Platelet Aggregation Testing in Platelet-Rich Plasma

Description of Procedures With the Aim to Develop Standards in the Field

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Abstract

Platelet function testing consisting of platelet aggregation and secretion often is requested in the clinical evaluation of patients with bleeding problems. At present, there are no uniform clinical laboratory standards for the performance or interpretation of these studies. The present report describes one laboratory's methods and interpretations of platelet aggregation and secretion studies of platelet-rich plasma using each of the common platelet agonists. Diagnostic categories for the evaluation of the platelet function testing are presented. The diagnostic categories then are applied to the evaluation of 61 patients referred to our medical center for these studies. The aims of this report are to present clinical platelet aggregation and secretion studies and to provide a working schema to evaluate these results. Our intent is to stimulate interest in the development of professional guidelines for platelet function testing in the clinical laboratory.

Platelet function testing commonly is requested at many institutions. However, there is no accepted practice as to how these studies are to be performed and interpreted. Although there are highly suggestive platelet function patterns for Glanzmann thrombasthenia and Bernard-Soulier syndrome (BSS), these entities are rare and do not make up the vast majority of platelet function defects seen in practice. The present report describes one institution's approach to categorizing platelet aggregation and secretion defects commonly referred for an evaluation. We hope to open a debate on the development of criteria for good laboratory practice for platelet aggregation and secretion studies.

The role of platelets in hemostasis sequentially involves their adherence to sites of vessel injury, activation of internal signaling pathways, aggregation to form plugs, and acceleration and localization of the coagulation reactions that eventually form thrombin. Agents that physiologically activate platelets in vivo include adenosine diphosphate (ADP), epinephrine, collagen, thromboxane A_2 (TxA_2), serotonin, and thrombin. Platelets have receptors for each of these agonists and a major integrin receptor ($\alpha_2\beta_3$ integrin [glycoprotein IIb/IIIa, CD41/CD61]) for the adhesive glycoproteins (GPs), fibrinogen, von Willebrand factor (vWF), thrombospondin, fibronectin, and vitronectin and the GPIb-IX-V complex for vWF. All of these agonists synergize to activate platelets. Platelet function may be impaired if any of the pathways mediated by these agonists and/or their receptors is defective.

ADP is considered a mild platelet agonist. It also is present in dense granules of platelets. Two G-protein-coupled purinergic (P2) receptors contribute to platelet aggregation initiated by ADP. The P2Y$_1$ receptor activates phospholipase C, induces shape change, and initiates primary wave platelet aggregation through calcium mobilization. The P2Y$_{12}$ receptor is considered

the major ADP receptor mediating the full platelet aggregation response to ADP through inhibition of adenyl cyclase and stabilization of the platelet aggregates. $P2Y_{12}$ also is the target of the antiplatelet drugs clopidogrel (Plavix) and ticlopidine and the ADP receptor antagonist AR-C66096.[1-4] In addition to these 2 purinergic receptors on platelets, the $P2X_1$ inotropic receptor is an ion gate channel present on platelets. Although it has been postulated that $P2X_1$ potentiates platelet activation in combination with other platelet agonists, the function of $P2X_1$ is not understood fully.[5]

The receptor on platelets for epinephrine is the α_2-adrenergic receptor. Epinephrine causes inhibition of adenyl cyclase and releases calcium ions from endoplasmic reticulum through inositol phosphate 3 and phosphorylation of pleckstrin by protein kinase C through the diacylglycerol pathways.[6] Like ADP, it is considered a weak platelet agonist because it has aggregation-dependent secretion.[7] However, defects in signaling through the platelet α-adrenergic receptor have been associated with bleeding states.[8]

Platelet arachidonic acid is the precursor of TxA_2 and hydroxyl fatty acids. Various platelet agonists mobilize calcium through G-protein-coupled receptors. Calcium activates phospholipase A_2, which liberates arachidonic acid from phosphatidylcholine and phosphatidylethanolamine. Calcium also activates myosin light-chain kinase. Arachidonic acid then is converted to TxA_2 by cyclooxygenase and thromboxane synthase, which further mobilizes calcium from intracellular storage sites. TxA_2 and activated myosin light-chain kinase together lead to platelet coagulant activation by stimulating secretion of products of platelet granules, allowing tenase and prothrombinase formation.[9]

Collagens are important for platelet adhesion and subsequent activation on the extracellular matrix of the denuded endothelium.[10] At present, 2 major platelet receptors for collagen have been identified. One of the receptors is GPIa/IIa ($\alpha_2\beta_1$ integrin), which contributes to platelet adhesion. The other collagen receptor is GPVI, which is responsible for platelet signaling and activation leading to TxA_2 formation.[11] These collagen receptors have synergistic activity.

Finally, thrombin is the most potent physiologic agonist of platelets. Protease-activated receptors 1 (PAR1) and 4 (PAR4) are activated by thrombin. These proteins belong to a family of 4 known 7-transmembrane G-protein-coupled receptors that are activated by a single cleavage within the amino terminal, extracellular region of the receptors.[12-15] Binding and cleavage of PAR1 by thrombin results in the exposure of a new amino terminus that subsequently touches down to a transmembrane domain of the receptor to activate $G_{\alpha q}$ subunits that initiate signal transduction. Subsequent activation of PAR4 results in sustained signal propagation and might be required for optimized platelet activation.[15,16]

Platelet aggregation and secretion studies are qualitative tests for platelet function. These assays were first described during the early 1960s and have remained largely unchanged.[17,18] Moreover, a number of reviews have described how to perform these studies.[19-21] The most frequently used testing for platelet function is that of measuring platelet aggregation following addition of agonists, including ADP, epinephrine, collagen, and arachidonic acid.

Platelet activation in response to γ-thrombin or the thrombin receptor activation peptide for PAR1 (SFLLRN [single letter amino acid abbreviation]) also is tested in clinical laboratories. γ-Thrombin is used in the clinical laboratory because it does not bind fibrinogen and, therefore, will not clot the plasma in platelet-rich plasma (PRP). It stimulates both PAR1 and PAR4 on human platelets. The peptide SFLLRN is the newly exposed amino terminus of thrombin-activated PAR1 and can be used as an inducer of thrombin stimulation of platelets through PAR1 signaling.[12] Stimulation of human PAR4 also can be achieved by its agonist peptide, AYPGFK, which activates the PAR4 signaling pathway in platelets.[22] Platelets washed free of suspension plasma can be studied using α-thrombin. However, the addition of α-thrombin to PRP will clot the plasma and activate the platelets.

Platelets in plasma also are examined for their ability to agglutinate or activate when treated with ristocetin. Initially, ristocetin and vWF adhere to the GPIb-IX-V complex on the platelet membrane. This event allows for platelet-platelet interactions by vWF with subsequent agglutination. During the close contact of agglutinating platelets, there is phosphorylation of intracellular platelet proteins with subsequent platelet activation and release of their granule contents (eg, fibrinogen) and secondary platelet aggregation.

In general, correlations have been drawn between the platelet responses to each of these agonists and specific defects of their function. Currently there is no consensus on how platelet function assays should be performed to evaluate patients. Each institution follows its own practices. Unified standards of sample collection, processing, test performance, reference values, and interpretation do not exist. In this report, we present how platelet aggregation and secretion testing is performed at the University of Michigan Coagulation Laboratory (Ann Arbor). Our goal is to present one approach for the clinical testing of platelet function to serve as a template for discussion about establishing clinical laboratory standards for this commonly performed assay.

