Review

PROGRESS IN THE ASSESSMENT OF PLATELET FUNCTION

Platelets play a key role in both haemostasis and thrombosis. The accurate measurement of platelet function is critical for not only identifying patients with either platelet dysfunction or hyperfunction, but is becoming increasingly important for the monitoring of modern anti-platelet therapy. The history of platelet function testing really began only 120 years ago when platelets were first identified as distinct cells (and not fragments) in the blood and it was demonstrated that they were vital for both haemostasis and thrombosis in vivo (Bizzozero, 1882). However, despite these remarkably early observations, for the majority of this century the only means of assessing platelet function were a small number of fairly unreliable tests (e.g. manual platelet counting, inspection of the blood film, the vivo bleeding time, the Hess test and qualitative ‘snow storm’ tilt tube techniques). One of the major problems facing scientists working in the platelet field has been the enormous difficulty in simulating the complexity of either primary or global haemostasis. In addition, because of their inherent sensitivity, platelets are prone to artefactual in vitro activation. Early attempts to simulate haemostasis in vitro included methods where platelets were counted before and after exposure to foreign surfaces (e.g. glass columns) or monitoring thrombus formation within closed plastic tube loops. Many of these early tests remained largely as research tools because of their technical difficulty and were thus restricted to specialized centres. The ability to test platelet function accurately in the routine laboratory remained fairly primitive until the widespread introduction of platelet aggregometry (Born, 1962). Remarkably, despite our rapid increases in knowledge in platelet biochemistry/biology since the 1960s, further advances in laboratory platelet analysis have still lagged behind both the functional and molecular analysis of the coagulation/fibrinolytic system. Table I illustrates the small number of tests that were available up to the late 1980s. However, the recent availability of new reliable platelet function analysers, flow cytometry and molecular biological techniques are beginning to change our approach to platelet function analysis. Table II lists a number of relatively new platelet function tests that are now available. The introduction of these new methodologies into our existing portfolio of platelet and haemostatic tests will increase the efficiency of the laboratory diagnosis of platelet dysfunction/hyperfunction. The improvement in technology will also result in more point-of-care testing, enable clinicians to monitor anti-platelet therapy accurately and may provide novel measurements that could predict either thrombosis or bleeding. Only a brief history and recent major advances in the analysis of platelet function are covered in this review. Given the large amount of literature on this subject, I apologize if there are any omissions. Advances in the molecular biology of either platelet receptor polymorphisms or platelet function defects are beyond the scope of this article and are adequately covered elsewhere (Bray, 1999; Nurden, 1999).

ADVANCES IN PLATELET COUNTING

The peripheral platelet count will continue to be utilized as a first-line basic test of platelet function. Before the widespread introduction of impedance counters the platelet count was performed manually. The manual count using phase-contrast microscopy is the oldest means of enumerating platelets and, remarkably, is still used as the gold standard international reference method (Brecher et al, 1953; England et al, 1988). The reference procedure is imprecise with typical coefficient of variations (CVs) in the range of 15–25% and is thus totally impractical for calibrating routine blood analysers (Harrison et al, 2000). Recently, a new immunoplatelet counting procedure has been proposed as the new international reference method (see below). Although modern impedance counters are rapid, precise and reproducible, they tend to overestimate the platelet count in samples that contain cellular debris, e.g. thalassaemia, thrombocytopenic purpura (TTP). Conversely, in patients with large platelets (e.g. macrothrombocytopenia) the platelet count is underestimated (Ault, 1996). The accurate estimation of the peripheral blood platelet count is becoming extremely relevant given that the platelet transfusion threshold is now set at 10^9/l (Gmur et al, 1991; Rebulla et al, 1997; Ancliff & Machin, 1998; Norfolk et al, 1998). More recent advances in commercial analysers have led to the development of optical counting methods where platelets are either identified by their light scattering properties or their fluorescence after addition of a suitable dye (Stanworth et al, 1999; Briggs et al, 2000). These new methods increase the accuracy of the count as both normal and large-sized platelets are easily discriminated from noise and other cell populations. Recent evidence confirms that optical counters agree more closely with a newly proposed platelet counting reference procedure (Harrison et al, 2000). In the latter method, platelets are identified via flow cytometric analysis of samples that have been incubated with a monoclonal antibody (e.g. anti-CD61) (Davis & Bigelow, 1999; Harrison et al, 2000). The platelet count is derived by dividing the number of fluorescent platelets by the number of collected red blood

