

The Minimum Concentration of Fibrinogen Needed for Platelet Aggregation using ADP

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OBJECTIVE: Determine the minimum concentration of plasma fibrinogen needed to stimulate the aggregation of platelets, collected from normal subjects, using ADP.

DESIGN: Platelet rich plasmas (300×10^9 platelets/L) were made and adjusted to final fibrinogen concentrations of 75, 19, 5, and 0 mg/dL using fibrinogen free serum. Each fibrinogen concentration in all twelve subjects was aggregated with ADP.

SETTING: Research laboratory in the Department of Clinical Laboratory Science at Saint Louis University.

PARTICIPANTS: Twelve healthy volunteers of both genders, between the ages of 18 and 60 years who were not pregnant and weighed at least 110 pounds were included in the study. Subjects were excluded from the study if they had ingested aspirin within one week prior to blood collection. In addition, subjects with a history of bleeding disorders such as afibrinogenemia, hypofibrinogenemia, von Willebrand disease, and Bernard-Soulier disease were rejected from the study.

MAIN OUTCOME MEASURES: Platelet aggregation tracings were analyzed for amplitude and compared across plasma fibrinogen concentrations. In addition, the type of curve (monophasic vs. biphasic), smoothness and aggregation stability were also noted.

RESULTS: The results show that aggregation occurred with every dilution of fibrinogen tested and that the amplitude of the aggregation curves appears not to be dependent on plasma fibrinogen.

CONCLUSIONS: The results indicate that platelets from healthy individuals previously exposed to normal fibrinogen levels will aggregate equally well in decreasing plasma fibrinogen concentrations and even in the absence of plasma fibrinogen using ADP as the aggregator.

ABBREVIATIONS: ADP = adenosine diphosphate; PPP = platelet poor plasma; PRP = platelet rich plasma; vWF = von Willebrand Factor.

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INDEX TERMS: ADP; aggregation; fibrinogen; platelets.

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Platelets are produced in the bone marrow and normally range between 150 to 440×10^9 /L in whole blood samples. When activated by vessel injury, platelets aggregate to form a platelet plug that is stabilized by a fibrin network. Research has shown that fibrinogen is essential for platelet aggregation but the literature does not suggest minimum plasma fibrinogen concentrations necessary to achieve this function.

The process of platelet plug formation involves three discrete steps; adhesion, the release reaction, and aggregation.¹ Platelets first adhere to the newly exposed collagen and other subendothelial fibers, within the subendothelium of the broken vessel wall. von Willebrand Factor (vWF) is a multimeric protein complex present in normal circulating blood that binds to the collagen fibers and to the glycoprotein Ib found on the platelet surface causing platelets to adhere to the site of vessel injury.²

Once the platelets have attached to the vessel wall, the contents of the dense and alpha granules are released by the stimulation of thromboxane A_2 , ADP, or thrombin. Dense granules release ADP, ATP, serotonin, epinephrine, norepinephrine, and calcium. ADP attracts more platelets to the injury site and causes the platelets to undergo a shape change exposing the previously nonfunctional glycoprotein IIb-IIIa, also known as integrin $\alpha_{11b}\beta_3$ or the fibrinogen receptor. Calcium facilitates fibrinogen attachment to the glycoprotein IIb-IIIa receptors and will result in platelet aggregation. Serotonin is a vasoconstrictor and uses agonists, such as ADP, epinephrine, and norepinephrine, to promote platelet aggregation. Alpha granules release various adhesion proteins including fibrinogen, thrombospondin, fibronectin, and vWF.³

There are two steps in the process of platelet aggregation termed primary and secondary aggregation. In primary aggregation fibrinogen attaches to glycoprotein IIb-IIIa and crosslinks platelets. It has been shown that fibrinogen attachment is essential for platelet aggregation.²⁻⁷ Methods of flow cytometry and ¹²⁵I-labeled fibrinogen have been used to show that fibrinogen binds with glycoprotein IIb-IIIa in platelet aggregation.^{4,7-9} Following the introduction of ADP, the α and γ chains of fibrinogen cause glycoprotein IIb-IIIa receptors to be exposed. The D domain of the fibrinogen molecule will attach to either the IIb or IIIa region of the platelet. Aggregation can not occur in the absence of either calcium or fibrinogen.^{4,10,11} The result is a loose network of adhering platelets, forming the first wave toward the formation of the primary hemostatic plug.

