Primary haemostasis and its assessment by laboratory tests

A. J. Reininger
Abteilung Transfusionsmedizin und Hämostaseologie, Klinik für Anästhesiologie, Klinikum der Ludwig-Maximilians-Universität, München, Germany

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Summary
Platelets constantly patrol the inner surface of blood vessels searching leaks to be sealed, in order to prevent blood loss. When they detect a vessel injury their action can be divided into three phases. Adhesion: The platelets adhere to the injured vessel wall via their receptors glycoprotein (GP) Ib and integrin α2β3 (GPIIb/IIIa), which are present on the platelet membrane as well as on the vessel wall, fibrinogen and von Willebrand factor (VWF), fibrinogen, and others. Aggregation: Platelets stick to each other through fibrinogen bridging integrin α2β3 (GPIIb/IIIa) on adjacent platelets. Secretion: During activation the content of platelet granules is released by exocytosis, thus augmenting and propagating formation of a haemostatic plug or thrombus.

Laboratory tests mimic one or several aspects of these three phases to obtain reliable data on a patients platelet function. In this overview essays, test principles, and pitfalls are presented.

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Defects in primary haemostasis result from the inability of thrombocytes or platelets to adhere to an injured vessel wall. Thrombocytes or platelets are anucleated cytoplasmic fragments of megakaryocytes released from the bone marrow into the blood. The fluid organ blood is in constant motion to deliver cells, chemical substances and heat into all regions of the organism. Platelets flowing constantly along the vessel wall monitor the vasculature for defects since leakage cannot be tolerated and they react immediately upon vessel injury with adhesion and aggregation.

Usually, the inner lining of blood vessels consist of endothelial cells, which under normal conditions form an interface towards the flowing blood resistant to adhesion of circulating cells. Disruption of the continuous endothelial layer exposes subendothelial matrix proteins triggering a fast platelet response leading to firm adhesion. Collagen of various types (e.g. type I, III) is the major component of the subendothelium. Soluble multimeric von Willebrand factor (VWF) can bind via its A3 domain to the collagen fibers (1) and thus mediates the initial arrest of fast flowing platelets. The central interaction occurs between the platelet receptor glycoprotein (GP) Ibα and the VWF A1 domain. This requires no prior platelet activation and is only transient. The receptor is present in the platelet membrane as the complex GP Ibα-IX-V.

Permanent adhesion is obtained by subsequent binding of other platelet receptors, e.g. integrin α2β3 (GPIIb/IIIa), integrin α2β1, GP VI, with their respective ligands, and needs activation and calcium dependent intracellular signalling. The immobilized platelets then change their shape, spread, and attract further platelets from the flowing blood. Apart from adhesion the latter platelet-platelet interaction is referred to as aggregation.

Primary haemostasis denotes the concerted action of all these processes:
- initial platelet arrest,
- platelet adhesion, and
- platelet aggregation.

This is the first line of defense against bleeding from an injured vessel. The activated platelets present in such aggregates exhibit a membrane surface rich in phosphatidylserine, which is an acceleration factor for blood coagulation and not found in resting platelets (2). In particular, assembly and activation of tissue factor, factor VII, the tenase complex and the prothrombinase complex are fostered by activated membranes, and thus mediate secondary haemostasis. The final result is a fibrin stabilized platelet aggregate that occludes the vessel injury and prevents deleterious leakage of blood. The prothrombotic response is simultaneously limited to the so-called region of interest by potent antithrombotic and anticoagulatory counter-regulation.

Since platelets can not distinguish between a traumatized and a pathologically altered vessel, a chronic degenerative process like atherosclerosis with a ruptured plaque can trigger a physiological mechanism leading to fatal ischaemia. The effect of flow is reflected in the morphology of the thrombi (3):
Arterial thrombi formed under high shear conditions are rich in platelets and are stabilized by fibrin (white thrombi), whereas venous thrombi formed under low shear conditions consist of a dense fibrin mesh with fewer platelets and many erythrocyte inclusions (red thrombi).

