Fibrinogen-Independent Aggregation and Deaggregation of Human Platelets: Studies in Two Afibrinogenemic Patients

By M. Cattaneo, R.L. Kinlough-Rathbone, A. Lecchi, C. Bevilacqua, M.A. Packham, and J.F. Mustard

Platelets from two afibrinogenemic patients were used to determine whether fibrinogen is essential for platelet aggregation and to examine whether released fibrinogen contributes to the stabilization of platelet aggregates when platelets have been induced to aggregate and release their granule contents by stimulation with thrombin. The addition of adenosine diphosphate (ADP) to platelet-rich plasma (PRP) or to suspensions of washed platelets from the afibrinogenemic patients caused the formation of small aggregates, which was either not inhibited or only slightly inhibited by the F(ab')2 fragments of an antibody to fibrinogen but was inhibited by an antibody (10E5) to glycoprotein IIb/IIIa. Thus there is a component of ADP-induced platelet aggregation that is not dependent on fibrinogen or other plasma proteins but is dependent on glycoprotein IIb/IIIa.

Fibrinogen is believed to be important in human platelet aggregation because platelets from patients with afibrinogenemia aggregate poorly in response to adenosine diphosphate (ADP), and washed human platelets also aggregate poorly in response to ADP if they are suspended in a medium without added fibrinogen. In addition, thrombasthenic platelets that lack the fibrinogen receptor glycoprotein IIb/IIIa do not aggregate in response to any agonist and antibodies directed against glycoprotein IIb/IIIa inhibit platelet aggregation and the binding of fibrinogen. However, despite these observations it is not known whether fibrinogen is essential for platelet aggregation. Several observations indicate that a small extent of ADP-induced aggregation occurs without the addition of fibrinogen to suspensions of washed human platelets in a medium containing 2 mmol/L Ca2+ and 1 mmol/L Mg2+, in which ADP does not cause appreciable release of platelet granule contents, and in which suspensions of washed platelets from rabbits aggregate extensively in response to ADP without the addition of fibrinogen to their suspending medium.

When aggregation is induced by agents that cause the release of granule contents, released fibrinogen is thought to be responsible for aggregation. Tollesen and Majerus reported that Fab fragments of an antibody to fibrinogen inhibited thrombin-induced aggregation. However, although Legrand et al. also found that Fab fragments of an antibody to fibrinogen were able to partially inhibit platelet aggregation caused by very low concentrations of thrombin, high concentrations were not inhibited. Further evidence that aggregation can occur without fibrinogen includes the observations of Turitto et al. who have shown that platelets from the native blood of an afibrinogenemic patient form large aggregates in a Baumgartner flow chamber, and the studies of Soria et al., showing that washed platelets from afibrinogenemic subjects aggregate normally in response to thrombin.

It has not been established whether fibrinogen plays a major role in preventing platelet deaggregation. When platelets aggregate without releasing granule contents, deaggregation occurs readily, and the fibrinogen that has been bound during aggregation readily dissociates. In contrast, when the release of granule contents takes place, platelets do not deaggregate readily, and even when they are induced to deaggregate, only some of the bound fibrinogen dissociates.

There was little difference in the extent of aggregation and the release of granule contents of normal and afibrinogenemic platelets in response to the release-inducing agents collagen, platelet-activating factor (PAF), sodium arachidonate, or thrombin. With normal or afibrinogenemic platelets, aggregation by thrombin (0.2 U/mL or higher) was not inhibited by the Fab'1 fragments of an antibody to human fibrinogen. Deaggregation by combinations of inhibitors of platelets aggregated by 1 U/mL thrombin showed no difference between platelets from afibrinogenemic and control subjects, indicating that released fibrinogen does not make a major contribution to the stabilization of platelet aggregates formed by thrombin stimulation.