Secondary aggregation begins when the platelets release their granular contents. These substances are agonists and continue the aggregation process by stimulating platelets to bind to one another. Actin and other contractile proteins within the pseudopods of platelets produce platelet retraction that prevents further blood loss and facilitates healing. This results in a large, irreversible, and stable platelet plug.^{2,12}

DISEASES AND CONDITIONS AFFECTING PLATELET AGGREGATION

Certain diseases such as Bernard-Soulier, von Willebrand disease, Glanzmann thrombasthenia, storage pool disease, and fibrinogen deficiencies will interfere with platelet aggregation by various mechanisms. Platelets fail to adhere properly in Bernard-Soulier and von Willebrand disease. Glycoprotein Ib is absent in Bernard-Soulier disease; therefore initial platelet attachment to collagen fails to occur.

In von Willebrand disease subjects have decreased production of the vWF platelet factor. Without vWF the attachment of the platelet receptor, glycoprotein Ib, to collagen can not occur. In both Bernard-Soulier and von Willebrand disease, aggregation is not affected by ADP, collagen, and epinephrine but abrogated by ristocetin. In Glanzmann thrombasthenia glycoprotein IIb-IIIa is absent or mutated and platelets will not aggregate following the addition of ADP, thrombin, collagen, or epinephrine but do aggregate with ristocetin. Normally fibrinogen binds to glycoprotein IIb-IIIa in primary aggregation, but cannot bind in the absence of this receptor.

In storage pool disease platelets are deficient in dense granules. Subjects have an increased bleeding time, a monophasic platelet aggregation curve with ADP and epinephrine, and no response to collagen. In gray platelet syndrome alpha granules, which contain fibrinogen, thrombospondin, fibronectin, and vWF are deficient, but platelet aggregation studies remain normal.¹⁰

Of the disease states involving fibrinogen, namely afibrinogenemia, hypofibrinogenemia, and dysfibrinogenemia, only afibrino-

genemia affects platelet aggregation. Afibrinogenemia is homozygous and results in the absence of fibrinogen. In heterozygous hypofibrinogenemia, plasma fibrinogen levels are usually between 20 and 100 mg/dL which is sufficient to support normal platelet aggregation. In dysfibrinogenemia there is a genetic mutation resulting in abnormal fibrinogen structure but platelet aggregation is usually not affected.

Drugs and patient characteristics can also affect platelet aggregation. The ingestion of aspirin interferes with aggregation by preventing platelets from synthesizing thromboxane A₂.^{10,11} Decreased thromboxane A₂ reduces platelet aggregation because granular contents within the platelet are not released appropriately. It takes up to ten days for aggregation studies to return to normal after the ingestion of aspirin.¹² It has also been shown that there are gender differences for glycoprotein IIb-IIIa function. Premenopausal women have more active glycoprotein receptors than young men.¹³ Certain glycoprotein IIb-IIIa inhibitors, such as c7E3 (abciximab), are used clinically to inhibit platelet aggregation. However, further studies have shown that in low concentrations c7E3 induces platelet aggregation and at high concentrations blocks platelet aggregation.⁹