Materials and Methods

Equipment

Platelet aggregation was performed on a Dual Channel Aggregometer (model 440, Chronolog, Havertown, PA). Chart recordings were monitored on a Kipp-Zone Chart Recorder (Fisher Scientific, Chicago, IL). Secretion of carbon

14 (^{14}C)-serotonin from activated labeled platelets was measured by a Tricor Scintillation counter (model 6891, Tricor, Elk Grove Village, IL).

Reagents

ADP and epinephrine were purchased from Sigma Chemical (St Louis, MO) and placed in small 400-μmol/L stock aliquots that were frozen at –70°C until use. ADP was diluted to concentrations of 1 to 10 μmol/L in the aggregation cuvette filled with PRP for testing, and epinephrine was used at a final concentration from 0.1 to 10 μmol/L. These samples were thawed once and used on the day of experimentation; any remaining material was eliminated after completion of studies.

Arachidonic acid (Bio/Data, Horsham, PA) was divided into aliquots of 5 mg/mL stock (final concentration, 500 μg/mL in assay). Collagen (Chronolog) was stored as a 1 mg/mL stock. Its final concentration in the platelet function assay was 5 μg/mL. Ristocetin was purchased from Helena Chemical, Collierville, TN. A 23.8 mg/mL stock solution was made and used at a final concentration of 0.6 to 1.2 mg/mL when testing platelet responsiveness to this agent. γ-Thrombin was purchased from Enzyme Research Laboratories, South Bend, IN. A concentration of 29 μmol/L of stock was frozen in 25-μL aliquots, thawed once, and used in final concentrations ranging from 14 to 166 nmol/L. The unused remaining γ-thrombin was discarded. ^{14}C-serotonin (50 μCi per vial; Amersham, Arlington Heights, IL) was placed into 20-μL aliquots that were stored until use.

Preassay Variables

Because numerous medications can affect platelet function testing, patients for study were requested to avoid all medications for 10 days before assay. Patients also were asked not to drink coffee on the day of the test and to fast for at least 4 hours before testing.

PRP Preparation

Whole blood was anticoagulated with 3.2 g% of sodium citrate (sodium citrate/whole blood ratio, 1:10) by collecting blood directly into a plastic polypropylene syringe containing the anticoagulant, followed by placing aliquots into polypropylene tubes for centrifugation. Collection, transport, and centrifugation were performed at room temperature.

PRP was prepared by centrifugation of anticoagulated blood at 180g for 10 minutes at room temperature. After preparing PRP, the platelets were labeled with ^{14}C-serotonin and incubated 30 minutes at 37°C in capped tubes (see "^{14}C-Serotonin Labeling"). The cloudy yellow supernatant containing the platelets was removed carefully with a disposable plastic pipette and placed into a clean polypropylene tube and capped. Care was taken not to disturb the WBC and RBC cell layers when removing the PRP. Platelet-poor plasma (PPP)

was prepared by centrifuging the remaining sample at 1,200g for 20 minutes at room temperature.[23]

Hemolyzed and lipemic specimens might interfere with the light transmission on the platelet aggregometer. Platelet function testing should be completed within 3 hours after blood collection. The PRP was adjusted to a platelet count of 200 to 350 × 10^3/μL (200-350 × 10^9/L) after addition of PPP. Clinical samples should be assayed with similar platelet counts in the normal range. Platelet counts less than 100 × 10^3/μL (100 × 10^9/L) are not optimal for these functional tests.

^{14}C-Serotonin Labeling

PRP samples for secretion studies were incubated with ^{14}C-serotonin for 30 minutes at 37°C in a capped tube.[24,25] ^{14}C-serotonin was added to the PRP suspension such that the total number of counts per minute was between 5,000 and 7,000. At the end of the incubation, the ^{14}C-serotonin–labeled PRP was treated with imipramine (final concentration, 2 μmol/L) to prevent reuptake of any lost serotonin.[26] At the conclusion of platelet aggregation studies, 200 μL of ^{14}C-serotonin–labeled activated platelets from the aggregometer cuvette were added to a conical centrifugation tube containing 50 μL of formalin-EDTA solution (formalin, 135 μmol/L concentration; EDTA, 5 mmol/L concentration). The formalin-EDTA solution was present to prevent loss of platelet granule constituents from the close contact of the platelets during centrifugation.[27] The tubes were centrifuged at 12,000g for 5 minutes at room temperature in a tabletop microcentrifuge. Before patient studies, 2 tubes of ^{14}C-serotonin–labeled PRP without centrifugation were counted to represent total counts. Two additional tubes of PRP that were not treated with a platelet agonist and for which the supernatant was prepared by centrifugation were put aside to be counted on the scintillation counter and used in the calculations for platelet serotonin uptake and secretion. The time from the collection of sample to beginning platelet aggregation studies usually was less than 1 hour.

Platelet Aggregation and Secretion Studies

Platelet aggregation was determined by measuring the change in the optical density (ie, light transmittance) of stirred PRP after addition of the aggregating agent to the aggregometer cuvette. Platelet aggregation occurs only if the PRP in the aggregometer cuvette is stirred, usually at the rate of 800 to 1,200 rpm.

A Teflon-coated magnetic stirrer was used that was recommended by the equipment manufacturer. The stirring speed and nature of the stirrer might vary by manufacturer of the equipment. The aggregometer was standardized by placing the patient's PPP sample in one channel to represent 100% light transmittance and the patient's PRP sample in another channel representing 0% transmittance. The increase in light transmittance from 0% to 100% is reflected on the chart recorder as the aggregometer tracing. Usually, the baseline of the patient's PRP was adjusted to be at the 10% chart deflection level, and the

patient's PPP baseline was adjusted to the 90% level on the chart recorder. The light transmittance of the PRP relative to the PPP blank was recorded automatically. When an aggregating agent is added to the PRP, platelet aggregates form, and this event results in an increase in the light transmittance, which is recorded and used as an index of platelet aggregation.

Generally, each platelet aggregation should run for at least 5 minutes and occasionally up to 10 minutes. The chart speed of the recorder varies by equipment, but it should be sufficiently fast to see the change in the shape of the aggregation tracing, usually 2 cm or more per minute. This amount of time allows for observation of primary and second wave aggregation for ADP and epinephrine and the determination of late responders to epinephrine, collagen, and γ-thrombin. In general, the direction of chart deflection in platelet aggregation depends on how the chart recorder and aggregometer interface.

After each aggregation test was done, 200 μL of the activated ^{14}C-serotonin–labeled PRP was transferred to tubes containing the formalin-EDTA solution as described. The supernatant of the activated PRP then was transferred to a bottle of scintillation fluid for counting on the β scintillation counter. As an alternative, adenosine triphosphate release from platelets can be measured directly in an aggregometer cuvette using luciferase in a lumi-aggregometer.[7] This latter method is a nonradioactive means of simultaneously measuring platelet aggregation and secretion.

Calculations of Platelet Aggregation and Secretion

The percentage of aggregation was determined as the percentage of chart deflection between 10% and 90% at its highest average point of deflection on the chart recorder as follows:

Percentage of Aggregation = (Highest Average Point of Deflection – 20)/80 × 100.