MATERIALS AND METHODS

Materials. 14C-serotonin (14C-5-hydroxytryptamine-3'-creatinine sulfate, 50 mCi/mmol) was from Amersham Corporation, Arlington Heights, Ill. Bovine serum albumin, Pentex fraction V, was from Miles Laboratories, Elkhart, IN. Apyrase was prepared from potatoes by the method of Molnar and Lorand. The activity of this preparation in the final platelet-suspending medium was such that the enzyme (1 μL/mL) converted 0.25 μmol/L adenosine triphosphate (ATP) to adenosine monophosphate (AMP) within 120 seconds at 37°C. Human fibrinogen (grade L, AB Kabi, Stockholm, Sweden) was pretreated with diisopropylfluorophosphate (DFP, Sigma Chemical Co, St. Louis) and was partially purified by the method of Lawrie et al. Chymotrypsin, ADP, collagen, and sodium arachidonate were from Sigma. Hirudin was from Pentapharma (Basel, Switzerland). Synthetic platelet-activating factor (PAF, from the Centro Emofilia E Trombosi, Ospedale Policlinico, Milano, Italy; the Department of Pathology, McMaster University, Hamilton, Ontario; and the Department of Biochemistry, University of Toronto, Ontario.

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1-0-octadecyl-2-acetyl-sn-glyceryl-3-phosphoryl-choline) was from Bachem Feinchemikalien (Bubendorf, Switzerland). Thrombin was from Miles and PGE, from the Upjohn Company (Kalamazoo, MI). A monoclonal antibody (MoAb) to GPIIb/IIa (10E5) was kindly provided by Dr Barry Coller (Stony Brook, NY) and was dialyzed against 0.14 mol/L NaCl solution before use. F(ab')2 fragments of an antibody to human fibrinogen raised in goats were from Cappel (Malvern, PA); F(ab')2 fragments of nonimmune IgG were from the same source. Sodium arachidonate and PGE, were prepared as previously described.\textsuperscript{38,39} Acid-soluble collagen was prepared by the method of Cazenave et al.\textsuperscript{40} All other solutions were dissolved in modified Tyrode's solution. Citrated platelet-rich plasma (PRP) and suspensions of washed human platelets were prepared as described previously.\textsuperscript{34,41}

Aggregation and release. Platelet aggregation and the release of \textsuperscript{14}C-serotonin from prelabeled platelets was measured as described previously.\textsuperscript{41,42} Release of ATP from platelets was measured in a lumi-aggregometer (Chronolog, Haverton, PA).

Platelet count. The platelet count in PRP was adjusted to 350,000/\muL; in suspensions of washed platelets it was 500,000/\muL.

Fibrinogen assay. Platelet and plasma fibrinogen were measured using a fibrinopeptide A assay (FPA) kit (Mallinckrodt, St. Louis). FPA was freed from the fibrinogen by incubation with thrombin (4 U/mL) for two hours at 37°C. For measurement of platelet fibrinogen, platelets were treated with Triton X100, and the lysate was incubated with thrombin.

Characteristics of patients. Both patients with afibrinogenemia were young females (18 and 19 years of age) with a history of bleeding episodes dating from early childhood. Their bleeding times were prolonged (12 minutes and 18 minutes respectively, control 2.5 minutes) and both the prothrombin times and partial thromboplastin times were greater than 600 seconds (controls 13 seconds and 28 seconds respectively). Neither patient had detectable amounts of plasma fibrinogen, as measured by the fibrin polymerization time (Bonomelli, Milan, Italy).

RESULTS

Fibrinogen concentrations. The concentrations of fibrinogen in the platelets of the afibrinogenemics and normal subjects are shown in Table 1. Both patients had plasma fibrinogen concentrations of approximately 3 \mug/mL.

Citrated PRP. The addition of ADP to citrated PRP prepared from the blood of the two afibrinogenemic patients caused the formation of small platelet aggregates; the addition of fibrinogen enhanced the response (Fig 1). Collagen, PAF, or sodium arachidonate induced more extensive aggregation of afibrinogenemic platelets, although the extent of aggregation was somewhat less than in PRP prepared from normal subjects (Fig 2). Aggregation of the afibrinogenemic platelets in response to arachidonate occurred after a lag phase, whereas normal platelets aggregated more rapidly. The amount of ATP released in response to the agonists was similar to or less than the amount released from normal platelets under the conditions used (Fig 2).