PLATELET AGGREGOMETRY

Platelet aggregation is measured spectrophotometrically using a platelet aggregometer and various aggregating reagents including ADP, epinephrine, collagen, and ristocetin. Aggregation is measured for a maximum of 15 minutes following the addition of the aggregating reagent, in our case, ADP. As platelets aggregate a shape change occurs, going from a flat discoid shape to a spherical shape with pseudopodia, becoming 'sticky' and forming aggregates. The optical density then decreases as more light is transmitted around the aggregates as compared to individual platelets in suspension. Changes in optical density are graphed on a chart recorder. The expected response of 0.45 mL of PRP to 20 mL ADP at a concentration between 1 to 2 mM is a biphasic curve.¹⁴ The period before ADP is added is designated as the baseline reading corresponding to 0% transmittance. As the platelets begin to form pseudopods there is an initial increase in absorbance. The primary wave represents the aggregation due to the addition of ADP. Once the platelets begin to release endogenous ADP, a secondary wave of aggregation is produced.¹⁰

RESEARCH DESIGN

The primary goal of this project was to determine the minimum concentration of plasma fibrinogen needed to stimulate platelet aggregation by testing PRP with decreasing levels of plasma fibrinogen while maintaining platelet numbers. Platelet aggregation tracings of the PRP from 12 healthy individuals, adjusted to five different plasma fibrinogen concentrations, were the primary parameter measured. A fibrinogen assay was performed on the plasma of each donor in order to determine the initial plasma fibrinogen concentration and the amount of serum needed to dilute the plasma to the fibrinogen levels established for testing. A platelet count

was performed on the PRP tubes from each donor and adjusted so that each tube tested contained approximately 300×10^9 platelets/L. This was done to standardize the platelet number for aggregometry. ADP was added to each dilution and aggregation tracings were collected and compared at varying fibrinogen levels.

Fibrinogen has been shown to be essential for platelet aggregation by binding the glycoprotein IIb-IIIa complex on the platelet surface.^{4,7-11} Another group demonstrated that the velocity of platelet aggregation decreases with lower plasma fibrinogen concentrations, and that platelet aggregation can, under certain circumstances, be supported by fibrinogen released from alpha granules in the absence of plasma fibrinogen.^{4,15,16} However, in these experiments, aggregometry was not employed as a measure of aggregation and platelets were stimulated with thrombin, a direct secretagogue, thus bypassing the two-stage aggregation process.

This work seeks to clarify the effect of decreasing plasma fibrinogen concentrations on platelet aggregation using aggregometry as the measure of aggregation and an aggregator (ADP) that typically produces a biphasic curve. The information gathered from this study can be used to determine the minimum replacement therapies needed to restore platelet function in patients with afibrinogenemia and to help determine the relative contributions of plasma fibrinogen and platelet defects to abnormal platelet aggregation tracings.

MATERIALS AND METHODS

Specimen collection

Venipuncture was performed on 12 healthy individuals using standard phlebotomy technique and the Vacutainer Blood Collection System. The donors were asked to complete a questionnaire for validation of their health status, and to review and sign a study consent form. The venipuncture procedure was performed on three individuals each morning. Samples were collected in three 4.5 mL draw sodium citrate tubes (3.8%) using the discard tube technique.

Preparation of PPP, PRP, and serum

The Drucker Centrifuge Model L-708 was used for all samples. One citrate blood sample was centrifuged for ten minutes at $1800 \times g$. The plasma was removed and stored as platelet poor plasma (PPP). The other two citrate blood samples were centrifuged for ten minutes at $300 \times g$. This supernant was removed, pooled, and stored as platelet rich plasma (PRP). Red topped tubes were collected in advance of the study from two healthy donors and allowed to clot. These tubes were centrifuged for ten minutes at $1800 \times g$. The serum from these samples was pooled, passed through two sodium citrate tubes at the appropriate 9:1 ratio, stored, and used to dilute PRP to achieve the decreasing fibrinogen levels.

Platelet count

A platelet count was performed on the PRP of each subject using the standard unopette and hemacytometer method. The platelet

count was adjusted to $300 \times 10^9/L$ by adding homologous plasma or by centrifuging and removing plasma.

Fibrinogen analysis

Fibrinogen assays were performed on PPP and serum using a BBL FibroSystem Fibrometer. A 1:10 dilution was made on each donor sample using Owrens Buffer solution. Clot time was measured by adding 100ml Baxter Thrombin reagent (concentration 100 units/mL) to 200ml of the diluted sample. The clot time was applied to a standard curve to determine fibrinogen concentration.