Another way to quantify platelet aggregation is to present the initial rate of aggregation per minute. This form of calculation is determined by drawing a tangent to the initial deflection of the aggregometer tracing after shape change and calculating the slope of the tangent per unit of time (usually per minute). Initial studies with ^{14}C-serotonin can assess the uptake of the radiochemical into platelet dense granules. ^{14}C-serotonin uptake is calculated as follows:

Percentage of Serotonin Uptake = (Mean Total Count – Mean Supernatant Count)/Mean Total Count × 100

Platelet secretion of the ^{14}C-serotonin is calculated as follows:

Percentage of Serotonin Secretion = (Sample Count – Mean Supernatant Count)/(Mean Total Count – Mean Supernatant Count) × 100.

Determination of Normal Values for Platelet Aggregation and Secretion

Normal values for platelet aggregation and secretion were obtained by testing platelets from 23 healthy individuals without abnormalities of the hemostatic system (11 men, 12 women). All were hospital staff members. None of the subjects studied had taken any medication for at least 10 days before testing by history. None of the women was taking oral contraceptives when blood samples were obtained. No healthy donor had a diagnosis of a chronic disease.

For the purpose of choosing evaluable healthy donors, results for individuals whose platelets showed impaired response to epinephrine were excluded from the determination of "normal" values. For each agonist, platelet aggregation and secretion responses from the donor platelets were obtained, and the mean value and range of normal response (mean ± 2 SD) were determined. The serotonin uptake for this population also was determined. The results of aggregation and secretion studies in response to all agonists that fell outside the determined range of normal in our study population were considered abnormal for the purpose of classifying disorders.

Classification of Platelet Function Defects

Working diagnostic criteria were developed for individual patient samples referred for a platelet function evaluation based on the results of the normal sample evaluation. An *aspirin-like defect* was diagnosed when a patient's results fell outside our normal range of response to 500 μg/mL of arachidonic acid regardless of whether the patient also had defects in ADP- and/or epinephrine-induced platelet activation. These individuals did not have defects in collagen- or thrombin-induced platelet activation. An *ADP- or epinephrine-like defect* was diagnosed when only ADP- or epinephrine-induced platelet aggregation was abnormal, respectively, and arachidonic acid–, collagen-, and thrombin-induced platelet activation were normal. A *weak platelet agonist defect* was assessed when both ADP- and epinephrine-induced platelet activation, but not arachidonic acid–, collagen-, and thrombin-induced platelet activation, were abnormal. *Pan-secretion platelet function defects* were determined when ADP-, epinephrine-, and collagen-induced platelet activation fell outside the normal values for the laboratory. Last, a diagnosis of a *storage pool disorder* was made when the serotonin content of platelets was below the lower limit of the range of serotonin uptake (ie, <75%) in our healthy subjects.

Results

Initial efforts were to describe normal aggregation tracings after PRP was exposed to ADP, epinephrine, collagen, arachidonic acid, γ-thrombin, or ristocetin. Some of the variables of platelet aggregation were mentioned in the "Materials and Methods" section. Variables in the performance of platelet aggregation studies have been reviewed carefully.[28]

ADP-Induced Platelet Aggregation

❚Image 1❚ is an example of changes in light transmission during normal platelet aggregation induced by ADP. As shown in Image 1 (left), platelets first are stabilized in the cuvette. The baseline of light transmission before the instillation of the agonist is indicated by "1." Once the agonist is added, as indicated by the spike labeled "2," there is an initial shape change (indicated by "3"), resulting in a decrease in light transmittance followed by a primary wave of aggregation (indicated by "4"). If the stimulus is not sufficiently strong, the platelets will disaggregate (data not shown). Alternatively, if the stimulus is strong, the secondary wave of ADP-induced platelet aggregation arises, indicated by "5" when there is a release of platelet granule contents that include fibrinogen, serotonin, thromboxane, and ADP that potentiate the primary aggregation response (Image 1).[7]

When potent agonists such as collagen or thrombin are used, there is only 1 curve, and no distinction between primary and secondary wave aggregation is noted because platelet stimulus-response coupling occurs before any aggregation response.[7] Alternatively, with the so-called weaker agonists, ADP and epinephrine, a primary wave aggregation response occurs leading to internal platelet signal transduction that

allows for the release of granule contents necessary to promote the secondary wave of aggregation seen.

The threshold for ADP-induced platelet aggregation in most healthy populations is between 1 and 7.5 μmol/L. With higher concentrations of ADP, a single combined primary and secondary aggregation wave is produced and the inflection between the 2 curves can be missed (Image 1, right). We usually start with a 5 μmol/L concentration of ADP in testing aggregation. If no response is seen, higher concentrations of ADP, up to 10 μmol/L, are added. Alternatively, if a full tracing is seen at a 5 μmol/L concentration of ADP, lower concentrations of agonists are added to map out the threshold point between primary and secondary platelet aggregation. The lowest concentration of ADP giving both primary and secondary wave aggregation and adequate secretion of the ^{14}C-serotonin label, as determined by falling within our reference range, is the threshold concentration ❚Table 1❚.

Epinephrine-Induced Platelet Aggregation

Epinephrine also is considered a weak agonist that aggregates platelets ❚Image 2❚. Shape change usually is not noted for epinephrine-induced platelet aggregation. Image 2 shows that after an initial stabilization of the baseline (indicated by
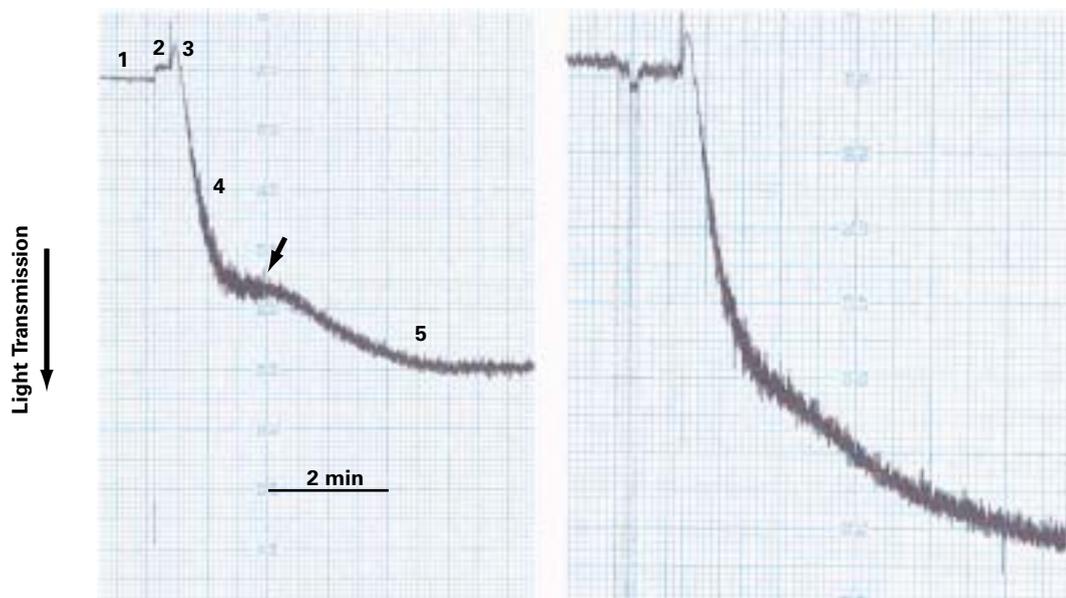

❚Image 1❚ Adenosine diphosphate (ADP)-induced platelet activation. Left, 5 μmol/L concentration of ADP. The baseline of light transmission before the instillation of the agonist is below "1." The "2" to the left of the spike indicates when the platelet agonist ADP was added. The decrease in light transmittance to the left of the "3" indicates platelet shape change as a result of ADP binding to platelets. The curve with increasing light transmittance to the left of "4" indicates primary wave aggregation. The slight positive inflection at the bottom of the "4" curve at the arrow indicates the beginning of secondary wave platelet aggregation. The curve below "5" is the secondary wave of aggregation. Right, 7.5 μmol/L concentration of ADP. All waveforms as seen in the left part of the image are seen except the inflection that begins the second wave of platelet aggregation. This image is a representative image of ADP-induced platelet aggregation at and above the threshold for secondary wave platelet aggregation.

