Abbreviations: AA = arachidonic acid; ADP = adenosine diphosphate; ATP = adenosine triphosphate; GP = glycoprotein; NSAID = nonsteroidal anti-inflammatory drug; PAR = protease-activatable receptors; PRP = platelet-rich plasma; VWF = von Willebrand factor.

Index Terms: aggregometry; lumiaggregometry; platelets.

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George Fritsma MS MT(ASCP) is the Focus: Hemorrhagic Platelet Disorders editor.

Learning objectives
Upon completion of this article, the reader will be able to:
1. collect blood specimens for platelet aggregometry.
2. prepare platelet rich plasma for optical aggregometry.
3. recount the history and applications of the bleeding time test.
4. illustrate the principle of platelet aggregometry and lumiaggregometry.
5. apply several platelet aggregation agonists and compare results.

The Focus section seeks to publish relevant and timely continuing education for clinical laboratory practitioners. Section editors, topics, and authors are selected in advance to cover current areas of interest in each discipline. Readers can obtain continuing education credit (CE) through P.A.C.E.* by completing the tearout form/examination questions included in each issue of Clin Lab Sci and mailing it with the appropriate fee to the address designated on the form. Suggestions for future Focus topics and authors, and manuscripts appropriate for CE credit are encouraged. Direct all inquiries to the Clin Lab Sci Editorial Office, IC Ink, 858 Saint Annes Drive, Iowa City IA 52245. (319) 354-3861, (319) 338-1016 (fax). ic.ink@mchsi.com

Platelets are central to primary hemostasis, and platelet disorders manifest themselves with mucocutaneous (systemic) bleeding: petechiae, purpura, epistaxis (nosebleed), hematemesis (vomiting blood), and menorrhagia (uncontrolled menses). The accompanying article by Dr. Larry Brace entitled “Thrombocytopenia” describes the most common conditions in which platelet counts fall to hemorrhagic levels. In “Qualitative Platelet Disorders”, Dr. Brace describes hemorrhagic disorders in which platelet count is normal or mildly reduced but function is compromised. This introductory article describes platelet aggregometry and lumiaggregometry, the current laboratory means for diagnosing platelet disorders. These articles are adapted from chapters 43, 44, and 45 of Rodak BF, Fritsma GA, and Doig K, editors. Hematology: Clinical Principles and Applications, 3rd ed. Philadelphia: Saunders; In press. The textbook is due for publication March, 2007.

To determine the cause for mucocutaneous bleeding, a platelet count is performed, and the blood film is reviewed before beginning platelet function tests (see “Thrombocytopenia”).1 Functional platelet abnormalities are suspected when bleeding is present but the platelet count exceeds 50,000/μL (see “Qualitative Platelet Disorders”). Acquired platelet defects are associated with liver disease, renal disease, myeloproliferative disorders, myelodysplastic syndromes, myeloma, and drug therapy. Hereditary platelet functional disorders are less common, but provide models for physiological study. Platelet morphology is often a clue; Bernard-Soulier syndrome is associated with mild thrombocytopenia and large, gray platelets. The presence of large platelets with an elevated mean platelet volume often indicates rapid platelet turnover, such as in immune thrombocytopenic purpura or thrombotic thrombocytopenic purpura. Giant or bizarre platelets are seen in myeloproliferative disorders, acute leukemia, and myelodysplastic syndromes.

Bleeding time test for platelet function
The original test for platelet function is the bleeding time, still...
occasionally helpful for diagnosing an unexplored bleeding disorder, although it fails as a screen.2 Developed by Duke in 1910,3 modified by Ivy in 1941,4 and standardized by Mielke in 1969;5 the bleeding time fails to control for critical non-platelet variables of intra-capillary pressure, skin thickness, and length and depth of the wound.

The scientist places a blood pressure cuff on the upper arm and inflates it to 40 mm Hg to control intra-capillary pressure in the forearm. A site on the volar surface of the forearm distal from the antecubital crease is selected and cleansed. The skin thickness in this area varies only slightly among individuals, so results are theoretically comparable, provided the area is free of superficial blood vessels, rashes, hair, and scar tissue. A spring-loaded bleeding time lancet that produces a wound five mm long and one mm deep is placed firmly on the site parallel to the long axis of the arm and triggered. Simplate (Organon Teknika Corp, Durham NC) and Surgicutt (International Technidyne Corp, Edison NJ) are suitable bleeding time lancets. A stopwatch is started at the moment the lancet is triggered.6 The scientist safely discards the lancet and blots the wound with filter paper every 30 seconds, taking care that a fresh portion of the paper is exposed with each blot and that the paper contacts only the blood. Touching the wound dislodges the platelet plug and prolongs the result. Blotting continues until the bleeding stops, the watch is stopped, and the interval is recorded. The scientist places a pressure or “butterfly” bandage over the site to reduce scarring.