Preparing fibrinogen concentrations in PRP

Pooled serum was used to dilute the fibrinogen to the final concentrations: 75 mg/dL, 19 mg/dL, 5 mg/dL, and 0 mg/dL. The PRP tubes were initially diluted to 150 mg/dL by removing the appropriate amount of plasma and adding the same volume of serum. Following the initial dilution, the PRP tubes containing 150 mg/dL of fibrinogen were centrifuged as before and 0.25 mL of the sample was removed and 0.25 mL of serum added. This process was repeated to achieve the final plasma fibrinogen concentrations listed above. To make the 0 mg/dL fibrinogen sample, the platelets were washed twice with 3.5 mL of saline containing no preservatives. The saline was decanted and 0.4 mL of serum added to the platelet pellet to reach a final volume of 0.5 mL. In this system, serum was used instead of saline to replace the plasma in order to create a natural, yet fibrinogen free environment for the platelets.

Aggregation assay

Platelet Aggregation Profiler Model PAP-2A was used for aggregation studies. Assays were performed on 12 healthy individuals using Bio/Data ADP solution as the agonist. The stock concentration of ADP was $2 \times 10^{-4}/mL$ and working concentration $4 \times 10^{-5}/mL$. The instrument self zeros by comparing PPP and PRP, both of which were allowed to preheat to $37^\circ C$. Into glass cuvettes, 0.5 mL of PPP and 0.45 mL of PRP was added. A small magnet was placed in each cuvette to keep the platelets in suspension. An undiluted and four fibrinogen dilutions of 75 mg/dL, 19 mg/dL, 5 mg/dL, and 0 mg/dL were aggregated by adding 50 ml ADP working solution, and recorded on a chart recorder using heat sensitive paper.

DATA ANALYSIS

In order to interpret the differences in platelet aggregation between the fibrinogen dilutions, aggregation amplitude was measured. The amplitude was determined by measuring the difference between the baseline, where the ADP was added, and the point at which maximum aggregation was achieved. Curve type (monophasic vs. biphasic), smoothness, and aggregation stability was also noted.

RESULTS

Amplitude

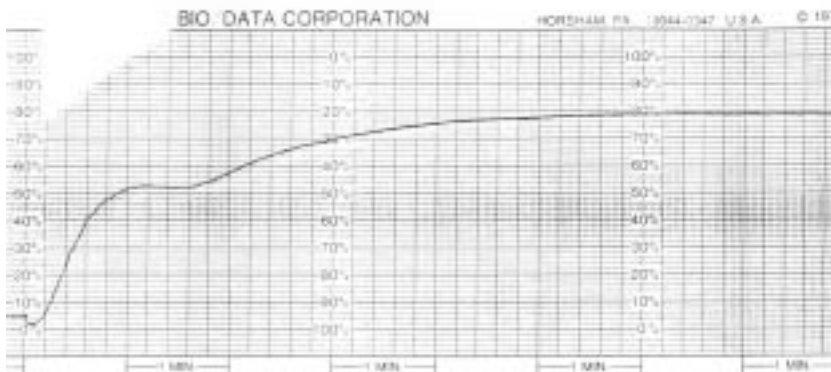
Amplitude was measured on a total of 54 curves. Of these 54, three had an amplitude between 19% and 39%, twelve between

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Table 1. Platelet aggregation with decreasing fibrinogen concentrations

Fibrinogen concentration	Mean amplitude	Curve type	Smoothness	Stability
Undiluted, n = 12 mean = 375 mg/dL	77.5%	6 biphasic 6 monophasic	11 smooth 1 jagged	6 stable 6 slight disaggregation
75 mg/dL, n = 11	62.7%	0 biphasic 11 monophasic	6 smooth 6 jagged	11 stable 0 slight disaggregation
19 mg/dL, n = 11	54.6%	0 biphasic 11 monophasic	5 smooth 6 jagged	10 stable 1 slight disaggregation
5 mg/dL, n = 12	57.1%	0 biphasic 12 monophasic	7 smooth 5 jagged	12 stable 0 slight disaggregation
0 mg/dL, n = 8	60.3%	0 biphasic 8 monophasic	5 smooth 3 jagged	8 stable 0 slight disaggregation
Totals:		6 biphasic 48 monophasic	47 stable 21 jagged	7 slight disaggregation