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cell events (RBC ratio) and multiplying by the known RBC count provided by a suitable calibrated impedance analyser. As the method is independent of both pipetting and dilution artefacts, providing sufficient platelets are counted and samples optimally diluted to control for RBC:platelet and RBC:RBC coincidence events, the derived counts are not only highly accurate, but precise, with typical CVs in the order of 5% (Davis & Bigelow, 1999; Harrison et al., 2000). Given the clear superiority of the RBC ratio to the manual count, the International Council for Standardization in Haematology/International Society for Laboratory Haematology (ICSH/ISLH) have recently proposed that the method could become the new international reference method. This method can also be potentially transferred to modern cell counters such as the Abbott Cell Dyn 4000 (Ault et al., 1997; Kickler et al., 1998). Given these improvements in both the reference method and the introduction of optical counting, it is therefore possible that increasing the accuracy of platelet counting in severe thrombocytopenia could result in a further reduction in the platelet transfusion threshold down to as low as $5 \times 10^9/l$, without an associated increase in bleeding risk. This could result in further dramatic savings in transfusion budgets by decreasing the frequency of unnecessary blood transfusions.

**Platelet count ratio**
Platelets within whole blood, when stimulated by agonists, form aggregates with a reduction in the number of free platelets. By counting the number of platelets in a control sample (usually EDTA) and a sample that has been activated

### Table I. List of established platelet function tests.

<table>
<thead>
<tr>
<th>Platelet function test</th>
<th>Aspects of platelet function measured</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bleeding time</td>
<td>In vivo screening test</td>
<td>Physiological</td>
<td>Insensitive, invasive &amp; high inter-operator CV</td>
</tr>
<tr>
<td>Aggregometry - turbidometric methods</td>
<td>Responsiveness to panel of agonists</td>
<td>Diagnostic</td>
<td>Labour intensive, non-physiological</td>
</tr>
<tr>
<td>Aggregometry – impedance methods</td>
<td>Responsiveness to panel of agonists</td>
<td>Whole blood test</td>
<td>Insensitive</td>
</tr>
<tr>
<td>Aggregometry &amp; luminescence</td>
<td>Combined aggregation and ADP release</td>
<td>More information</td>
<td>Semi-quantitative</td>
</tr>
<tr>
<td>Adenine nucleotides</td>
<td>Stored and released ADP</td>
<td>Sensitive</td>
<td>Specialized equipment</td>
</tr>
<tr>
<td>Thromboelastography (TEG)</td>
<td>Global haemostasis</td>
<td>Predicts bleeding</td>
<td>Measures clot properties only, insensitive to aspirin</td>
</tr>
<tr>
<td>Glass filterometer</td>
<td>High shear platelet function</td>
<td>Simple</td>
<td>Requires blood counter</td>
</tr>
<tr>
<td>Platelet release markers, e.g. βTG PF4</td>
<td>In vivo platelet function markers</td>
<td>Simple, systemic measure of platelet activation</td>
<td>Prone to artefact</td>
</tr>
</tbody>
</table>

