Comparison of Fibrinogen Association With Normal and Thrombasthenic Platelets on Exposure to ADP or Chymotrypsin

By J. F. Mustard, R. L. Kinlough-Rathbone, M. A. Packham, D. W. Perry, E. J. Harfenist, and K. R. M. Pai

Although 125I-fibrinogen becomes associated with washed platelets from normal human subjects during ADP-induced shape change and aggregation, 125I-fibrinogen did not become associated with washed platelets from a thrombasthenic subject during ADP-induced shape change and the platelets did not aggregate. Platelets from control and thrombasthenic subjects were treated with chymotrypsin, which is known to degrade platelet membrane glycoproteins. More 125I-fibrinogen became associated with chymotrypsin-pretreated platelets from normal subjects than with untreated platelets, and fibrinogen caused the enzyme-treated platelets to aggregate. 125I-fibrinogen did not become associated with chymotrypsin-pretreated thrombasthenic platelets, and fibrinogen did not aggregate them. Thus, there appears to be a defect in thrombasthenic platelets that prevents the association of fibrinogen with them.

Although washed human platelets change shape in response to adenosine diphosphate (ADP) in the absence of fibrinogen in the suspending medium, they do not aggregate unless fibrinogen is present. It has recently been shown that 125I-fibrinogen becomes associated with human platelets during ADP-induced shape change and aggregation; the fibrinogen is lost when the platelets deaggregate. Thrombasthenic platelets change shape but do not aggregate in response to ADP and have been shown to have abnormalities of their membrane glycoproteins II and III. Several investigators have reported that less fibrinogen is associated with thrombasthenic platelets than with normal platelets. In this study, we have investigated the possibility that the functional defect of thrombasthenic platelets may be an inability to become associated with fibrinogen upon exposure to ADP.

MATERIALS AND METHODS

Adenosine diphosphate and bovine tendon collagen were obtained from Sigma Chemical Company, St. Louis, Mo.; chymotrypsin from Worthington Biochemical Corp., Freehold, N.J.; and bovine thrombin from Parke Davis and Co., Detroit, Mich. All solutes were dissolved in either 0.85% saline solution or modified Tyrode solution (no calcium or magnesium). Suspensions of finely divided collagen were prepared as previously described. Human fibrinogen (Grade L, Kabi) was treated with diisopropylfluorophosphate (DFP, Sigma) to inactivate any procoagulant material. Fibrinogen was labeled with 125I (Amersham/Searle, Arlington Heights, Ill.) using iodine monochloride. The specific radioactivity of the 125I-fibrinogen preparation was 33,312 cpm/μg. When this fibrinogen was clotted...
with thrombin, 93%-94% of the radioactivity was present in the clot. We demonstrated previously that \(^{125}\)I-fibrinogen behaved similarly to unlabeled fibrinogen in supporting ADP-induced aggregation and that possible contamination of the \(^{125}\)I-fibrinogen with plasminogen or factor VIII could not be responsible for the observed binding of radioactive material to platelets. Furthermore, fibrinogen prepared by a different method in which low solubility proteins, such as cold-insoluble globulin or factor XIII, were removed from plasma by prefractionation before the fibrinogen was salted out gave similar results to Kabi fibrinogen.\(^7\) Kao et al.\(^8\) have recently demonstrated that factor VIII/von Willebrand factor does not bind to human platelets unless ristocetin is present.