The reference interval for the Mielke bleeding time is two minutes to nine minutes. The bleeding time is prolonged in thrombocytopenia, qualitative platelet dysfunction, von Willebrand disease, a fibrinogenemia, severe hypofibrinogenemia, and vascular disorders. A single dose of aspirin causes a measurable prolongation of the bleeding time in about 50% of normal individuals. Owing to its poor predictive value for bleeding and its tendency to scar, the bleeding time has been largely replaced by optical and whole-blood platelet aggregometry, lumiaggregometry, and near-patient coagulometers.

SPECIMENS FOR PLATELET AGGREGOMETRY

Blood for platelet aggregometry or lumiaggregometry is collected in 3.2% sodium citrate and held at 18°C to 24°C until testing, as chilling destroys platelet activity. Aggregometry must be completed within three hours of specimen collection.7,8

Optical platelet aggregometers are designed to test platelet-rich plasma (PRP): plasma with a platelet count of 200 to 300 × 10^9/L. The freshly collected blood is centrifuged at 50g for 30 minutes with the stopper in place to maintain the pH. The supernatant PRP is transferred by plastic pipette to a clean plastic tube, and the tube is sealed and stored at 18°C to 24°C until the test is begun. PRP aggregometry is initiated 30 minutes after centrifugation and completed within three hours of the time of collection.9 To produce sufficient PRP, the original specimen must provide 12 mL to 15 mL of whole blood.10

For whole-blood lumiaggregometry, the fresh uncentrifuged blood specimen is mixed 1:1 with physiologic saline and tested immediately. If the platelet count is less than 200 × 10^9/L, the specimen is tested undiluted.11

PLATELET AGGREGOMETRY

Functional platelets adhere to subendothelial collagen, aggregate with each other, and secrete the contents of their α granules and dense granules. Normal adhesion requires intact platelet membranes and plasma von Willebrand factor. Normal aggregation requires that platelet membranes are intact, that plasma fibrinogen is available and that normal secretions come from platelet organelles. Platelet adhesion, aggregation, and secretion are assessed using in vitro platelet aggregometry.
Optical platelet aggregometry
An optical aggregometer measures platelet aggregation in PRP. The operator pipettes aliquots to several cuvettes, drops a plasticized stir bar into each, and allows the aliquots to warm undisturbed to 37°C for ten minutes. The first cuvette, containing PRP and stir bar, is transferred to the instrument's reaction well. The operator starts the stirring device and recording device, often a desktop computer. The stir bar turns at 800 RPM to 1200 RPM, keeping the platelets in suspension. After a few seconds, an agonist (aggregating agent) is pipetted forcibly into the specimen to start the reaction. Aggregation is complete in six minutes to ten minutes.

The aggregometer directs focused light through the sample cuvette to a photomultiplier (Figure 1). Light transmittance (absorbance) is monitored continuously and recorded (Figure 2). As the PRP is stirred, the recorder first stabilizes to print the baseline, near 0% transmission. After the agonist is added, the platelets' shape changes from discoid to irregular and small aggregates form. The intensity of the transmitted light increases moderately. As the platelets secrete granule contents, larger aggregates form, more light passes, and the tracing moves toward 100% light transmittance. Abnormalities are reflected in diminished aggregation.

Whole-blood platelet aggregometry
In whole-blood aggregometry, aggregation is measured by electrical impedance. Parallel electrodes that produce a small direct current are suspended within the saline-diluted blood. As aggregation occurs, platelets collect on the electrodes, impeding the current (Figure 3). The change is amplified and recorded.

Whole-blood aggregometry eliminates the need for PRP. The operator pipettes an aliquot of properly mixed whole blood to the cuvette and adds an equal volume of physiologic saline and a stir bar. After the suspension has warmed to 37°C, the cuvette is placed in the reaction well, the agonist is added, and the electrodes are placed within the mixture. A whole-blood aggregometry tracing resembles a PRP tracing.

Platelet lumiaggregometry
The lumiaggregometer may be used to measure platelet aggregation and secretion simultaneously. The instrument, available from Chrono-log Corporation (Havertown PA) records aggregation and secretion of dense granule adenosine triphosphate (ATP). Released ATP oxidizes a firefly-derived luciferin-luciferase reagent (Chrono-lume; Chrono-log Corporation) to generate proportional chemiluminescence. The instrument detects, amplifies, and records the light emission.