Figure 1. Biphasic and smooth curve



40% and 49%, seven between 50% and 59%, nine between 60% and 69%, eight between 70% and 79%, eight between 80% and 89%, and seven between 90% and 100%. In general, there was no trend in amplitude between the various dilutions (Table 1).

Curve type

The two curve types observed were

biphasic and monophasic. Of the 54 aggregations, six were biphasic, all from undiluted samples, and 48 monophasic (Table 1). A typical biphasic curve showed aggregation for the first minute and then leveled off for the next minute before a second wave of aggregation began (Figure 1). Monophasic curves showed only one wave of aggregation (Figure 2).

Smoothness

Aggregation graphs were described as either smooth or jagged. Smooth aggregations had little or no noise throughout the entire run (Figure 1). Jagged aggregations showed a variety of peaks and valleys (Figure 3). Of the 54 observations, 34 were smooth and 21 jagged (Table 1).

Stability

After aggregation had occurred, two trends were observed throughout the run, stability or slight disaggregation. The stable curves would remain horizontal with no increase or decrease in absorbance (Figure 2). Slight disaggregation was observed in some plots over time (Figure 4). Of the 54 data points, 47 were stable and seven showed slight disaggregation (Table 1).

DISCUSSION

The goal of this research project was to determine the minimum concentration of plasma fibrinogen necessary for platelet aggregation. Aggregation was observed in all 53 data points, showing a similar pattern with no recognizable trends. This information indicates that decreasing plasma fibrinogen concentrations does not abrogate

aggregation nor produce a consistent rise or fall in the amplitude of platelet aggregation tracings. The data also suggest that under these circumstances aggregation will occur in the absence of plasma fibrinogen.

Amplitude

The concentration of fibrinogen did not appear to affect the amplitude of aggregation to an appreciable degree. In some cases amplitude was lower in the 75 mg/dL dilution as compared to the undiluted sample, but would often show higher amplitude in the remaining dilutions. In five of the twelve cases the undiluted sample did not show the highest amplitude. Variations in amplitude appear to be related more to random error in the protocol as opposed to fibrinogen concentrations. Therefore, the amplitude of platelet aggregation curves does not appear to be a function of plasma fibrinogen concentration when tested under these conditions.

Curve type

In an attempt to induce biphasic curves in pilot samples prior to data collection, various dilutions of ADP were added to donor PRP. ADP dilutions of 2×10^{-4} , 1×10^{-4} , 4×10^{-5} , 2×10^{-5} , 2×10^{-6} mL were tested with PRP from two different healthy volunteers. No biphasic curves were produced in any of the samples from this ADP concentration experiment. The 4×10^{-5} mL dilution was selected for data collection because it gave the smoothest curve with the greatest amplitude and was within the concentration range recommended by the manufacturer. Six of the twelve undiluted samples among the study group showed biphasic aggregation. The others displayed only monophasic aggregation for reasons we were unable to determine. It is possible that below normal fibrinogen levels may have contributed to the lack of biphasic curves in all the diluted samples.