### Table II. List of new platelet function tests.

<table>
<thead>
<tr>
<th>Platelet function test</th>
<th>Aspects of platelet function measured</th>
<th>Advantages</th>
<th>Disadvantages</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clot signature analyser (CSA)</td>
<td>Global haemostasis. High shear platelet function</td>
<td>Measures global haemostatic function</td>
<td>Cost of cartridges</td>
</tr>
<tr>
<td>Cone and plate analyser (CPA)</td>
<td>High shear platelet adhesion/aggregation</td>
<td>Small volume of blood, physiological</td>
<td>Requires image analysis</td>
</tr>
<tr>
<td>Flow cytometry</td>
<td>Platelet glycoproteins, activation markers, platelet function</td>
<td>Whole blood test, flexible, wide variety of tests, powerful</td>
<td>Specialized operator, Expensive equipment</td>
</tr>
<tr>
<td>ICHOR</td>
<td>Platelet counting pre and post activation</td>
<td>Simple point-of-care instrument</td>
<td>Preparation of reagents</td>
</tr>
<tr>
<td>Haemodyne</td>
<td>Platelet contractile force</td>
<td>Rapid and simple</td>
<td>Measures clot properties only</td>
</tr>
<tr>
<td>Hemostasus device</td>
<td>Platelet procoagulant activity</td>
<td>Simple</td>
<td>Insensitive to aspirin and GpIb function</td>
</tr>
<tr>
<td>Laser platelet aggregometer (PA-200)</td>
<td>Platelet microaggregates</td>
<td>Sensitive, study hyperfunction</td>
<td>Little widespread experience</td>
</tr>
<tr>
<td>Platelet function analyser (PFA-100)</td>
<td>High shear adhesion/aggregation</td>
<td>Rapid and simple screening, in vitro bleeding time, potential point-of-care instrument</td>
<td>Inflexible</td>
</tr>
<tr>
<td>Ultegra (RPFA) – rapid platelet function analyser</td>
<td>Gp IIb/IIIa dependent aggregation</td>
<td>Simple test for monitoring anti-GpIIb/IIIa therapy, Point-of-care instrument</td>
<td>Inflexible</td>
</tr>
<tr>
<td>Thrombotic status analyser (TSA)</td>
<td>High shear platelet function</td>
<td>Simple</td>
<td>Little widespread experience</td>
</tr>
</tbody>
</table>
with ADP it is possible to assess platelet function. This platelet count ratio correlates well with platelet-rich plasma (PRP) aggregation (Nicholson et al., 1998), requires a blood counter and the reagents are commercially available in kit form (Plateletworks; Array Medical, Somerville, NJ, USA). A very simple whole blood platelet function assay utilizing the ICHOR Point of Care Haematology Counter has recently been developed to count samples pre- and post activation (Carville et al., 1999). This instrument provides a possible means of assessing the platelet count and function in a near patient format within an acute care environment. Platelet counting can also be performed pre- and post exposure of samples to foreign surfaces (e.g. glass columns, see below).

ADVANCES IN PLATELET AGGREGOMETRY

Classic platelet aggregometry

In the 1960s, the invention of platelet aggregometry revolutionized our ability to identify and diagnose platelet function defects (Born, 1962). This soon became the ‘gold standard’ for platelet function testing. Blood is centrifuged gently to obtain PRP, which is stirred in a cuvette at 37°C between a light source and a photocell within the aggregometry instrument. Upon addition of an agonist, platelets aggregate and the transmission of light increases and is detected and recorded on a chart with time. The addition of a panel of platelet agonists (e.g. collagen, ADP, adrenaline, etc.) at a range of concentrations, triggers classical platelet responses including shape change, primary and secondary aggregation. The recorded response depends upon the normal functioning of the platelet, the presence of inhibitors and the concentration of agonist and, thus, facilitates the detection of classical platelet disorders based upon the pattern of aggregation traces. Although an enormous amount of information can be obtained with aggregometry, the test is labour intensive, requires sample preparation, quality control and a fair degree of technical expertise both to perform the test and interpret the results. Although the development of modern aggregometers with multichannel capability, computer control and the ability to measure ADP release via luminescence has improved the technology, the test will still be limited to specialized pathology laboratories. Other developments, including whole blood aggregation, detection of microaggregates by light scattering and the development of a point-of-care instrument for monitoring anti-platelet therapy, are discussed below.

Whole blood aggregometry

One of the problems with platelet aggregation is the requirement for the separation of platelets from the rest of the blood. Not only is this time consuming, but the removal of other blood constituents that affect platelet function may influence the result. In response to this, the measurement of platelet aggregation within whole blood by electrical impedance was invented (Cardinal & Flower, 1980). Similar to PRP aggregation, whole blood is stirred at 37°C between two platinum wire electrodes set at a fixed distance (Mackie et al., 1984). The electrode immediately becomes covered in platelets with further adhesion of platelet aggregates after the addition of agonists. The mass of platelets that sticks onto the electrodes changes the impedance with time and is monitored on a chart recorder. One problem is that the electrodes must be carefully cleaned after each test. Comparative studies with turbidimetric aggregometry gives similar results, but suggests that the test does not compare well when monitoring anti-platelet therapy (Mascelli et al., 1997; Nicholson et al., 1998; Storey et al., 1999). Indeed, the results suggest that turbidimetric aggregometry underestimates both the degree and duration of inhibition by anti-Gp IIb/IIIa therapy (e.g. abciximab) (Mascelli et al., 1997). Within each donor, variation tends to be slightly higher than conventional aggregometry and the test still requires technical expertise and is relatively expensive. The test can be combined with simultaneous luminescence measurements in order to monitor the release of ADP during aggregation.