"1"), primary wave aggregation (indicated by "3") begins after the instillation of the agonist (indicated by "2"), followed by secondary wave aggregation (indicated by "4"). The starting concentration of epinephrine to determine its threshold for platelet activation usually is 5 μmol/L. If the tracing shows primary and secondary waves, the threshold below a 5 μmol/L concentration is determined. Alternatively, if no response or a blunted response is seen at a 5 μmol/L concentration of epinephrine, concentrations up to 10 μmol/L are tested to determine the threshold response to the agonist.

Collagen- and Arachidonic Acid–Induced Platelet Aggregation

Platelet aggregation in response to collagen typically displays a longer lag phase from the instillation of the agonist (indicated by "2") to shape change (indicated by "3") than that seen with ADP or thrombin (indicated by "4") ❚**Image 3**❚ (left). Collagen does not induce a biphasic change in aggregometer tracings as do ADP and epinephrine (Image 3, left). Collagen at 5 μg/mL is considered a potent agonist that should induce platelet activation in all but the most seriously impaired platelets. Lower doses of collagen could be used (eg, 1.25 μg/mL) to induce platelet activation, but defects in ADP or epinephrine pathways (eg, aspirin treatment) also might impair collagen-induced platelet activation at this concentration.

The addition of arachidonic acid examines whether platelets are able to produce thromboxanes (Image 3, right). A final concentration of 500 μg/mL of arachidonic acid often is used in platelet function testing with a single response wave observed (indicated by "4") (Image 3, right). The addition of arachidonic acid (indicated by "2") induces a shape change (indicated by "3") from the initial stabilization of the baseline
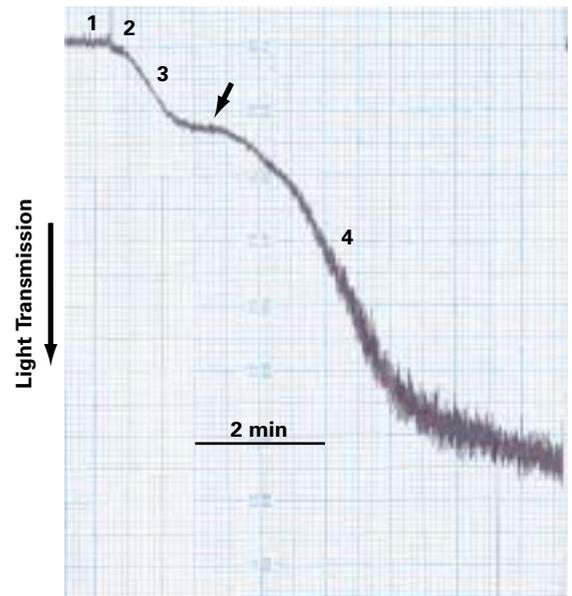

❚**Image 2**❚ Epinephrine-induced platelet activation. The baseline of light transmittance is indicated by "1," followed by the instillation of the agonist epinephrine indicated by the spike to the left of "2." The tracing below "3" shows the curve of primary wave aggregation. The inflection indicated by the arrow and tracing below "4" show the initiation and propagation of secondary wave aggregation, respectively. The image is a representative tracing of epinephrine-induced platelet aggregation.

(indicated by "1"). Arachidonic acid–induced platelet activation is blocked by aspirin treatment. Confirmation that the arachidonic acid pathway is inhibited by aspirin or another agent can be obtained by using the prostaglandin analog U46619 as an additional platelet agonist (data not shown).

❚**Table 1**❚
Normal Values for Platelet Function Studies*

	Mean ± SD†	Range
Arachidonic acid aggregation, % (500 μg/mL)	69 ± 16	36-101
Arachidonic acid secretion, %	35 ± 16	2-67
ADP threshold for aggregation, μmol/L	5.5 ± 1.7	1-7.5
ADP secretion, %	47 ± 11	24-69
Epinephrine threshold for aggregation, μmol/L	5.8 ± 1.6	0.5-10
Epinephrine secretion, %	41 ± 19	16-66
Collagen aggregation, % (5 μg/mL)	80 ± 6.4	63-84
Collagen secretion, %	68 ± 16	41-83
γ-Thrombin aggregation, %‡	74 ± 7.8	58-89
γ-Thrombin secretion, %	56 ± 16	23-88
Serotonin uptake, %	90.7 ± 3.6	75-97
Ristocetin, 1.2 mg/mL	Positive	—
Ristocetin, 0.6 mg/mL	Negative	—

ADP, adenosine diphosphate.

* Data were generated by the examination of samples from 23 individuals who were healthy by history and had not been taking medication recently when samples were obtained for study. All individuals included in the evaluation responded to epinephrine-induced platelet activation.
† For results from 23 healthy individuals.
‡ The γ-thrombin concentration for the threshold varied from 14 to 166 nmol/L. The values represent the percentage of aggregation seen at the threshold thrombin concentration.
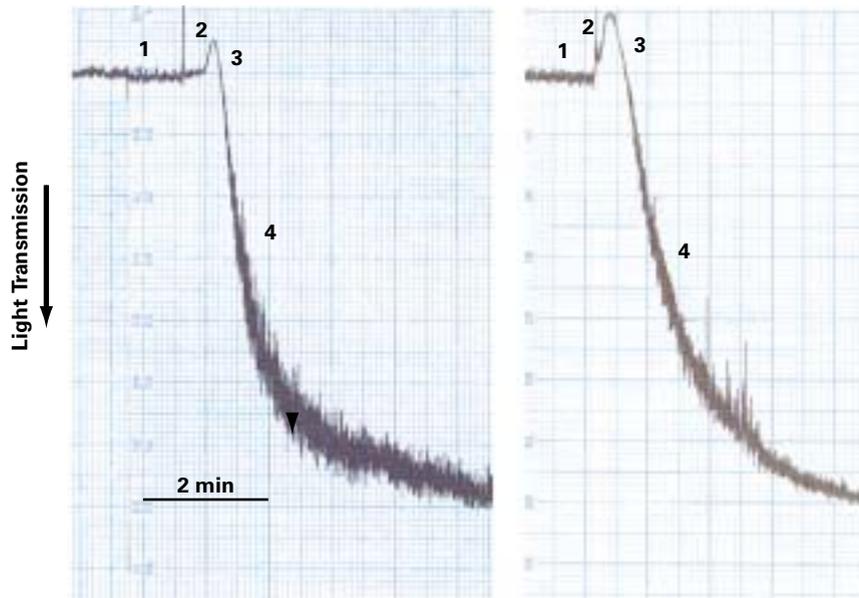
Image 3 Collagen-induced (left) and arachidonic acid–induced (right) platelet activation. Platelet aggregation in response to collagen (5 μg/mL) is characterized by a prolonged lag phase between the addition of the collagen, a spike to the left of "2," and shape change, to the left of "3." A single wave response is shown below "4." "1" represents the stabilization of the baseline. Platelet aggregation in response to arachidonic acid (500 μg/mL) is shown after stabilization of the baseline, indicated by "1." The instillation of the agonist arachidonic acid is indicated by the spike to the right of "2." The induction of shape change is to the left of "3," and the single wave aggregation response is shown by the curve labeled "4." These images are representative of collagen- and arachidonic acid–induced platelet aggregation, respectively.