Smoothness

In all cases of a given fibrinogen concentration, some curves were smooth while others were jagged. We attempted to resolve this issue by maintaining a minimum sample volume of 0.5 mL in both the PRP

Figure 2. Monophasic curve

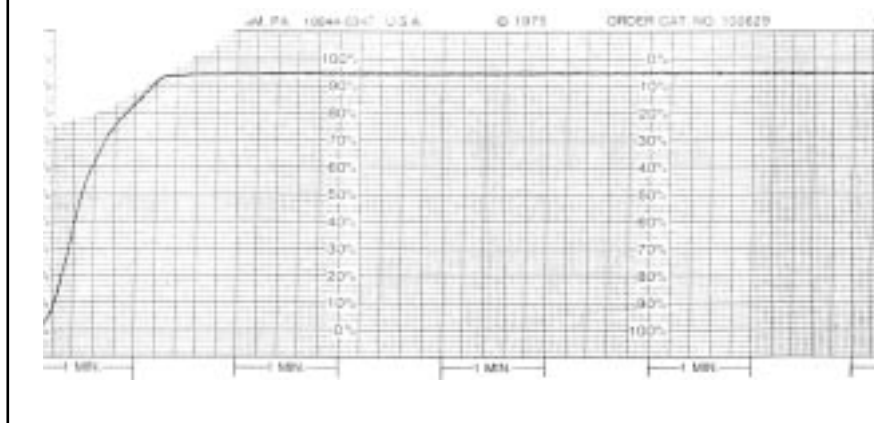


Figure 3. Jagged curve

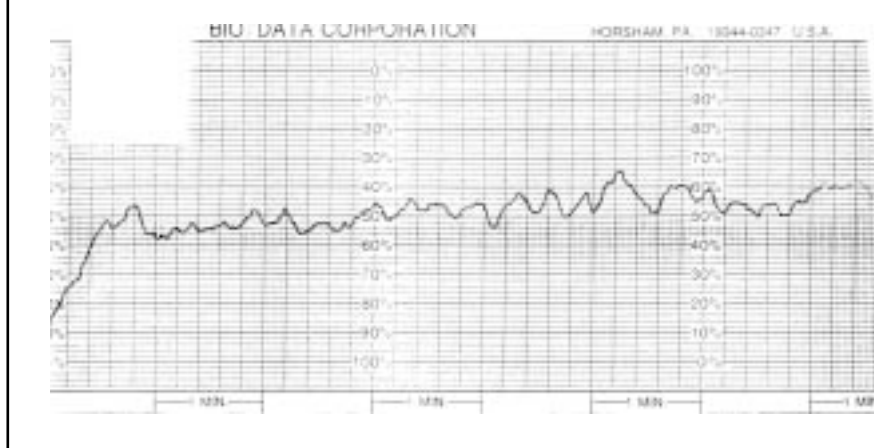
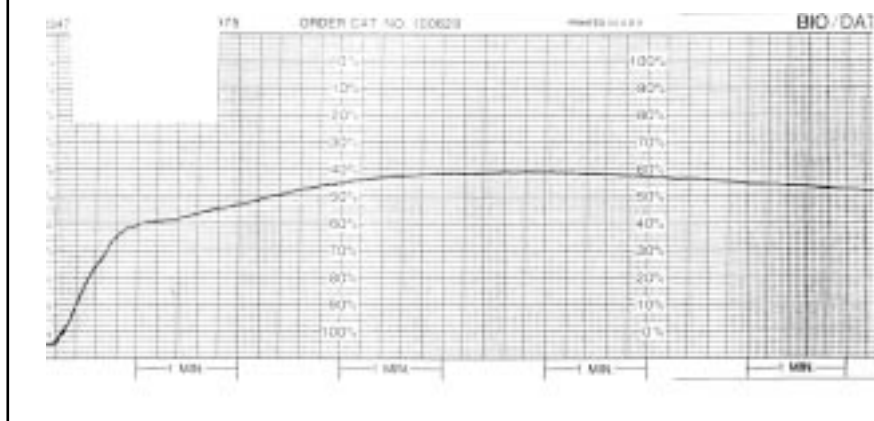


Figure 4. Slight disaggregation



and PPP cuvettes. However, jagged curves were still observed after this attempt. In a few cases small microfibrin pieces were observed in the dilutions and we felt these could interfere with the homogeneous distribution of the platelets in suspension. In all subsequent aggregations wooden applicator sticks were applied to gather fibrin, but fibrin was rarely found and jagged curves were still observed. Although the cause of jagged curves was undetermined, we assume it is background noise due to poorly suspended platelets or the presence of small bubbles.