Further experiments were done to establish that the \(^{125}\)I that binds to platelets during ADP-induced shape change and aggregation is due to \(^{125}\)I-labeled fibrinogen rather than to a minor labeled impurity present in the fibrinogen preparation. In these experiments, rabbit platelets were aggregated with ADP (1.8 \(\mu\)M) in the presence of \(^{125}\)I-fibrinogen prepared from human fibrinogen (Kabi). The supernate was recovered and incubated for 1 hr to allow the apyrase in the suspending medium to degrade the ADP. A portion of the supernate was added to fresh platelets that were then aggregated by the addition of more ADP. Four such cycles were carried out (i.e., the supernate from one cycle was used as the source of \(^{125}\)I-fibrinogen in the subsequent cycle). In control experiments, the \(^{125}\)I-fibrinogen used had been taken through the same procedures, but had not been exposed to platelets. In a representative experiment, at cycle 3, 2.8% and 2.6% of the previously adsorbed and control \(^{125}\)I-fibrinogens, respectively (initial concentration, 0.016 mg/ml), were bound by the platelets; at cycle 4, the platelets bound 2.7% (previously adsorbed) and 2.8% (control) of the \(^{125}\)I-fibrinogens (initial concentration, 0.0026 mg/ml). Thus, the amounts of \(^{125}\)I bound by the platelets during each cycle were the same regardless of whether a small percentage of the radioactivity had been removed from the \(^{125}\)I-fibrinogen preparations by previous exposure to platelets during ADP-induced aggregation. Therefore, it was concluded that the platelets were not selectively removing an appreciable portion of a minor labeled impurity that could have been responsible for the \(^{125}\)I bound to the platelets during each cycle, but that the labeled material that associates with the platelets during aggregation is \(^{125}\)I-fibrinogen.

The patient with thrombasthenia was a 17-yr-old youth with a long-standing history of easy bruising and recurrent bleeding from nasal and buccal mucosae. There was no family history of a bleeding tendency. The diagnosis of Glanzmann's thrombasthenia was made on the basis of the failure of the platelets to aggregate in response to ADP, collagen, or epinephrine, associated with a prolonged Ivy bleeding time (greater than 30 min) and the history of a bleeding tendency.

Suspensions of washed platelets in Tyrode solution, containing 0.35% albumin and apyrase (pH 7.35), were prepared as described previously\(^7\) using blood from a control subject and blood from the patient. The platelets from 200 ml of platelet-rich plasma were labeled in the first washing fluid by incubation for 15 min at 37°C with 2 \(\mu\)Ci \(^{14}\)C-serotonin (5-hydroxytryptamine creatinine sulfate-\(^{14}\)C, approximately 50 \(\mu\)Ci/\(\mu\)mole; Amersham/Searle). Platelets prepared from the blood of each individual were treated in two ways: (1) The platelets were treated with chymotrypsin (final concentration 8 U/ml) for 30 min at 37°C, washed once in Tyrode-albumin solution containing apyrase to remove the chymotrypsin, and finally resuspended in Tyrode-albumin solution containing apyrase. (2) A suspension of platelets from each individual was subjected to the same centrifugation procedures but was not exposed to chymotrypsin. Membrane glycoprotein changes caused by the treatment of normal platelets with chymotrypsin were assessed by SDS-polyacrylamide gel electrophoresis, followed by periodic acid Schiff (PAS) staining by methods described elsewhere.\(^27\) It was not possible to obtain enough blood from the thrombasthenic patient to do similar glycoprotein studies. Under the conditions used, glycoproteins III and IV of normal platelets were not susceptible to proteolysis, whereas nearly all of the PAS-staining material disappeared from the positions of glycoproteins I and II. (Glycoprotein numbering by the system of Phillips and Agin.\(^28\))

The platelet count in the final suspending medium was adjusted to 500,000/cu mm. Aggregation in response to ADP, collagen, or thrombin was studied by measuring light transmission through a stirred suspension in an aggregometer (Payton Associates, Scarborough, Ontario, Canada).\(^29\) The sensitivity of the aggregometer was adjusted so that the oscillations in light transmission characteristic of disc-shaped platelets could be observed. The initial decrease in light transmission after addition of an aggregating agent and the reduction in the oscillations in light transmission indicated a change in shape of the platelets from discs to a more rounded form with pseudopods.\(^30\) The amounts of all materials added to the platelet suspensions are expressed as final concentrations after all additions. The extent of the release reaction from platelets exposed to aggregating stimuli was determined by measuring the amount of \(^{14}\)C-serotonin in the supernate obtained by centrifuging the platelet suspension for 60 sec in an
Eppendorf centrifuge (Brinkmann, Rexdale, Ontario) 3 min after the addition of the aggregating agent. This value was expressed as a percentage of the total radioactivity in the platelet suspension.29