Table 1. Platelet Fibrinogen Concentrations in Patients With Afibrinogenemia and in Control Subjects

<table>
<thead>
<tr>
<th>Subjects</th>
<th>n</th>
<th>Platelet Fibrinogen \mu g/10^{12} Platelets</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient I</td>
<td>1</td>
<td>14.2</td>
</tr>
<tr>
<td>Patient II</td>
<td>3</td>
<td>8.6 ± 2.1</td>
</tr>
<tr>
<td>Controls</td>
<td>9</td>
<td>312.0 ± 17.0</td>
</tr>
</tbody>
</table>

Mean values ± SEM.

To investigate the possible contribution to the extent of ADP-induced aggregation of the small amount of fibrinogen in afibrinogenemic plasma, the effect of F(ab')2 fragments of an antibody to human fibrinogen was investigated. There was no inhibitory effect on aggregation induced by 2 \mu mol/L ADP in comparison with control samples to which nonimmune F(ab')2 fragments had been added; aggregation curves with the nonimmune fragments were superimposable on aggregation curves to which no F(ab')2 fragments were added (Fig 3A). Addition of 8 \mu g/mL of fibrinogen to afibrinogenemic PRP enhanced aggregation to some extent, and this enhancement was eliminated by the F(ab')2 fragments of the antibody to fibrinogen (Fig 3B). Again, the aggregation curve obtained with nonimmune control F(ab')2 fragments was superimposable on the aggregation curve obtained with only 8 \mu g/mL fibrinogen added before the ADP.

Suspensions of washed platelets. For these experiments the platelets were resuspended in Tyrode-albumin solution, which is a balanced salt solution containing 2 mmol/L...
PLATELET AGGREGATION WITHOUT FIBRINOGEN

Fig 3. Platelet aggregation induced by ADP (2 µmol/L) in citrated PRP obtained from patient II. (A) Comparison of the effect of F(ab')2 fragments of an antibody to fibrinogen (F(ab')2 AF: 220 µg/mL) with F(ab')2 fragments of nonimmune IgG (F(ab')2 NI: 250 µg/mL). The aggregation curve obtained without the addition of F(ab')2 fragments was superimposable on the F(ab')2 NI curve. Tyrode's solution (TYR) was added to maintain constant volumes. The additions to control samples to maintain constant volumes. The additions to control samples were made at the points indicated by the arrows. The concentration of ADP used was 10 µmol/L. ADP but had no effect on the shape change of the platelets.

Calcium, 1 mmol/L magnesium, 0.35% albumin and apyrase. In this medium ADP in the presence of fibrinogen causes only the primary phase of aggregation of normal platelets, regardless of the concentration of ADP used.10-14; thromboxane formation does not occur, and there is little or no release of the contents of the platelet storage granules.33

Figure 4 shows that ADP caused washed platelets from both an afibrinogenemic patient and from a normal subject to aggregate to a small extent. Microscopic examination confirmed that some of the platelets were present as small aggregates. The addition of fibrinogen before ADP enhanced the aggregation response and resulted in the formation of large aggregates (Fig 4). In all cases aggregation was followed by deaggregation.

With both the normal and afibrinogenemic platelets the small amount of aggregation that was observed in the absence of fibrinogen was either not inhibited or only slightly inhibited by the F(ab')2 fragments of an antibody to fibrinogen that completely inhibited the potentiation of aggregation caused by the addition of fibrinogen (3 to 11 µg/mL) (Fig 5). (Control experiments were done with nonimmune F(ab')2 fragments.) However, an antibody to glycoprotein IIb/IIIa (10E5) at a final concentration of 10 µg/mL completely inhibited platelet aggregation induced by 10 to 20 µmol/L ADP but had no effect on the shape change of the platelets.