The entire array of interactions and processes just described occurs at the interface between flowing blood and the stationary thrombogenic surface of the injured blood vessel. A fact often underestimated in laboratory tests that usually try to mimic the in vivo situation, is the major role of flow in bringing the reaction partners together. This is well illustrated by the phenomenon that anaemia, e.g. a haematocrit of <30%, can increase bleeding time (4). The rheological or haemodynamic explanation is, that the diminished effect of erythrocyte axial migration at low red cell counts leads to reduced platelet concentration in the boundary layer adjacent to the vessel wall (5–7).

In addition, experimental in vitro studies showed that blood flow over a surface coated with VWF led to a dense layer of adherent platelets that was not changed when platelet counts of 325,000/µl, 170,000/µl and 37,000/µl were used. Only drastic reduction to 5000 platelets/µl diminished surface coverage with platelets (3). This flow induced abundance of platelets in the boundary layer at the wall also illustrates the finding that severe bleeding in patients usually occurs with platelet counts less than 10,000/µl (8).

**Platelet count**

Although in most cases platelet counts are performed electronically, manual counting is required in some situations:

- very low platelet counts (<5000 platelets/µl),
- EDTA-induced platelet agglutination or questionable previous electronic count.

For manual counting whole blood samples are collected by venipuncture in EDTA or citrate anticoagulant, and are stable up to 4 hours at room temperature. Mixing the blood with 1% ammonium oxalate haemolyses the red cells and yields a suspension of platelets in plasma, which is placed in a counting chamber (e.g. according to Neubauer). Overfilling or underfilling the chamber are sources for false platelet counts. Phase contrast microscopy is necessary to visualize the platelets that sediment onto a grid etched into the chamber surface. The height of the chamber multiplied with the area of the grid squares gives the respective volume.

The basic principle for electronic cell counting is the change in electric impedance caused by the passage of the cells through a small orifice in the measuring unit. The term impedance describes electric resistance when alternating current is used. Since the changes in impedance depend on cell size, whole blood can be used and all blood cells can be measured in one run. This usually counts more than 100 times the number of cells counted manually, thus increasing accuracy. Particles or debris of roughly the size of platelets can lead to erroneous platelet counts. Automated repeated measurements through one or more apertures are compared by the analyzer to minimize errors. In addition to a histogram of size distribution of platelets the mean platelet volume (MPV) is also determined and helpful for example in diagnosis of thrombocytopenia (e. g. Wiskott-Aldrich syndrome). The lower limit for an accurate and precise platelet count is in the range of 2000 to 4000 platelets/µl, with manual counts performing a little better at the very low end but without effect on the clinical significance (9).

**Bleeding time**

Determination of bleeding time is the only in vivo test of platelet function. Although burdened with high intra- and interindividual variability with regard to patients and operators, it has shed light on the process of haemostasis and has still useful applications. Routine preoperative screening has not proven helpful whereas bleeding time has its value in defined clinical situations such as congenital or acquired thrombocytopenopathies, or for assessing the effectiveness of platelet transfusions.

In principal, the closure time of a prick or incision injury of the skin by a platelet rich thrombus is measured. Since this is dependent on how fast a platelet aggregate forms and how stable it is, the count and functionality of the platelets are decisive factors. With decreasing platelet counts from 100,000 to 10,000/µl the bleeding time prolongs. Several methods have developed over the decades which differ in location where the injury is performed (Duke: ear lobe; Ivy: lower arm), whether constant pressure is applied (Ivy: pressure cuff with 40 mmHg on upper arm), and whether the blood is swabbed or flows freely into sterile water into which the ear lobe is immersed (Marx and Ressels) (10, 11).

The bleeding time gives a rough information and detects only severe defects. It should be performed when bleeding diathesis is suspected, von Willebrand syndrome has to be assessed, or a decision for platelet concentrate transfusion is necessary. Bleeding time measurement is hard to standardize, time and personnel consuming, scars can form, and incision depth is crucial. 60–70% of patients with von Willebrand disease show normal bleeding time (12, 13), heterozygous Glanzmann’s thrombasthenia shows prolonged bleeding time, whereas mild forms display values within the reference range. Other congenital platelet function defects affecting bleeding time include:

- Bernard-Soulier syndrome,
- storage pool disease (Hermansky-Pudlak),
- Wiskott-Aldrich syndrome.