Lumiaggregometry may be performed using whole blood or PRP. The operator adds an ATP standard to the first aliquot, then adds luciferin-luciferase and tests for full luminescence. A second aliquot is prepared with the luciferin-luciferase, an agonist is added, and the specimen is monitored simultaneously for aggregation and secretion. Thrombin is typically the first agonist used because it reliably induces full secretion. The luminescence induced by thrombin addition is measured, recorded, and used for comparison with the luminescence produced by other agonists. Secretion induced by other agonists produces luminescence at about 50% of that resulting from thrombin stimulus. Figure 4 depicts simultaneous aggregation and secretion responses to thrombin.

AGONISTS (ACTIVATING AGENTS) USED IN AGGREGOMETRY
The agonists used most in clinical practice are thrombin, adenosine diphosphate (ADP), epinephrine, collagen, arachidonic acid, and ristocetin. Table 1 lists representative concentrations and the receptors and activation pathways tested by each. Small volumes (two μL to five μL) of concentrated agonist are used so that they have little dilutional effect.
Thrombin
Thrombin cleaves two platelet membrane protease-activatable receptors (PAR), PAR-1 and PAR-2. Thrombin also cleaves glycoprotein (GP) Ibα and GP V. Activation involves G proteins and the eicosanoid and the diacylglycerol pathways and results in full secretion and aggregation. In lumiaggregometry, the operator ordinarily begins with one U/mL of thrombin. In normal platelets, thrombin induces the release of one to two $\mu$mol/L of ATP, detected by the firefly luciferin-luciferase luminescence assay. Other agonists, such as one or five $\mu$g/mL collagen, induce the release of 0.5 to 1 $\mu$mol/L ATP. Thrombin-induced secretion may be diminished to less than one $\mu$mol/L in storage pool deficiency (see “Qualitative Platelet Disorders”), but is relatively unaffected by membrane disorders or enzyme deficiencies.

Adenosine diphosphate
ADP binds the membrane receptors P2Y$_1$ and P2Y$_{12}$. ADP-induced platelet activation relies on the G protein response and the eicosanoid synthesis pathway to suppress adenyl cyclase activity and induce calcium mobilization to the platelet cytosol. The resulting increase in cytosolic free calcium mediates platelet activation and induces secretion of dense granule-stored ADP, which activates neighboring platelets.

ADP has been the most commonly used agonist, particularly in systems that measure only aggregation. When testing normal specimens, the ADP concentration may be adjusted to between one $\mu$mol/L and ten $\mu$mol/L to induce “biphasic” aggregation (Figure 3). At ADP concentrations near one $\mu$mol/L, platelets achieve primary aggregation, followed by disaggregation, a deflection from baseline that lasts one to two minutes and returns to baseline. Primary aggregation involves shape change with formation of microaggregates, both reversible. Secondary aggregation is full aggregate formation after release of platelet ADP. At ADP concentrations near ten $\mu$mol/L, there is simultaneous irreversible shape change, secretion, and formation of aggregates, resulting in a monophasic curve and full deflection of the tracing. In a biphasic tracing, reagent ADP induces shape change that is followed by a brief flattening of the curve called lag phase just before secretion of endogenous ADP begins.

Clinical laboratory scientists titer ADP concentration to generate the biphasic curve. This enables them to use aggregometry alone to detect platelet secretion, upon which the secondary wave depends. Lumiaggregometry provides a direct measure of secretion, as described above.

Aggregation to ADP (usually at five $\mu$mol/L) is diminished in platelet membrane disorders, cyclooxygenase pathway enzyme deficiencies, nonsteroidal anti-inflammatory drug (NSAID) and aspirin therapy and clopidogrel (Plavix®) therapy.

Epinephrine
Epinephrine binds platelet $\alpha$-adrenergic receptors, identical to muscle receptors, and activates the platelet through the same metabolic pathways as exogenous ADP. The results of epinephrine-induced aggregation match those of ADP except that epinephrine cannot induce aggregation in storage pool disorder or release defects no matter how high its concentration. The platelets of about 10% of normal individuals do not respond to epinephrine.

Collagen
Collagen binds GP Ia/IIa and GP VI, but induces no primary wave. After a lag of 30 to 60 seconds, aggregation begins, and a monophasic curve develops. Aggregation to collagen at one or five $\mu$g/mL requires intact membrane receptors, G protein integrity, and normal eicosanoid pathway function. Loss of collagen-induced aggregation may indicate a membrane abnormality, secretion defect, or the effects of aspirin and other NSAIDs.