Stability

In general, most runs displayed a stable line of transmittance after aggregation had occurred. Of the runs showing slight disaggregation, 86% of them were in the undiluted samples. The degree of disaggregation was insufficient to be described as total disaggregation. The presence or extent of disaggregation was also not a reflection of fibrinogen concentration. Slight disaggregation, like the jagged curves, appeared to be a random event.

The data show that aggregation will occur in the absence of detectable plasma fibrinogen. It was observed that even samples washed free of fibrinogen produced aggregation curves of similar amplitude, stability, and smoothness, when compared to samples with higher fibrinogen levels. Therefore platelet aggregation must either be supported by the release of stored fibrinogen, or by fibrinogen that was previously attached to the platelets.

Previous studies conclude that fibrinogen is needed for platelet aggregation.³⁻⁷ Other studies indicate that glycoprotein IIb-IIIa receptors are not exposed on resting platelets but are induced in the presence of ADP.^{2,10,12} Given this information, our data suggest that one possible explanation may be that enough glycoprotein IIb-IIIa receptors may be exposed on the surface of resting platelets to bind sufficient fibrinogen, before ADP stimulation, to induce strong aggregation in low or absent plasma fibrinogen levels. It is possible that the centrifugation process may have resulted in platelet stimulation and glycoprotein IIb-IIIa exposure. However, this is unlikely because in the samples containing 0 mg/dL of fibrinogen, the tubes were centrifuged only twice and in 3.5ml of preservative free saline. It is also improbable that sufficient plasma fibrinogen may be present in the test system because fibrinogen is absent in normal serum, none was detected using a fibrinogen assay, and platelets were washed in the 0 mg/dL samples.¹⁰

Another possible explanation is that sufficient amounts of fibrinogen to support platelet aggregation may have been released from the alpha granules. However, this is unlikely because a lag phase was not observed on the aggregation curves of the fibrinogen free samples. Not only does fibrinogen dependent aggregation precede the release reaction but the dense bodies are secreted before the alpha granules. One would therefore expect a delay in aggregation until the alpha granules have released stored

fibrinogen to allow aggregation to proceed. Regardless of the mechanism, our data suggest that very little fibrinogen is needed for platelet aggregation.

We hypothesize that normal resting platelets, exposed to an abundant fibrinogen environment, will bind sufficient fibrinogen for normal platelet aggregation even when all fibrinogen is subsequently removed from the plasma. Most of the previous studies that suggest the necessity of fibrinogen for platelet aggregation were performed on afibrinogenemic patients. The platelets in these patients were never exposed to a normal fibrinogen environment unless the patient received replacement therapy prior to testing. Therefore, fibrinogen could not have been bound to the platelets prior to testing. It is also possible that primary aggregation may be induced by some serum substance other than fibrinogen, such as vWF, that can stimulate secretion and the release of stored fibrinogen from the alpha granules.

In conclusion we suggest that platelet aggregation studies using ADP can be performed on patients with dramatically decreased fibrinogen levels without affecting the interpretation of the aggregation tracing. However, platelet aggregation assays should not be performed on patients with afibrinogenemia because their platelets would not be exposed to plasma fibrinogen and their alpha granules would be devoid of fibrinogen. In addition, the administration of fibrinogen replacement therapies should be adjusted to correct the clotting defect since so little is needed to induce platelet aggregation.

This research has provided us with a better understanding of platelets and aggregation curves at various dilutions of fibrinogen. It is our hope to repeat this protocol on more subjects so statistical analysis can be performed to test the hypotheses stated above. We also plan to test for the presence of fibrinogen and glycoprotein IIb-IIIa on the surface of washed resting platelets using fluorescent antibodies and to elute fibrinogen, if present, from the surface of normal resting platelets to test the effect on platelet aggregation. We hypothesize that successful and complete elution of bound fibrinogen from the platelet surface will delay or totally abrogate platelet aggregation.

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