Acquired forms are associated with severe uremia, liver cirrhosis, high levels of monoclonal immunoglobulins, and platelet active drugs, such as abciximab, clopidogrel, acetylsalicylic acid, ticlopidine, prostacyclines. Adverse reaction to drugs can also cause prolonged bleeding time (10, 11).

**Cave:** Severe thrombocytopenias and platelet function defects can cause drastic bleeding at the incision site.
Platelet aggregation

Platelet function disorders are difficult to evaluate, tests are laborious and expensive. In order to confirm a diagnosis, additional tests are needed. The use of platelet aggregation as a laboratory test for platelet function was developed by Gustav V. R. Born in 1962 (14). The original method still in use (Apact®, LABi Tec GmbH, Ahrensburg, Germany; Platelet Aggregation Profiler®, Bio/Data, Horsham, PA, USA) measures the transmittance of light in turbid platelet rich plasma (PRP). When the platelets aggregate after stimulation with various agonists, the turbidity decreases and more light passes through the PRP. These changes in light transmittance can be analyzed and correlated with platelet shape change, aggregation and desaggregation if present. Weak agonists such as epinephrine or ADP at low concentration show a biphasic aggregation curve, i.e. an initial shape change wave followed by the aggregation wave. Strong agonists like collagen, thrombin or ADP at high concentration mostly override the first phase of shape change and generate a rapid aggregation response. Ristocetin is an agonist that causes platelets to stick together (agglutination) independent of a metabolic response even when platelets have been fixated with formaldehyde. It is a measure for platelet glycoprotein Ib receptor interaction with the plasma protein VWF.

Aggregation tests have some technical pitfalls (15).

- The platelet count must be adjusted to approximately 200,000 to 250,000 platelets/μl to obtain a reproducible aggregation response reflecting the biological function. This may require additional dilution of concentrated PRP with plasma and thus 10 ml of whole blood need to be drawn.
- After blood sampling and centrifugation the PRP should sit for 30 min to allow the platelets to quiet down before agonists are added.
- The type of anticoagulant (e.g. citrate, buffered citrate, heparin) affects the results.

Platelet aggregation can also be measured in whole blood, which allows to assess platelet function in the presence of erythrocytes and leukocytes and preventing artefactual platelet activation due to the separation steps. The whole blood method detects the electrical impedance between metal electrodes submerged in the blood (Chrono-log platelet aggregometers; Multiplate® – Multiple Platelet Function Analyzer, Dynabyte, Munich, Germany). The kinetics of impedance changes reflect the aggregation of platelets on the pins after addition of agonists. Although whole blood aggregometry is less cumbersome and time consuming, since preparation of PRP and PPP is not necessary – a more abundant and robust body of literature exists on PRP aggregometry in order to detect platelet disorders.

The release of platelet granule content occurs during aggregation and can be enzymatically determined via luminescence measurement. Thus platelet activation, secretion, and storage disease can be assessed.

Determinants of aggregometry are the platelet function disorders, which are rarely congenital but mostly acquired. Von Willebrand disease in the true sense is not a platelet function disorder but a quantitative or qualitative defect of the plasma protein VWF synthesized in the endothelial cells. The link with platelet function is the capability of the soluble VWF to bind to the collagen presented at an injury of the vessel wall and thus arrest platelets from the blood flow. Although ristocetin induced aggregation is dependent on VWF binding to GP Ib and can thus detect VWF deficiency, von Willebrand disease is best diagnosed by quantitation of VWF antigen, ristocetin cofactor activity and molecular assessment of VWF multimer composition. In a rare form known as platelet-type von Willebrand disease high-molecular-weight multimers of VWF show an increased affinity to GP Ib, due to a mutation in the latter. Consequently, VWF is depleted from the plasma and the platelets with already prebound VWF are hypersensitive to ristocetin induced aggregation. The same phenotype – of increased affinity and hypersensitivity to ristocetin – occurs with von Willebrand disease type 2B although here the mutation concerns the VWF molecule (16, 17).