The percentage of $^{125}$I-fibrinogen that became associated with the platelets was compared with the percentage of trivalent $^{51}$Cr that remained trapped with the platelets. Trivalent $^{51}$Cr was prepared by adding 1 vol of 10 mM sodium ascorbate (prepared immediately before use by adjustment of ascorbic acid to pH 7 with 2N NaOH) to an equal volume of disodium $^{51}$chromate (NEN Canada, Ltd., Lachine, Quebec, specific activity 200-500 $\mu$Ci/$\mu$g of chromium). Labeled fibrinogen and trivalent $^{51}$Cr solutions were mixed together before they were added to the platelet suspensions. After the addition of the mixture of radiolabeled fibrinogen and trivalent $^{51}$Cr, ADP was added to the stirred platelet suspension in an aggregometer cuvette. Either before or after the addition of ADP, the contents of the cuvette were rapidly transferred to a centrifuge tube and immediately centrifuged for 60 sec in an Eppendorf centrifuge. About 11 sec elapsed between the removal of the cuvette and the attainment of maximum centrifuge speed. The supernatant fluid was removed immediately, and the radioactivity of each isotope in the platelet pellet was determined in a well-type gamma scintillation counter.

$^{125}$I-fibrinogen associated with the platelets was calculated in the following way:

$$\text{Percent association} = \frac{\text{I}}{\text{Total }^{125}\text{I in platelet suspension}} \times 100$$

We have previously established the suitability of trivalent $^{51}$Cr as a space marker.9

RESULTS

Suspensions of washed platelets from the thrombasthenic patient did not aggregate in response to ADP in the presence of fibrinogen (Fig. 1). In contrast, the suspension of washed platelets from the control subject aggregated strongly in response to 4.3 $\mu$M ADP in the presence of fibrinogen (Fig. 1). Neither platelets from the control subject nor platelets from the patient with thrombasthenia released $^{14}$C-serotonin in response to ADP. The thrombasthenic platelets did not aggregate in response to thrombin (0.9 U/ml) or collagen, although they changed shape and released 41% and 5%, respectively, of their $^{14}$C-serotonin. The control platelets released 55% and 45% of their $^{14}$C-serotonin under the same conditions.

Platelets from the control subject that had been pretreated with chymotrypsin
aggregated in response to fibrinogen (1.9 mg/ml), whereas chymotrypsin-treated platelets from the thrombasthenic patient did not (Fig. 2).

\(^{125}\text{I}-\text{fibrinogen, added to the platelet suspension before ADP, did not become associated to a detectable extent either with the platelets from the control subject or with the thrombasthenic platelets. During ADP-induced shape change and aggregation, the }^{125}\text{I}-\text{fibrinogen became associated with the control platelets but not with the thrombasthenic platelets, and the thrombasthenic platelets did not aggregate (Table 1). When platelets from the control subject were pretreated with chymotrypsin, some radiolabeled fibrinogen became associated with them, but with the low concentration of }^{125}\text{I}-\text{fibrinogen used (0.13 mg/ml), appreciable aggregation did not occur until ADP was added. During ADP-induced shape change and aggregation, more }^{125}\text{I}-\text{fibrinogen became associated with the enzyme-treated platelets than with untreated control platelets. Chymotrypsin-treated platelets from the thrombasthenic patient did not take up radiolabeled fibrinogen even upon exposure to ADP (Table 1). Both untreated and chymotrypsin-treated platelets from the control subject aggregated in response to ADP in the presence of the radiolabeled fibrinogen, whereas the platelets from the thrombasthenic subject

