however, with the report by Martin et al. (30) that at low concentrations of thrombin, 90% of the total thrombin is free in solution with 10% bound to the platelet surface. In the experiments with added tissue thromboplastin there was no difference between PPP and PRP, compatible with thrombin generation independent of the platelet surface in this situation. The concentrations of thrombin found in the plasma in these experiments are similar to those reported by Shuman and Majerus (19) and by Shuman and Levine (13, 21). They are, however, 400-fold higher than the concentrations of thrombin which would be calculated to be required to generate FPA at the rate seen in the control experiments reported here, based on studies of FPA generation after addition of thrombin to whole blood (31). The reason for this difference is not readily apparent, and resolution of this question may require the development of new methods for quantitation of small quantities of thrombin.

In conclusion, it appears that the two pathways by which thrombin can be formed lead to different patterns of platelet release. The intrinsic pathway leads to thrombin formation in association with the platelet membrane and the extrinsic or tissue thromboplastin mediated system leads to thrombin formation in the plasma. Thrombin generated by the intrinsic pathway would be expected to stimulate platelet release and fibrin formation simultaneously, whereas thrombin generated via tissue-thromboplastin mediated reactions would be expected to cause fibrin formation before platelet release. Finally, in situations where thrombosis occurs in areas of endothelial loss, platelet release stimulated by subendothelial collagen would be expected to precede evidence of fibrin formation.

ACKNOWLEDGMENTS

The authors wish to acknowledge the excellent technical assistance of Suzanne Rose.

This work was supported by research grants from the National Institutes of Health (HL-15486, HL-21006). Dr. K. L. Kaplan is a Senior Investigator of the New York Heart Association.

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