Glanzmann thrombasthenia is characterized by a defect in the platelet receptor integrin α2β3 (GPIIb/IIIa), which leads to an impaired binding of fibrinogen to the receptor. Since fibrinogen acts as bridge between juxtaposed platelets connecting them via their integrin α2β3 receptors (GPIIb/IIIa) to form an aggregate, this defect results in decreased aggregation, i.e. almost no response to collagen, ADP, epinephrine but full response to ristocetin. The ristocetin response is only dependent on the GP Ib receptor. A defect in the GP Ib-IX-V complex found in the Bernard-Soulier syndrome results in a defective binding of VWF to this receptor and thus in a diminished ristocetin response in the aggregation test, whereas all other agonists lead to normal aggregation curves. Clinically, both defects are combined with a bleeding diathesis (15).

Platelet storage pool diseases are disorders where the dense (delta) or the alpha granules of the platelets are missing. The former is also termed Hermansky-Pudlak syndrome, the latter grey platelet syndrome. A combined defect is possible. The aggregation response of platelets from patients with storage pool disease is typically characterized by a reduced second aggregation wave to weak stimuli due to the missing content of the granules, i.e. ADP from dense granules and beta-thromboglobulin or platelet factor 4 (PF4) from alpha granules. Flow cytometry should be used instead of aggregometry to obtain a more reliable diagnosis for storage pool disease.

Acquired platelet disorders are frequently caused by drugs or uraemia (15). The widely used acetylsalicylic acid and many other non-steroidal anti-inflammatory drugs (NSAIDs) inhibit the platelet enzyme cyclooxygenase, which converts arachidonic acid into thromboxane (TX) A2. TXA2 is a short-living platelet agonist. The effect of a single dose of acetylsalicylic acid persists for 5 to 7 days, since it irreversibly inhibits the cyclooxygenase. Only the appearance of newly synthesized platelets in the circulation abates its effect. NSAIDs can either act by reversibly inhibiting the cyclooxygenase or by other modes of action. A list of diseases and drugs causing acquired platelet disorders is given in Table 1.
Flow cytometry

Flow cytometry is a technique to measure the characteristics of a large number of cells (>100,000 per minute) in a short period of time and in a small volume (approximately 30 µl sample for a one minute measurement). Prior to flow the cells of interest are labeled with monoclonal or polyclonal antibodies conjugated to specific fluorochromes. Labeling can be performed in whole blood, platelet rich plasma, or buffer solution. With subsequent fixation the platelets remain stable and lysis of red cells clears the solution from erythrocytes interfering with flow cytometric analysis. The core of the flow cytometer consists of a flow cell in which a thin thread of fluid passes through the focus of a laser beam. Within this thread of fluid the cells are aligned like pearls on a string and the flow is further stabilized by an additional sheath fluid. The fluorochrome is excited by the laser wavelength and emits light at a longer wavelength, which is then detected by an array of photomultipliers. In addition, the light strayed in the direction of the laser beam and at a 90 degree angle are also detected as forward and side scatter. They indicate the size of the cells as well as their granularity.

Although platelets are analyzed in their physiological milieu, manipulation is minimal, and the activation state of the platelets can be determined, there are drawbacks: Flow cytometers are expensive with regard to purchase and maintenance; they require experienced personnel; almost daily use is recommended.

The measurement of platelet activation in whole blood and in the absence of exogenous stimulants can be performed by using markers (e.g. monoclonal antibodies) that detect the activation dependent conformational changes of integrin α2β3 receptors (GPIIb/IIIa), the exposure of granule membrane proteins on the platelet surface such as P-selectin or CD63, or the exposure of phosphatidylserine on the outer leaflet of the platelet membrane via annexin V. Platelet derived microparticles are elusive and their detection via flow cytometry requires diligence in calibrating the instrumentation and careful preanalytical preparation of the samples to prevent microparticle formation due to handling of the blood sample.

Monocyte-platelet and neutrophil-platelet aggregates are formed via the P-selectin receptor mediated adhesion of platelets to the respective leukocyte ligand PSGL-1 (P-selectin glycoprotein ligand 1). The aggregates, which are determined by their size (forward scatter), can be readily identified by using specific labels for both platelets and monocytes or platelets and neutrophils. For more details see literature (18).