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<th>Table 1. Comparison of Fibrinogen Uptake by Normal and Thrombasthenic Platelets on Exposure to ADP</th>
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<tr>
<td>Percent (^{125}\text{I}-\text{Fibrinogen Associated With Platelets}^*</td>
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<td>Thrombasthenic subject</td>
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*Mean values for duplicate samples at each interval. Samples were stirred for 30 sec with \(^{125}\text{I}-\text{fibrinogen before addition of ADP. Neither the chymotrypsin-treated nor the untreated platelets from the control subject aggregated appreciably during stirring with this low concentration of }^{125}\text{I}-\text{fibrinogen (0.13 mg/ml) but the addition of ADP (10 \(\mu\text{M}\)) caused aggregation. Platelet count 500,000/cu mm.}
†Shape change and aggregation refer to the responses of the untreated control platelets to ADP in the presence of fibrinogen. Samples of the other platelets were taken at the same times as the samples of untreated platelets from the control subject.
‡Shape change only was observed in response to ADP (10 \(\mu\text{M}\)).
changed shape in response to ADP (as indicated by the change in light transmission through the platelet suspension) but they did not aggregate.

**DISCUSSION**

The suspension of washed platelets from the thrombasthenic subject changed shape in response to ADP, collagen, or thrombin but did not aggregate. Thrombin and collagen caused release of $^{14}$C-serotonin from these platelets. These responses are similar to those described for thrombasthenic platelets in citrated platelet-rich plasma.12-14

There are a number of reports that less fibrinogen is associated with thrombasthenic platelets than with normal platelets.18-22 Nachman and Marcus18 observed that thrombasthenic platelets were less responsive than normal platelets to an antibody against fibrinogen. Taylor and Zucker31 also found that thrombasthenic platelets were not aggregated by antifibrinogen.

In this study, $^{125}$I-fibrinogen did not become associated to a detectable extent with the thrombasthenic platelets when they were exposed to ADP. In contrast, as shown previously,9 $^{125}$I-fibrinogen became associated with the platelets from the control subject during ADP-induced shape change and aggregation. It is not possible to determine from these observations whether the thrombasthenic platelets do not aggregate because fibrinogen cannot become associated with them or whether the fibrinogen does not become associated with them because of their inability to aggregate. It is also not known whether the failure of fibrinogen to associate with thrombasthenic platelets is related to the abnormalities of their membrane glycoproteins15-17,32 or whether the binding site for fibrinogen is missing.

More $^{125}$I-fibrinogen becomes associated with chymotrypsin-treated platelets from normal subjects than with untreated platelets. During ADP-induced shape change and aggregation, more $^{125}$I-fibrinogen becomes associated with the enzyme-treated platelets than with untreated platelets. Thrombasthenic platelets pretreated with chymotrypsin did not take up $^{125}$I-fibrinogen even during exposure to ADP. Chymotrypsin cleaves membrane glycoproteins from platelets.27,33-36 We have previously shown33,36,37 that fibrinogen aggregates chymotrypsin-treated rabbit or human platelets, and similar results have been obtained with pronase-treated human platelets.38 In the present study, fibrinogen caused aggregation of chymotrypsin-treated platelets from a normal human subject, but it did not aggregate chymotrypsin-treated platelets from the thrombasthenic patient. It seems likely that normal disc-shaped platelets do not have available fibrinogen-binding sites but that these are revealed either when the platelets change shape in response to ADP or when glycopeptides are removed from membrane glycoproteins by treatment with proteolytic enzymes. Either the glycoproteins that prevent fibrinogen binding to normal platelets are not readily removed from thrombasthenic platelets by treatment with chymotrypsin, or a binding site for fibrinogen is not present on thrombasthenic platelets. The latter explanation seems more plausible.

Niewiarowski et al.39 have reported similar findings in studies of the interaction of $^{125}$I-fibrinogen with pronase-treated normal and thrombasthenic platelets and have also concluded that thrombasthenic platelets may be deficient in a fibrinogen receptor.
ACKNOWLEDGMENT

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