Platelet Aggregation Testing in Platelet-Rich Plasma

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

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Key Words: Platelet aggregation and secretion; Platelet disorders; Platelet function testing

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 A2 (TxA2), serotonin, and thrombin. Platelets have receptors for each of these agonists and a major integrin receptor (α2β3 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 P2Y1 receptor activates phospholipase C, induces shape change, and initiates primary wave platelet aggregation through calcium mobilization. The P2Y12 receptor is considered...
the major ADP receptor mediating the full platelet aggregation response to ADP through inhibition of adeny1 cyclase and stabilization of the platelet aggregates. P2Y12 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 P2X1, inotropic receptor is an ion gate channel present on platelets. Although it has been postulated that P2X1 potentiates platelet activation in combination with other platelet agonists, the function of P2X1 is not understood fully.5

The receptor on platelets for epinephrine is the α2-adrenergic receptor. Epinephrine causes inhibition of adeny1 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.3

Platelet arachidonic acid is the precursor of TxA2 and hydroxyl fatty acids. Various platelet agonists mobilize calcium through G-protein-coupled receptors. Calcium activates phospholipase A2, which liberates arachidonic acid from phosphatidylcholine and phosphatidylethanolamine. Calcium also activates myosin light-chain kinase. Arachidonic acid then is converted to TxA2 by cyclooxygenase and thromboxane synthase, which further mobilizes calcium from intracellular storage sites. TxA2 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 (α2β1 integrin), which contributes to platelet adhesion. The other collagen receptor is GPVI, which is responsible for platelet signaling and activation leading to TxA2 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 Gαq 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
and incubated 30 minutes at 37°C in capped tubes (see "14C-
serotonin for 30 minutes at 37°C in a capped tube.24,25 14C-sero-
totonin was added to the PRP suspension such that the total num-
er of counts per minute was between 5,000 and 7,000. At the end of the incubation, the 14C-serotonin–labeled PRP was treated
with imipramine (final concentration, 2 µmol/L) to prevent reup-
take of any lost serotonin.26 At the conclusion of platelet aggrega-
tion studies, 200 µL of 14C-serotonin–labeled activated platelets
from the aggregometer cuvette were added to a conical centrifuga-
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 14C-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 centrifu-
gation 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.

14C-Serotonin Labeling

PRP samples for secretion studies were incubated with 14C-
serotonin for 30 minutes at 37°C in a capped tube.24,25 14C-sero-
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er of counts per minute was between 5,000 and 7,000. At the end of the incubation, the 14C-serotonin–labeled PRP was treated
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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 aggre-
gometer cuvette is stirred, usually at the rate of 800 to 1,200 rpm.

A Teflon-coated magnetic stirrer was used that was recom-
manded by the equipment manufacturer. The stirring speed and
nature of the stirrer might vary by manufacturer of the equip-
ment. 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 transmitt-
cence 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

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.

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.

Preassay Variables

Because numerous medications can affect platelet func-
tion 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 14C-serotonin
and incubated 30 minutes at 37°C in capped tubes (see “14C-
Serotonin Labeling”). The cloudy yellow supernatant contain-
ing the platelets was removed carefully with a disposable plas-
tic 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)
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:

\[
\text{Percentage of Aggregation} = \left( \frac{\text{Highest Average Point of Deflection} - 20}{80} \right) \times 100.
\]

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:

\[
\text{Percentage of Serotonin Uptake} = \left( \frac{\text{Mean Total Count} - \text{Mean Supernatant Count}}{\text{Mean Total Count}} \times 100 \right)
\]

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

\[
\text{Percentage of Serotonin Secretion} = \left( \frac{\text{Sample Count} - \text{Mean Supernatant Count}}{\text{Mean Total Count} - \text{Mean Supernatant Count}} \times 100 \right).
\]

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 \(\mu\)g/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, \(\gamma\)-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 14C-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...
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 (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*