Normal values for collagen and arachidonic acid in our laboratory are shown in Table 1.

Thrombin-Induced Platelet Aggregation

Thrombin activates 2 receptors on platelets, PAR1 and PAR4. However, the activation of these receptors is not seen specifically in an aggregometer tracing. There is little published clinical experience of the use of γ-thrombin to assess platelet activation in patient samples. γ-Thrombin activates platelets in PRP by proteolyzing PAR4 and PAR1. The amount of γ-thrombin necessary to induce threshold platelet activation varies by the preparation of γ-thrombin and the individual's platelets. Platelet aggregation in response to low and high concentrations of γ-thrombin is shown in **Image 4**. In Image 4 (left), a 57 nmol/L concentration of human γ-thrombin was insufficient to induce platelet aggregation, although it induced shape change on binding. Alternatively, in Image 4 (right), the instillation of a 114 nmol/L concentration of γ-thrombin (indicated by "2") induced a shape change (indicated by "3"), followed by a single wave aggregation curve (indicated by "4").

Ristocetin-Induced Platelet Aggregation

Two concentrations of ristocetin were used to determine the presence and integrity of GPIbα-V-IX complex on platelets and vWF in the PRP **Image 5**. All normal platelets should respond to 1.2 mg/mL of ristocetin (Image 5, left) but not to 0.6 mg/mL (Image 5, right). The tracing seen with the addition of the higher concentration of ristocetin actually is initially platelet-platelet agglutination, which on close contact induces intracellular platelet activation and subsequent platelet aggregation.

Normal Platelet Function Studies

The mean values and range for aggregation and secretion for each of the platelet agonists is shown in Table 1. The mean agonist concentration that initiates primary and secondary wave aggregation (ie, threshold concentration) is given for ADP and epinephrine (Table 1). The threshold concentration for primary and secondary wave aggregation with ADP is quite narrow in a healthy population, usually from concentrations of 1 to 7.5 μmol/L (Table 1). The range of concentrations of epinephrine to induce both primary and secondary wave aggregation was wide, from 0.5 to 10 μmol/L (Table 1).

The range of ^{14}C-serotonin secretion for ADP and epinephrine at threshold second wave aggregation is given. These latter values are clinically useful because not infrequently, one cannot see the inflection of secondary wave aggregation with ADP. Knowing that the level of secretion is within the normal range at an agonist concentration between 1 and 7.5 μmol/L indicates that secondary wave aggregation must have occurred because ADP induces aggregation-dependent secretion.[7]
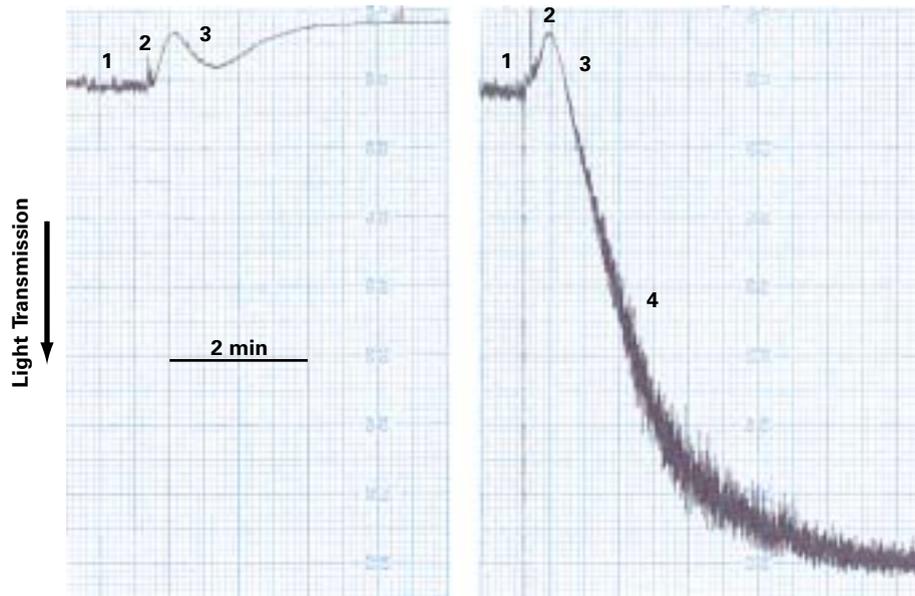
❚Image 4❚ Thrombin-induced platelet activation. Low concentration of γ-thrombin (57 nmol concentration; left) binds to platelets and elicits shape change (ie, the decrease in light transmittance) but is insufficient to induce platelet aggregation. A higher concentration of γ-thrombin (114 nmol/L concentration; right) after stabilization of the baseline as indicated by "1" and addition of the agonist indicated by the spike to the left of "2" induce platelet shape change shown to the left of "3" and a full aggregation response as shown by "4." The image is a representative tracing from a single platelet donor.
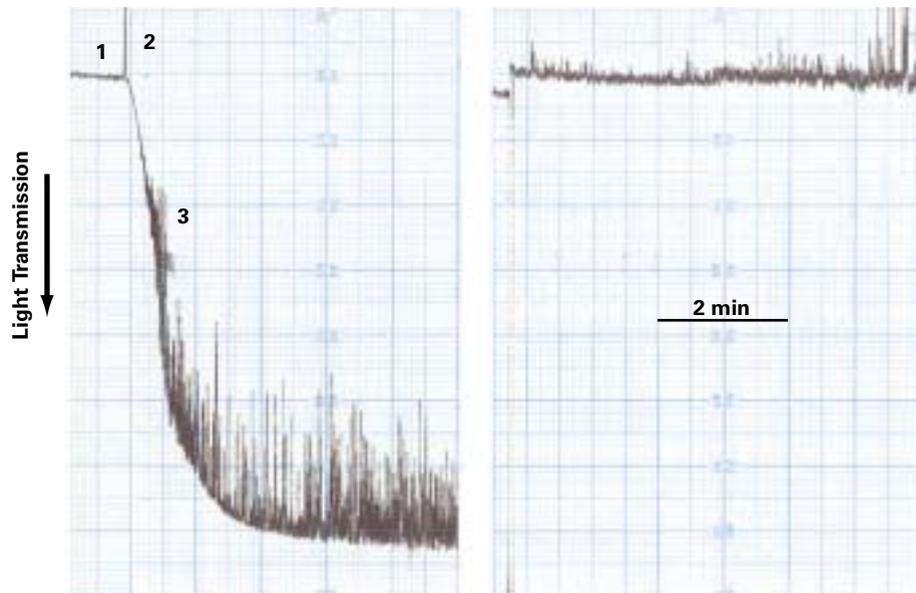

❚Image 5❚ Ristocetin-induced platelet activation. Normal platelets from healthy donors should have a platelet response to high-dose ristocetin (1.2 mg/mL; left) but not to the lower dose of ristocetin (0.6 mg/mL; right). The image is a representative tracing from a single donor. After stabilization of the baseline ("1") and addition of the agonist (spike to the left of "2"), a full agglutination-aggregation pattern is seen as indicated by "3." No shape change is seen with ristocetin-induced platelet activation.