There was little difference in the extent of aggregation and the release of granule contents of normal and afibrinogenemic platelets in response to thrombin (0.2 to 1.0 U/mL; Fig 6). In these experiments the addition of hirudin two minutes after thrombin did not lead to deaggregation of the platelets from either the normal or afibrinogenemic patient (data not shown). Aggregation by thrombin at a concentration of 0.2 U/mL or higher was not inhibited by the F(ab')2 fragments of an antibody to human fibrinogen (Fig 6).

Deaggregation. To investigate the possible role of fibrinogen in preventing deaggregation, we examined the effect of several combinations of inhibitors that we have previously shown cause the deaggregation of human platelets aggregated by thrombin.44 Figure 7 shows the effect of the combination of hirudin, EDTA, and chymotrypsin two minutes after the addition of thrombin to suspensions of washed platelets. The rate and extent of deaggregation was practically identical for the normal and afibrinogenemic platelets. Inhibitors that did not deaggregate either type of platelet in the presence of hirudin were PGE1 (10 µmol/L), chymotrypsin (10 U/mL), apyrase (40 µL/mL), EDTA (2.5 mmol/L), chymotrypsin plus PGE1, or chymotrypsin plus apyrase. No

Fig 4. Platelet aggregation induced by ADP (20 µmol/L) added to suspensions of washed platelets in a medium containing 2 mmol/L Ca**+, 1 mmol/L Mg**+, albumin (0.35%), and apyrase. Upper pairs of tracings: Normal platelets. Lower pairs of tracings: Afibrinogenemic platelets (Patient II). (a) Represents the response of platelets to ADP in the presence of added fibrinogen (FIB; 8 µg/mL). (b) Represents the response of platelets to ADP in the absence of added fibrinogen. Tyrode's solution (TYR) was added to control samples to maintain constant volumes. The additions were made at the points indicated by the arrows. The concentration of the F(ab')2 fragments of nonimmune IgG (F(ab')2 NI) was 150 µg/mL; the concentration of the F(ab')2 fragments of an antibody to fibrinogen (F(ab')2 AF) was 100 µg/mL.
The results from these studies show that fibrinogen in the ambient fluid is not necessary for ADP-induced platelet aggregation, although fibrinogen greatly enhances the extent of aggregation. This is in agreement with the results of Soria et al.\textsuperscript{1}

In citrated PRP from an afibrinogenemic patient and with suspensions of either normal or afibrinogenemic platelets without added fibrinogen, the formation of the small aggregates induced by ADP is not inhibited by F(ab')\textsubscript{2} fragments of an antibody to fibrinogen, although these fragments block the enhancement of aggregation produced by adding fibrinogen to the suspending fluid. The aggregation that occurs without added fibrinogen is blocked by an antibody (10E5) to glycoprotein IIb/IIIa, indicating that the receptor for adhesive proteins is involved in this response. Thus there is a component of platelet aggregation that is not dependent on plasma proteins such as fibrinogen but is dependent on glycoprotein IIb/IIIa.

Washed human platelets stimulated by ADP in the presence of physiologic concentrations of Ca\textsuperscript{2+} release little or no serotonin from their amine storage granules and only about 4 \(\mu\)g of fibrinogen per 10\textsuperscript{8} platelets from the alpha granules in response to 10 \(\mu\)mol/L ADP.\textsuperscript{29} The afibrinogenemic platelets had approximately 10 \(\mu\)g of fibrinogen per 10\textsuperscript{8} platelets. If this were in the granules and released in proportion to the release from normal platelets, the concentration of fibrinogen in the ambient fluid would be less than 0.05 \(\mu\)g/mL. This is insufficient to cause a detectable enhancement of ADP-induced aggregation, for which at least 3 \(\mu\)g/mL is necessary. Furthermore, the F(ab')\textsubscript{2} fragments of an antibody to fibrinogen did not inhibit the response to ADP. However, proteins other than fibrinogen, including thrombospondin, fibronectin, and von Willebrand factor (vWF) may have been released. Since fibronectin has not been found to support ADP-induced aggregation,\textsuperscript{45,46} it is unlikely to be involved in the aggregation observed. Recently de Marco and his associates\textsuperscript{47} reported that addition of vWF to afibrinogenemic citrated PRP supports ADP-induced aggregation of afibrinogenemic platelets. However, the concentrations of vWF required (up to 60 \(\mu\)g/mL) were higher than would be present in plasma even if all the platelet vWF were released. The observation that the antibody LJP5, an antibody to glycoprotein IIb/IIIa that blocks the binding of vWF but not of fibrinogen to IIb/IIIa, inhibits platelet aggregation in response to ADP and collagen in afibrinogenemic citrated PRP is in favor of vWF being involved.\textsuperscript{47} However, the concentration of vWF would be negligible in suspensions of washed human platelets in a medium containing 2 mmol/L Ca\textsuperscript{2+}, in which ADP causes very little release of \(\alpha\)-granule contents; in addition, vWF does not support ADP-induced aggregation in this medium.\textsuperscript{48}