<table>
<thead>
<tr>
<th>Test</th>
<th>Mean ± SD</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arachidonic acid aggregation, % (500 µg/mL)</td>
<td>69 ± 16</td>
<td>36-101</td>
</tr>
<tr>
<td>Arachidonic acid secretion, %</td>
<td>35 ± 16</td>
<td>2-67</td>
</tr>
<tr>
<td>ADP threshold for aggregation, µmol/L</td>
<td>5.5 ± 1.7</td>
<td>1-75</td>
</tr>
<tr>
<td>ADP secretion, %</td>
<td>47 ± 11</td>
<td>24-69</td>
</tr>
<tr>
<td>Epinephrine threshold for aggregation, µmol/L</td>
<td>5.8 ± 1.6</td>
<td>0.5-10</td>
</tr>
<tr>
<td>Epinephrine secretion, %</td>
<td>41 ± 19</td>
<td>16-66</td>
</tr>
<tr>
<td>Collagen aggregation, % (5 µg/mL)</td>
<td>80 ± 6.4</td>
<td>63-84</td>
</tr>
<tr>
<td>Collagen secretion, %</td>
<td>68 ± 16</td>
<td>41-83</td>
</tr>
<tr>
<td>γ-Thrombin aggregation, %</td>
<td>74 ± 28</td>
<td>58-89</td>
</tr>
<tr>
<td>γ-Thrombin secretion, %</td>
<td>56 ± 16</td>
<td>23-88</td>
</tr>
<tr>
<td>Serotonin uptake, %</td>
<td>90.7 ± 3.6</td>
<td>75-97</td>
</tr>
<tr>
<td>Ristocetin, 1.2 mg/mL</td>
<td>Positive</td>
<td>—</td>
</tr>
<tr>
<td>Ristocetin, 0.6 mg/mL</td>
<td>Negative</td>
<td>—</td>
</tr>
</tbody>
</table>

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.
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 14C-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
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, 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 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 14C-serotonin from previously labeled platelets. Setting up for the use of 14C labels might not be convenient for all laboratories. Measuring the secretion of platelet adenosine diphosphate 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

### Table 2

<table>
<thead>
<tr>
<th>Cluster of Platelet Agonists in Samples From 23 People</th>
<th>Mean ± SD Reaction Time (s)</th>
<th>Mean ± SD Initial Aggregation Rate (%/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Adenosine diphosphate</em></td>
<td>6.7 ± 1.8</td>
<td>770 ± 21.9</td>
</tr>
<tr>
<td><em>Epinephrine</em></td>
<td>—</td>
<td>174 ± 4.2</td>
</tr>
<tr>
<td><em>Collagen</em></td>
<td>32.0 ± 5.0</td>
<td>92.8 ± 173</td>
</tr>
<tr>
<td><em>Arachidonic acid</em></td>
<td>20.4 ± 8.5</td>
<td>51.3 ± 14.9</td>
</tr>
<tr>
<td><em>Thrombin</em></td>
<td>13.5 ± 2.1</td>
<td>61.0 ± 15.3</td>
</tr>
</tbody>
</table>

1 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.
2 Percentage of aggregation after shape change or baseline per minute in light transmittance.
3 Calculated with results for 21 people.

### Table 3

<table>
<thead>
<tr>
<th>Diagnosis*</th>
<th>No. of Patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td>24</td>
</tr>
<tr>
<td>Abnormal</td>
<td>37</td>
</tr>
<tr>
<td>Aspirin-like defect</td>
<td>12</td>
</tr>
<tr>
<td>ADP-only defect</td>
<td>4</td>
</tr>
<tr>
<td>Epinephrine-only defect</td>
<td>6</td>
</tr>
<tr>
<td>ADP and epinephrine defect</td>
<td>9</td>
</tr>
<tr>
<td>Pan-secretion defect</td>
<td>2</td>
</tr>
<tr>
<td>Storage pool disease</td>
<td>2</td>
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<tr>
<td>Medication</td>
<td>2</td>
</tr>
<tr>
<td>Total</td>
<td>61</td>
</tr>
</tbody>
</table>

ADP: adenosine diphosphate.
* See the “Materials and Methods” section for criteria for the diagnosis. 

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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 GPIα and GPIb. 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 (GPIb-IIIα, ie, α2β1β3 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 absent, 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. 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 phosphatidylerine on the outer layer of the membrane producing a procoagulant surface. 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.

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. 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. 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. 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 α2-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. 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.

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. It also is observed in up to 9% of platelet donors. 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. 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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