Aside from using flow cytometry in detecting platelet activation in clinical disorders – a task only suitable for specialized laboratories – it offers itself for analyzing the platelet receptor defects Glanzmann thrombasthenia, Bernard-Soulier syndrome as well as platelet storage pool disease as mentioned. A further application is the control of platelet concentrates produced in blood banks for contaminating leukocytes.

**Ultegra-RPFA**

The Ultegra-System Rapid Platelet Function Assay (RPFA, Accumetrics, San Diego, CA, USA) is a point-of-care assay developed to monitor the effect of the integrin α2β3 inhibitors abciximab (ReoPro®), tirofiban (Aggrastat®), and eptifibatide (Integrilin®), ASS, and clopidogrel (z.B. Plavix, Sanofi Synthelabo) thus allowing titration of these drugs instead of administering standard dosage. The measuring principle is turbidimetric determination of the agglutination of fibrinogen-coated polystyrene beads with platelets in whole blood in proportion to the number of unblocked integrin α2β3 receptors (GPIIb/IIIa).

The automated test is performed in 160 µl of whole blood anticoagulated with citrate or PPACK, a direct thrombin inhibitor. First reports of studies performed with the Ultegra-RPFA claim a correlation between clinical outcomes and test results that await further confirmation. (19)

**PFA-100®**

The PFA-100 (Dade/Behring, Schwalbach, Germany) is a specific system to analyze platelet function under flow conditions. The wall shear rates of approximately 5000/s are reached when 800 µl of citrated blood is sucked through an orifice 150 µm in diameter within a collagen coated membrane soaked with either ADP or epinephrine. Platelet adhesion and subsequent aggregation close the orifice and the blood flow stops. The kinetics of this simulated wound closure are recorded and the endpoint is given as closure time (CT). At the shear rates present in the PFA-100 the initial platelet arrest from fast flow is mediated by von Willebrand factor bound to the collagen and act-

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Primary haemostasis and laboratory tests

The following permanent adhesion and aggregation is dependent on the integrin α2bβ3 (GPIIb/IIIa). Therefore, the PFA-100 is a highly sensitive system for the detection of the von Willebrand-Jürgens syndrome as well as for the aggregation-blocking effect of acetylsalicylic acid.

Thus, when suspecting a platelet function defect, the PFA-100 is a valuable tool that can be usefully combined with an array of other tests. The therapeutic effect of DDAVP (desmopressin) that elevates VWF concentration in plasma by releasing it from endothelial cells can also be assessed with this technique. Perioperative use and application in intensive care are not recommended since platelet aggregates developed perioperatively as well as haemodilution can interfere with accurate test results. The CT will become abnormally prolonged when either the platelet count falls below 50 000/µl, or when the haematocrit is less than 25% (20).

DiaMed Impact®

The cone and plate analyzer DiaMed Impact (DiaMed, Ottobrunn, Germany) is a fully automated system for the point of care testing of platelet function in whole blood under arterial flow conditions (shear rates 1800/s). 200 µl citrated whole blood are sheared for 2 minutes in a cone and plate system made out of polystyrene. Plasma proteins adhere to the plate surface and allow platelets to attach. After washing off the blood the adherent platelets are fixed and stained. The degree of platelet adhesion and aggregation is measured via microscopy and image analysis, and the results are given as percentage surface coverage and average size in µm².

Due to the test conditions, the cone and plate assay appears sensitive for platelet abnormalities affecting the platelet integrin α2bβ3 (GPIIb/IIIa), e. g. Glanzmann thrombasthenia, anti-platelet drugs, platelet GPIb, von Willebrand’s disease and afibrinogenae mia. More extensive studies are needed to determine the usefulness of this test for the evaluation of a patient’s diathesis – may it be bleeding, thrombosis or therapeutic monitoring (21).

References


Correspondence to:
Priv.Do. Dr. med. Armin J. Reininger
Abteilung für Transfusionsmedizin und Hämostaseologie
Klinik für Anästhesiologie, Klinikum der Universität München
Ziemssensstr. 1, 80336 München, Germany
Tel.: +49(0)89/51 60–22 39, Fax: –2148
E-mail: Armin.Reininger@med.uni-muenchen.de