Because only a single concentration of arachidonic acid and collagen are used for these studies, percentage of aggregation, which is the percentage chart recorder deflection from baseline, is given for arachidonic acid and collagen. Arachidonic acid–induced platelet aggregation and secretion have values as low as 36% and 2%, respectively, in a normal sample (Table 1).

Although the threshold concentration of γ-thrombin-induced platelet aggregation was determined, the result is reported as percentage of aggregation at the threshold concentration

of the agonist. In our healthy population, threshold concentrations for γ-thrombin were quite variable over a wide range of concentrations. Therefore, we decided to present the percentage of aggregation at the threshold concentration of the agonist. Most healthy individuals have a serotonin uptake of 90% or more. However, 2 healthy individuals had a serotonin uptake as low as 75%, requiring us to use that value as the lower limit of normal, before we became concerned about a possible dense granule storage problem.

Additional data were generated from the studies on samples from healthy subjects ❘Table 2❘. For each agonist, the reaction time, which is the interval to aggregation from the instillation of the agonist to shape change, was determined. Collagen is known to have the longest reaction time (Table 2). Furthermore, the initial aggregation rate during the first minute was determined. Collagen and ADP had the steepest aggregation rates, and epinephrine had the slowest reaction time (Table 2).

Patient Studies

From January 2003 until March 2004, our laboratory performed a total of 61 tests of platelet aggregation and secretion on referred patients. Of these, 37 tests yielded abnormal responses, whereas results of the remaining 24 studies fell within our normal ranges ❘Table 3❘. Diagnoses were classified based on the criteria described in the "Materials and Methods" section. Of the 37 platelet aggregation tests with abnormal results, 12 patients had an aspirin-like defect characterized by an abnormal arachidonic acid response and variable defects in response to ADP and/or epinephrine. Nine patients had combined ADP and epinephrine defects, a so-called defect to weak platelet agonists, with a normal response to arachidonic acid, collagen, and thrombin. Six individuals had epinephrine-only defects but normal responses to arachidonic acid, ADP, collagen, and thrombin. Four individuals had defects only in ADP-induced platelet aggregation. Two individuals had low serotonin uptake, consistent with a storage pool disorder. One of these individuals subsequently was found to be taking a serotonin release inhibitor drug. Two referrals had a pan-secretion defect with abnormal aggregation and secretion to ADP and epinephrine and a reduced response to collagen. Last, 2 patients had acquired platelet function defects due to medication use that was revealed after close questioning.

Discussion

The present report aims to present in detail how one institution performs its platelet aggregation and secretion studies in the clinical laboratory. Platelet aggregation and secretion studies have many variables, some of which can be ameliorated by the reproducible performance of the assay.[28]

❘Table 2❘
Normal Values for Reaction Time and Initial Aggregation Rate of Platelet Agonists in Samples From 23 People

	Mean ± SD Reaction Time (s)[*]	Mean ± SD Initial Aggregation Rate (%/min)[†]
Adenosine diphosphate	6.7 ± 1.8	77.0 ± 21.9
Epinephrine	—	17.4 ± 4.2
Collagen	32.0 ± 5.0	92.8 ± 17.9
Arachidonic acid	20.4 ± 8.5	51.3 ± 14.9
Thrombin[‡]	13.5 ± 2.1	61.0 ± 15.3

[*] Seconds from the instillation of the agonist to the initiation of shape change. No reaction time is given for epinephrine because there was no shape change.
[†] Percentage of aggregation after shape change or baseline per minute in light transmittance.
[‡] Calculated with results for 21 people.

❘Table 3❘
Diagnosis of Platelet Function Defects in Patients Studied

Diagnosis[*]	No. of Patients
Normal	24
Abnormal	37
Aspirin-like defect	12
ADP-only defect	4
Epinephrine-only defect	6
ADP and epinephrine defect	9
Pan-secretion defect	2
Storage pool disease	2
Medication	2
Total	61

ADP, adenosine diphosphate.
[*] See the "Materials and Methods" section for criteria for the diagnoses.

The present investigation, unlike previous studies, relied heavily on the simultaneous performance of platelet aggregation and secretion studies, rather than platelet aggregation alone.[19] The secretion results support the findings seen on aggregation, serving as an independent, simultaneous report of the findings. The use of secretion results for interpretation is especially helpful in determining whether there is a second wave of aggregation to ADP.

In our laboratory, platelet secretion is measured by the liberation of ^{14}C-serotonin from previously labeled platelets. Setting up for the use of ^{14}C labels might not be convenient for all laboratories. Measuring the secretion of platelet adenosine triphosphate can be an alternative.[20,21]

Furthermore, this report, unlike previous reports, presents a simple, working descriptive classification of platelet function disorders that usually characterize the majority of disorders in patients being evaluated in the clinical laboratory.[19-21] This classification describes the clinical laboratory findings but does not diagnose specifically the functional defect in the patient's platelets.

Platelet function defects account for about 10% of cases in which patients have prolonged bleeding times not due to medication. Certain congenital defects in platelet function

have well-characterized patterns of abnormality in platelet function studies. These well-described platelet function defects include BSS and Glanzmann thrombasthenia. These congenital platelet function defects are rare but occasionally are seen at large referral centers that perform platelet function studies. Storage pool disorders also can be recognized in platelet function testing. Most patients with von Willebrand disease have normal platelet aggregation study results, except patients with markedly decreased vWF and patients with type 2B von Willebrand disease who have a heightened response to ristocetin, ie, response to a lower concentration of ristocetin.

Bernard-Soulier Syndrome

BSS arises from gene or protein defects in the GPIb-IX-V complex. Molecular defects have been shown in GPIbα, GPIbβ, and GPIX. These defects can cause totally absent expression of the complex or partial expression of the functional or dysfunctional complex on the surface of platelets. Laboratory features of BSS include thrombocytopenia, a prolonged bleeding time, giant platelets, and lack of platelet response to ristocetin. Responses to other agonists are normal, with the exception of low doses of thrombin. Acquired BSS can be seen as result of antibodies arising to medication.

Glanzmann Thrombasthenia

Glanzmann thrombasthenia is produced by reduction or defects in the fibrinogen receptor (GPIIb-IIIa, ie, $\alpha_{2b}\beta_3$ integrin) expression. It is characterized by lack of or reduction in platelet aggregation to all agonists because fibrinogen cannot bind to produce a platelet aggregate. Platelet responses to ristocetin and vWF are normal, and platelet secretion to collagen and thrombin remains existent, although at a reduced level because there is no additional burst in secretion resulting from platelet aggregation.

von Willebrand Disease

A certain minority of patients with von Willebrand disease have a characteristic platelet function pattern of reduced or absent ristocetin-induced platelet agglutination and activation with normal platelet responses to ADP, epinephrine, and collagen. The majority of patients with von Willebrand disease have a normal platelet aggregation phenotype. Platelet function studies cannot exclude any individual from the diagnosis of von Willebrand disease. Patients with type 2B von Willebrand disease characteristically respond to low-dose ristocetin, whereas healthy individuals and other patients do not.

Storage Pool Disease

Storage pool disease is a group of heterogeneous disorders that are characterized into 3 groups by electron microscopy: α granule storage pool disease (α-SPD), dense granule storage pool disease (δ-SPD), and both (αδ-SPD).