The response of afibrinogenemic platelets to thrombin (0.2 to 1 U/mL) or PAF (0.25 \(\mu\)mol/L) is practically indistinguishable from the response of normal platelets, indicating that released fibrinogen plays little part in the formation of platelet aggregates induced by these agonists. With collagen or sodium arachidonate the lack of releasable fibrinogen may contribute to the diminished aggregation response. One could also speculate that fibrinogen plays a greater part in aggregation that is largely dependent on thromboxane A\textsubscript{2}; thrombin and PAF can act through pathways that are largely independent of thromboxane A\textsubscript{2}.\textsuperscript{49-51} The finding that an antibody to fibrinogen did not inhibit platelet aggregation caused by 0.2 to 1 U/mL thrombin is in keeping with the observations of Legrand et al.\textsuperscript{50} and raises the possibility, as proposed by Soria et al.,\textsuperscript{1} of there being fibrinogen-independent mechanisms of platelet aggregation when platelets are stimulated with thrombin, and possibly with PAF.

We have previously shown that ADP-induced aggregation of human platelets is followed by rapid deaggregation if the release reaction does not occur.\textsuperscript{24} Fibrinogen binding is
associated with aggregation, and fibrinogen is lost from the surface of the platelets when they deaggregate. However, when the release reaction has taken place, agents such as PGE₁ or EDTA do not cause deaggregation, although they displace most of the bound fibrinogen (these agents do cause rapid reversal of ADP-induced aggregation under conditions in which the release reaction does not occur). It was possible to study the role of fibrinogen in forming stable platelet aggregates by comparing the susceptibility of the afibrinogenemic platelets and normal platelets to deaggregation after thrombin stimulation. Since the rate at which platelets from normal and afibrinogenemic subjects deaggregated was similar, it seems reasonable to conclude that fibrinogen is not necessary for the formation of stable platelet aggregates when the release-reaction has occurred. This is supported by the observation of Turitto and his associates who showed that large, stable, platelet aggregates formed on the subendothelium of rabbit aorta exposed to native afibrinogenemic blood in a Baumgartner flow chamber.

Thus ADP-induced aggregation can occur to a small extent without the presence of fibrinogen, but it is greatly enhanced by the addition of fibrinogen. Furthermore, with agonists that cause the release reaction, enhancement by fibrinogen is seen only with low concentrations of the agonist, and fibrinogen is not required to stabilize the platelet aggregates that form under these conditions.

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REFERENCES

27. Ardlie NG, Packham MA, Mustard JF: Adenosine diphos-
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33. Harfenist EJ, Packham MA, Mustard JF: Reversibility of the association of fibrinogen with rabbit platelets exposed to ADP. Blood 56:189, 1983


45. Zucker MB, Mosesson MW, Broekman MJ, Kaplan KL: Release of platelet fibronectin (cold-insoluble globulin) from alpha granules induced by thrombin or collagen; lack of requirement for plasma fibronectin in ADP-induced platelet aggregation. Blood 54:8, 1979


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