Light-scattering methods for monitoring platelet aggregation

Although platelet aggregometry is a relatively simple technique, there is an increasing amount of evidence suggesting that light transmission is not sensitive enough to monitor the formation of microaggregates or for monitoring platelet hyperfunction. The changes in transmission observed only reflect the formation of relatively large aggregates that are derived from microaggregates. Thus, a number of studies have demonstrated that turbidimetric measurements cannot detect all levels of platelet aggregation (Born & Hume, 1967; Frojmovic et al., 1983). For example, Thompson et al. (1986) suggested that aggregates with <100 platelets cannot be detected. Aggregometry is thus relatively insensitive for either detecting pre-existing aggregates or monitoring the early phase of aggregation. This may be particularly important for studying aggregates in patients with platelet hyperfunction. Similarly, whole blood aggregation impedance methods are also relatively insensitive to the presence of these aggregates.

Particle size analysis by flow cytometry is very accurate not only for the enumeration of platelets (see above), but also for use in monitoring formation of small aggregates during the early aggregation phase (Solis et al., 1975). By increasing the aperture size it is possible to detect larger aggregates. However, continuous measurement is not possible and disaggregation can also occur during dilution procedures if samples are not fixed.

Recently, a new aggregometer (PA-200, Kowa, Tokyo, Japan) has been developed that utilizes a combination of laser light scattering and aggregometry to monitor the continuous formation of platelet microaggregates (Ozaki et al., 1994; Yamamoto et al., 1995). This has significant advantages over semiquantitative systems using similar technology. The test is sensitive enough to detect aggregates containing only two or three platelets. The principle is very simple in that the intensity of light scatter is proportional to the size of the aggregates formed. The trick is to minimize multiple light scattering by focusing on a limited area of the PRP in the cuvette and detecting high intensity light scatter at 90 degrees by a series of lenses. Platelet aggregation assessed in this manner correlates well with conventional aggregometry, providing there are gross changes in light.
transmission. In contrast, however, the new aggregometer can detect small aggregates via changes in light scatter that are not detectable via light transmission in the conventional aggregometer. The data also suggests that small aggregates are formed prior to the formation of the macroaggregates that are detected in conventional instruments. These two phases have been shown to represent the primary and secondary aggregation responses. The instrument is thus an improvement of conventional technology in that it not only measures both the size and number of platelet aggregates, but also utilizes physiological concentrations of agonists (<1000 times those used in light transmission). This technology may prove very useful for detecting platelet hyper-reactivity in response to low concentrations of various agonists. For example, spontaneous formation of small aggregates (<100 platelets) has recently been observed in patients with acute coronary syndromes, but not in controls (Eto et al., 1998). Furthermore, upon agonist stimulation, the EC50 for epinephrine-induced primary aggregation was 50 times lower in these patients than in controls, while the EC50 for secondary aggregation was only two times lower. This suggests that platelet hypersensitivity is only detectable by primary aggregation of microaggregates in these patients. This technology may therefore prove very useful for studying hyperfunctionality of platelets in a variety of disease states including acute coronary syndromes, stroke and TTP.

The Ultegra rapid platelet function assay (RPA)