Platelet storage pool disorders are the most common inherited platelet function defect.

α-SPD, or gray platelet syndrome, is caused by a deficiency of α granules in megakaryocytes and platelets. The characteristic microscopic findings are enlarged, gray platelets devoid of normal α-granule staining. Patients with this disease have reduced quantities of platelet-specific proteins such as platelet factor 4/β-thromboglobulin.

δ-SPD can be a primary defect or, more commonly, a secondary defect that often is associated with congenital abnormalities such as Hermansky-Pudlak syndrome, Chédiak-Higashi syndrome, or Wiskott-Aldrich syndrome. It also can arise in acquired platelet disorders such as immune thrombocytopenia. Platelet function disorders seen in these patients include abnormal serotonin uptake owing to impaired storage in platelet dense granules and, often but not always, a lack of secondary wave aggregation in response to ADP or epinephrine.[29,30] However, when performing serotonin secretion studies, it is possible to observe that the percentage of secretion of the dense granule label is normal even though the actual uptake is reduced markedly.

Defective Platelet Procoagulant Activity

Defective platelet procoagulant activity (eg, Scott syndrome) is an extremely rare disorder caused by impaired activation of enzymes required for the exposure of the phosphatidylserine on the outer layer of the membrane producing a procoagulant surface.[31,32] Patients with Scott syndrome have severe bleeding disorders. The serum prothrombin time is abnormal, and platelet microvesiculation is impaired. Patients also have reduced factor Xa binding to the activated platelet surface.

Acquired Platelet Dysfunctions

There are an enormous number of causes of acquired platelet dysfunction. The most common cause is medication, prescribed or over-the-counter, which should be revealed in the patient's history. Patients should not undergo platelet function studies until the medications have been discontinued for a sufficient time. Similarly, a number of medical conditions can be associated with acquired platelet dysfunctions. The reader is referred to additional sources for a complete discussion of acquired platelet function disorders.[19-21,33]

Although the aforementioned hereditary platelet function defects are well characterized by platelet aggregometry, the conditions are quite rare. During the last year, only 2 of the 61 patients referred for platelet function studies had a history or platelet aggregation and secretion testing results consistent with a platelet function disorder as characterized in the preceding text (eg, reduced serotonin uptake consistent with storage pool disorder). In our hands, the majority of patients studied had defects that fell outside these classic platelet function

disorders. This result is distinguished from a study in the Netherlands that reported storage pool disorder as the most common platelet function defect seen there.[34] Most platelet function test abnormalities for patients not taking medication fall into the category of a defect in the intracellular signaling pathways of platelets and platelet secretion.[35] Some criteria need to be developed to classify these patients into various groups.

It has been noted that up to 15% of healthy people will exhibit only a primary wave aggregation response to epinephrine.[36-39] Other laboratories have found that incubation times of 10 minutes or longer detect more epinephrine responders than 5-minute incubation times. Some so-called healthy individuals are found to have a decreased or deficient number of α_2-adrenergic receptors in their platelets but are otherwise healthy. We did not include subjects with such characteristics in our determination of normal values. We found only 1 individual (1/24 [4%]) who otherwise was healthy and who exhibited a primary but no secondary wave aggregation response to epinephrine. Because our normal data were only from individuals who responded to epinephrine, one may argue that our "normal" population is skewed. This criticism is acceptable, but it is impossible to include nonresponders to epinephrine in a normal group. Alternatively, an individual with no response to epinephrine and no bleeding history should not be considered "abnormal" under current clinical testing. Any unusual epinephrine responses, including an absent secondary wave aggregation, should be evaluated carefully based on a thorough review of the patient's clinical history. Abnormal platelet aggregation stimulated by epinephrine frequently is observed in patients with chronic myeloproliferative disorders.[40] Patients with myeloproliferative syndromes also can show a large number of platelet abnormalities, including a decrease in the platelet content of serotonin and adenine nucleotides, defects in arachidonic metabolism or membrane glycoprotein, and abnormal aggregation with one or more agonists, with epinephrine the most frequently encountered.[41-43]

Of 37 patients with abnormal platelet aggregation studies, abnormal results for 12 were due to an aspirin-like defect. It is the most commonly seen platelet defect in patients referred for platelet studies, which is consistent with the findings in other studies.[44] It also is observed in up to 9% of platelet donors.[45] Most of these cases presumably are due to intake of cyclooxygenase inhibitors that is not remembered at the time of history taking and rarely to inherited defects in cyclooxygenase or thromboxane synthetase.[46] Certainty that an individual has a defect in the arachidonic acid pathway in platelets can be obtained by the use of the prostaglandin U46619.

There is no current agreement on the doses of agonists to be used in platelet function testing. We chose to determine threshold agonist doses for ADP and epinephrine as the minimal concentration of agonist to have primary and secondary wave aggregation. Some institutions use a single, higher concentration of ADP, eg, a 20 µmol/L concentration, thinking

that if the patient does not respond to this concentration of ADP, there must be a serious abnormality. However, the choice of agonist concentration to use is arbitrary.

Threshold doses also were used for γ-thrombin, but the result was reported as percentage of aggregation. This agonist is reported in this manner because the concentration of γ-thrombin for threshold aggregation might vary with the γ-thrombin preparation. γ-Thrombin is inherently unstable and might not be attractive for use as a routine reagent because its activity might vary over time, even when frozen in small aliquots at –70°C. Furthermore, some γ-thrombin preparations might be contaminated with α-thrombin, giving skewed results. As an alternative, use of the agonist peptides for PAR1 and PAR4 examines thrombin-induced signaling but not binding to and cleavage of the exodomains of its receptors. Therefore γ-thrombin is a more practical and global thrombin receptor activator, but it has inherent instability and, if not used in a critical manner, can be unreliable.

A higher concentration of collagen was used because mild platelet defects that interfere with ADP- or epinephrine-induced platelet aggregation also interfere with low-dose collagen-induced platelet aggregation.

Platelet aggregation and secretion studies are used routinely in specialized coagulation laboratories. However, there is no agreement as to how they should be performed, standardized, and reported. This fact usually makes interpretation of these platelet function data between laboratories impossible. Efforts should be made to create uniform means to study and interpret platelet aggregation and secretion studies.

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References

1. Mills DC. ADP receptors on platelets. *Thromb Haemost.* 1996;76:835-856.

2. Bauer SM. ADP receptor antagonists as antiplatelet therapeutics. *Expert Opin Emerg Drugs.* 2003;8:93-101.

3. Jantzen HM, Gousset L, Bhaskar V, et al. Evidence for two distinct G-protein-coupled ADP receptors mediating platelet activation. *Thromb Haemost.* 1999;81:111-117.

4. Hollopeter G, Jantzen HM, Vincent D, et al. Identification of the platelet ADP receptor targeted by antithrombotic drugs. *Nature.* 2001;409:202-207.

5. Erhardt JA, Pillarisetti K, Toomey JR. Potentiation of platelet activation through the stimulation of P2X1 receptors. *J Thromb Haemost.* 2003;1:2626-2635.

6. Wallace MA, Agarwal KC, Garcia-Sainz JA, et al. Alpha-adrenergic stimulation of phosphatidylinositol synthesis in human platelets as an alpha-2 effect secondary to platelet aggregation. *J Cell Biochem*. 1982;18:213-220.