<table>
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<th>Table 1. Platelet aggregometry agonists, reaction concentrations, and platelet receptors</th>
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Arachidonic acid
Arachidonic acid (AA) assesses the viability of the platelet eicosanoid synthesis pathway. Free AA at 500 ηmol/L is added to the specimen to induce a monophasic aggregometry tracing with virtually no lag phase. Aggregation is independent of membrane integrity. Deficiencies in eicosanoid pathway enzymes, including aspirin or NSAID-suppressed cyclooxygenase, result in reduced aggregation and secretion.

Ristocetin: ristocetin-induced platelet aggregation test
Although this test is called the ristocetin-induced platelet aggregation test, ristocetin actually induces an agglutination reaction because there is no shape change and little secretion. A normal result implies that von Willebrand factor (VWF) is present and the platelets possess a functional VWF receptor, GP Ib/V/IX.15

Ristocetin induces a monophasic tracing. Specimens from patients with von Willebrand disease (except subtype 2b) produce a reduced or absent ristocetin reaction to 10 mg/mL of ristocetin, although all other agonists generate normal tracings. Exogenous VWF from normal plasma restores the ristocetin aggregation reaction, confirming the diagnosis. Bernard-Soulier syndrome, a congenital abnormality of GP Ib/IX/V receptor, yields a diminished ristocetin reaction that is not corrected by addition of VWF. In subtype 2b von Willebrand disease, a VWF gain-of-function mutation, aggregation occurs even when reduced concentrations of ristocetin (one mg/mL) are used. Because of its varying penetration, the diagnosis of von Willebrand disease is elusive and requires repeated ristocetin-induced platelet aggregometry assays supplemented with additional assays such as ristocetin cofactor and VWF immunoassay.16,17

SUMMARY OF AGONIST RESPONSES IN VARIOUS PLATELET DISORDERS
Thrombin produces maximum ATP secretion through at least two membrane binding sites. Arachidonic acid tests for eicosanoid synthesis pathway deficiencies. Collagen, ADP, and epinephrine test for abnormalities in their respective membrane binding site and the eicosanoid synthesis pathway. Ristocetin checks for abnormalities of plasma VWF or GP Ib/IX/V. The following conditions may be detected through platelet lumiaggregometry.

Nonsteroidal anti-inflammatory drugs and platelet aggregometry
NSAIDs such as aspirin, ibuprofen, indomethacin, and sulfinpyrazone permanently inactivate or temporarily inhibit cyclooxygenase; prevent secretion of dense granule serotonin, ADP, and ATP; and suppress secondary aggregation.18,19 Secretion and aggregation in response to the agonists ADP, epinephrine, and in particular collagen and arachidonic acid are reduced, although thrombin produces a normal secretion response. Ristocetin induces normal aggregation. The physician or clinical laboratory scientist must instruct the patient to avoid all
FOCUS: HEMORRHAGIC PLATELET DISORDERS

drugs (if possible) for one week before blood is collected for aggregometry and particularly to avoid NSAIDs.

Platelet secretion defects: deficient eicosanoid pathway enzymes and aggregometry

Congenital or acquired deficiencies of cyclooxygenase or thromboxane synthase prevent secretion. Thrombin produces normal responses, but secretion and aggregation are diminished in response to ADP, epinephrine, collagen, and arachidonic acid. Because aggregation responses resemble those seen after the use of NSAIDs, release defects are often called aspirin-like disorders. Aggregation to collagen at one μg/mL is reduced by NSAIDs and secretion defects; however, collagen aggregation at five μg/mL is reduced in secretion defects, but not NSAIDs. A differential aggregation of 50% or greater between one μg/mL and five μg/mL of collagen may distinguish aspirin from aspirin-like disorder.20

Platelet storage pool defects and aggregometry

In a storage pool defect, ATP release in response to thrombin is reduced, as it is to ADP, epinephrine, arachidonic acid, and collagen.

Platelet membrane defects: thrombasthenia and aggregometry

Glanzmann thrombathenmia, a membrane defect characterized by dysfunction or loss of the GP IIb/IIIa receptor site, may be diagnosed by its characteristically diminished secretion and aggregation responses to all agonists with the exception of a modest response to arachidonic acid. Defects in additional receptor sites produce respective aggregometry results, for example, poor response to ADP in P2Y12 deficiency.

Acquired platelet disorders and aggregometry

Platelets are defective in acquired hematologic and systemic disorders, such as acute leukemias, aplastic anemias, myeloproliferative disorders, myelodysplastic syndromes, myeloma, uremia, liver disease, and ethanol abuse. Platelet aggregometry may be used to predict the risk of bleeding or thrombosis.21

REFERENCES