A modified platelet aggregometry device was developed in order to provide a simple and rapid functional means of monitoring anti-Gp IIb/IIIa therapy with various anti-platelet drugs (e.g. abciximab) (Coller et al., 1997). Data from recent clinical trials (e.g. evaluation of c7E3 for prevention of ischemic complications (EPIC), evaluation in percutaneous transluminal coronary angioplasty (PTCA) to improve long-term outcome with abciximab GpIIb/IIIa blockade (EPILOG)) have suggested that >80% of receptors need to be blocked to achieve significant clinical efficacy (EPIC investigators, 1994). Monitoring of receptor blockade is thus becoming crucial in order to ensure optimal dosaging and maximal clinical benefit within individual patients at risk of developing ischemic complications (e.g. during percutaneous coronary intervention) (Coller, 1998). Previous tests as such radiolabelled antibody binding assays, aggregometry and flow cytometry, although adequately suitable, are time consuming, expensive and require a fair degree of technical expertise and quality control within suitable pathology laboratories. Assays for monitoring Gp IIb/IIIa antagonists are reviewed by Cox (1998). Given the impending rapid increase in the use (both acute and chronic) of both oral and infused Gp IIb/IIIa antagonists, and the large number of potential samples that will be ultimately generated from cardiologists, there existed a demand for a simple, cheap and rapid method that could be utilized at the bedside/clinic in order to monitor anti-platelet therapy (Frelinger & Hillman, 1998; Smith et al., 1999). A simple functional assay also has additional advantages in that it not only assesses the goal of the therapy, but can assess all Gp IIb/IIIa antagonists in a standardized fashion (Kereiakes et al., 1999). The Ultegra rapid platelet function assay (RPA) (Accumetrics, San Diego, CA, USA) is based upon the principle that fibrinogen-coated beads will agglutinate in whole blood to proportion to the number of platelet GpIIb/IIIa receptors. A disposable cartridge contains fibrinogen-coated beads and a platelet activator (TRAP, thrombin receptor activating peptide) within reaction wells. To determine platelet function, a citrated whole blood sample is inserted into the cartridge within the instrument and results (expressed as percentage of inhibited platelets) are obtained within 1 min. TRAP activates platelets, resulting in GpIIb/IIIa exposure and binding of the fibrinogen-coated beads to the platelets that are not blocked with the drug in question. The instrument simply measures changes in light transmission automatically and thus the rate of platelet/bead aggregation. The degree of GpIIb/IIIa blockade can thus be calculated accurately and rapidly reported back to the clinician. Comparative evaluations demonstrate that the instrument mirrors both platelet aggregometry and the degree of GpIIb/IIIa blockade (Kereiakes et al., 1999; Smith et al., 1999). This instrument may also be useful for diagnosing Glanzmann’s thrombasthenia and monitoring other anti-platelet drugs (aspirin and clopidogrel). This test could potentially be used as a point-of-care instrument as it is very simple to use and utilizes a disposable cartridge-based system (Kereiakes et al., 1999). This represents a significant advance as the test could be performed reliably within different institutions without requiring either transport of sample, time delay or specialized personnel to perform the test.

PROBLEMS WITH THE IN VIVO BLEEDING TIME

The bleeding time invented by Duke (1912) was the first in vivo test of platelet function. Although the test is clinically useful and has been refined by the Ivy technique (Ivy et al., 1935; Mielke et al., 1969), it is poorly reproducible, invasive, insensitive and time consuming (Rodgers & Levin, 1990). The bleeding time is impractical to use during surgical procedures or serially and requires experienced operators to judge the subjective end-point of the test. However, even given these drawbacks, the bleeding time is still utilized as a first-line clinical screening tool and can be particularly useful for identifying patients with very severe haemostatic defects [e.g. von Willebrand’s disease (VWD), Glanzmann’s thrombasthenia] (Yardumian et al., 1986; The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force, 1988). The clear advantage of the bleeding time as a platelet test is that it studies natural haemostasis, does not require expensive specialized equipment and is not prone to anticoagulation artefacts. However, given the problems with the method, the introduction of reliable in vitro tests that simulate primary haemostasis in vitro within whole blood could eventually result in the bleeding time test being phased out.

INSTRUMENTS THAT SIMULATE PLATELET FUNCTION IN VITRO

Once aggregometry established itself as the gold standard of
platelet function analysis, it soon became apparent that the test does not mimic accurately all of the physiological processes of platelet adhesion, activation and aggregation that occur during haemostasis. In response to this problem, a number of investigators developed tests in an attempt to mimic or simulate the processes that occur during vessel wall damage. Many platelet adhesion tests have been developed to study shear-induced platelet activation (Proceedings of the Symposium on Novel Techniques Involving Platelet Activity in Flow, 1998). However, many of these techniques remain primarily as research tools because of their inherent complexity. The next sections will focus on instruments that have been or possibly will be commercialized into useful platelet function tests.