7. Charo IF, Feinman RD, Detwiler TC. Interrelations of platelet aggregation and secretion. *J Clin Invest*. 1977;60:866-873.

8. Rao AK, Willis J, Kowalska MA, et al. Differential requirements for platelet aggregation and inhibition of adenylate cyclase by epinephrine: studies of a familial platelet alpha 2-adrenergic receptor defect. *Blood*. 1988;71:494-501.

9. Clemetson KJ, Clemetson JM. Platelet collagen receptors. *Thromb Haemost*. 2001;86:189-197.

10. Santoro SA. Platelet surface collagen receptor polymorphisms: variable receptor expression and thrombotic/hemorrhagic risk. *Blood*. 1999;93:3575-3577.

11. Watson S, Berlanga O, Best D, et al. Update on collagen receptor interactions in platelets: is the two-state model still valid? *Platelets*. 2000;11:252-258.

12. Vu T-K, Hung DT, Wheaton VI, et al. Molecular cloning of a functional thrombin receptor reveals a novel proteolytic mechanism of receptor action. *Cell*. 1991;64:1057-1068.

13. Nystedt S, Emilsson K, Wahlestedt C, et al. Molecular cloning of a potential protease activated receptor. *Proc Natl Acad Sci U S A*. 1994;91:9208-9212.

14. Xu W-F, Andersen H, Whitmore TE, et al. Cloning and characterization of human protease activated receptor 4. *Proc Natl Acad Sci U S A*. 1998;95:6642-6646.

15. Khan ML, Zhen YW, Bigornia V, et al. A dual thrombin receptor system for platelet activation. *Nature*. 1998;394:690-694.

16. Covic L, Gresser AL, Kuliopulus A, et al. Biphasic kinetics of activation and signaling for PAR-1 and PAR-4 thrombin receptors in platelets. *Biochemistry*. 2000;39:5458-5467.

17. Born GVR. Aggregation of blood platelets by adenosine diphosphate and its reversal. *Nauchni Tr Vissh Med Inst Sofiia*. 1962;194:927-929.

18. O'Brian JR. Platelet aggregation, II: some results from a new method of study. *J Clin Pathol*. 1962;15:452-455.

19. Triplett DA. *Platelet Function: Laboratory Evaluation and Clinical Application*. Chicago, IL: ASCP Press; 1978.

20. Miller JL. Blood platelets. In: Henry JB, ed. *Clinical Diagnosis and Management by Laboratory Methods*. 20th ed. Philadelphia, PA: Saunders; 2001:632-641.

21. Miller JL. Congenital platelet dysfunction and von Willebrand disease. In: Shumacher HR, Rock WA, Stass SA, eds. *Handbook of Hematologic Pathology*. New York, NY: Marcel Dekker; 2000:591-610.

22. Faruqi TR, Weiss EJ, Shapiro MJ, et al. Structure-function analysis of protease-activated receptor 4 tethered ligand peptides. *J Biol Chem*. 2000;275:19728-19734.

23. Schmaier AH, Claypool W, Colman RW. Crotalocytin: recognition and purification of a timber rattlesnake platelet aggregating protein. *Blood*. 1980;56:1013-1019.

24. Colman RW, Schreiber AD. Effect of heterologous antibody on human platelets. *Blood*. 1976;48:119-131.

25. Jerushalmy Z, Zucker MB. Some effect of fibrinogen degradation products (FDP) on blood platelets. *Thromb Diath Haemorrh*. 1966;15:413-419.

26. Walsh PN, Gagnatelli G. Platelet antiheparin activity: storage site and release mechanism. *Blood*. 1974;44:157-168.

27. Schmaier AH, Zuckerberg A, Silverman C, et al. High-molecular weight kininogen. *J Clin Invest*. 1983;71:1477-1489.

28. Newhouse P, Clark C. The variability of platelet aggregation. In: Triplett DA, ed. *Platelet Function: Laboratory Evaluation and Clinical Application*. Chicago, IL: ASCP Press; 1978:63-107.

29. Weiss HJ, Chervenick PA, Zalusky R, et al. A familial defect in platelet function associated with impaired release of adenosine diphosphate. *N Engl J Med*. 1969;281:1264-1270.

30. Holmsen H, Weiss HJ. Hereditary defect in the platelet release reaction caused by a deficiency in the storage pool of platelet adenine nucleotides. *Br J Haematol*. 1970;19:643-649.

31. Weiss HJ. Scott syndrome: a disorder of platelet coagulant activity. *Semin Hematol*. 1994;31:312-319.

32. Stout JG, Basse F, Luhm RA, et al. Scott syndrome erythrocytes contain a membrane protein capable of mediating Ca2+-dependent transbilayer migration of membrane phospholipids. *J Clin Invest*. 1997;99:2232-2238.

33. Rao AK. Acquired qualitative platelet defects. In: Colman RW, Hirsh J, Marder VJ, et al, eds. *Hemostasis and Thrombosis*. Philadelphia, PA: Lippincott; 2001:905-919.

34. Nieuwenhuis HK, Akkerman JWN, Sixma JJ. Patients with a prolonged bleeding time and normal aggregation tests may have storage pool deficiency: studies on one hundred six patients. *Blood*. 1987;70:620-623.

35. Rao AK. Inherited defects in platelet signaling mechanisms. *J Thromb Haemost*. 2003;1:671-681.

36. Weiss HJ, Lages B. The response of platelets to epinephrine in storage pool deficiency: evidence pertaining to the role of adenosine diphosphate in mediating primary and secondary aggregation. *Blood*. 1988;72:1717-1725.

37. Kambayashi J, Shinoki N, Nakamura T, et al. Prevalence of impaired responsiveness to epinephrine in platelets among Japanese. *Thromb Res*. 1996;81:85-90.

38. Scrutton MC, Clare KA, Hutton RA, et al. Depressed responsiveness to adrenaline in platelets from apparently normal human donors: a familial trait. *Br J Haematol*. 1981;49:303-314.

39. Tamponi G, Pannocchia A, Arduino C, et al. Congenital deficiency of alpha-2-adrenoceptors on human platelets: description of two cases. *Thromb Haemost*. 1987;58:1012-1016.

40. Kaywin P, McDonough M, Insel P, et al. Platelet function in essential thrombocythemia: decreased epinephrine responsiveness associated with a deficiency of platelet alpha-adrenergic receptors. *N Engl J Med*. 1978;299:505-509.

41. Raman BK, Van Slyck EJ, Riddle J, et al. Platelet function and structure in myeloproliferative disease, myelodysplastic syndrome, and secondary thrombocytosis. *Am J Clin Pathol*.1989;91:647-655.

42. Holme S, Murphy S. Platelet abnormalities in myeloproliferative disorders. *Clin Lab Med*. 1990;10:873-888.

43. Avram S, Lupu A, Angelescu S, et al. Abnormalities of platelet aggregation in chronic myeloproliferative disorders. *J Cell Mol Med*. 2001;5:79-87.

44. Remaley AT, Kennedy JM, Laposata M. Evaluation of the clinical utility of platelet aggregation studies. *Am J Hematol*. 1989;31:188-193.

45. Jilma-Stohlawetz P, Hergovich N, Homoncik M, et al. Impaired platelet function among platelet donors. *Thromb Haemost*. 2001;86:880-886.

46. Horellou MH, Lecompte T, Lecrubier C, et al. Familial and constitutional bleeding disorder due to platelet cyclo-oxygenase deficiency. *Am J Hematol*. 1983;14:1-9.