Clot signature analyser (CSA)
Gorog & Ahmed (1984) developed an instrument called the Haemostatometer for testing global haemostasis. The principle of the methodology involves punching holes within a tube containing flowing non-anticoagulated blood under controlled conditions. Consequent platelet activation results in the formation of a primary haemostatic plug with clot stabilization via fibrin formation. Alternatively, the blood can also be simultaneously exposed to a small amount of collagen within the tube, which results in thrombus formation and complete stenosis of the tube. Xylum have developed a commercial form of this instrument called the clot signature analyser (CSA) that simultaneously measures both haemostatic plug formation in the ‘punch’ and collagen channels within a disposable plastic cassette (Li. C.K.N., et al, 1997; Conan et al, 1998). The advantage of this test is that it provides a global assessment of haemostasis using controlled conditions. The formation of the haemostatic plugs within both channels is shear-dependent (> 10 000/s in the punch channel and 6 200/s in the collagen channel) and relies upon functioning GPib, VWF and GpIIb/IIIa. Additionally, in the punch channel, a growing fibrin clot also eventually occludes the vessel after initial platelet plug formation that seals the punched holes. The test is therefore applicable to detecting abnormalities in both primary haemostasis and coagulation.

Thrombotic status analyser (TSA)
The thrombotic status analyser is an instrument that measures both thrombotic and thrombolytic activities within non-anticoagulated blood (Gorog & Kovacs, 1995). The principle of the test involves drawing whole blood through a capillary tube. The high haemodynamic forces induce platelet activation and vessel occlusion. After stabilization and upon compression of the platelet-rich thrombus, thrombolysis can also be measured. The test is capable of detecting patients with defective or excessive platelet function and monitoring both anti-platelet and thrombolytic therapy.

PEA-100
Kratzer and Born (1985) developed a new concept for testing platelets in vitro. The prototype instrument was named the Thrombostat-4000. In the mid-1990s, Dade/ Behring further developed the instrument into a commercial analyser called the PFA-100 or platelet function analyser (Kundu et al, 1995, 1996). The principle of the method is to expose platelets within citrated whole blood to high shear (5 000–6 000/s) within a capillary and then monitor the drop in flow rate as the platelets form a haemostatic plug within an aperture (150 μm) within the centre of a membrane coated with collagen and either ADP or epinephrine. The test records the validated parameter called the closure time (CT) and the in-flow rate and total volume of blood used in the test. The test is simple, rapid, does not require specialist training and is a potential screening tool for assessing patients with haemostatic abnormalities (Mammen et al, 1998; Harrison et al, 1999). The test is particularly advantageous in the paediatric setting (Carcio et al, 1998) and is a potential in vitro bleeding time test (Francis et al, 1999). As with the in vivo bleeding time, the test is sensitive to both the platelet count and haematocrit and thus it is recommended that a full blood count is performed with each test. Recent data confirms that the system is highly von Willebrand factor (VWF) dependent and is useful for not only evaluating patients with von Willebrand’s disease, but for monitoring their therapy with desmopressin (DDAVP) (Cattaneo et al, 1999a; Fressinaud et al, 1999). However, the test appears to be coagulation independent as blood from patients with Haemophilia A or B, factor VII deficiency or afibrinogenemia all exhibit normal closure times in the instrument. Comparison with the bleeding time confirms that the PFA-100 is more sensitive in detecting all subtypes of von Willebrand’s disease with a sensitivity of > 90%, apart from Type III VWD, where the haemostatic defect resides in the Factor VIII binding site on VWF (Fressinaud et al, 1998). Interestingly, factor VIII concentrates containing VWF or even VWF concentrates fail to correct the closure time in patients with Type III von Willebrand’s disease, suggesting that either platelet VWF is critical in the test or that the PFA-100 is very sensitive to the absence of highest molecular multimers in these concentrates (Cattaneo et al, 1999a; Fressinaud et al, 1999). The test may also be useful for the diagnosis and therapeutic monitoring of patients with platelet secretion defects (Cattaneo et al, 1999b). The collagen/epinephrine cartridge appears to be not only more sensitive to these defects, but also gives a prolonged closure time in either patients or normal donors who have ingested aspirin (Marshall et al, 1997; Harrison et al, 1999). The test may therefore prove to be beneficial in identifying patients who are aspirin insensitive. The PFA-100 may also be useful for monitoring other anti-platelet drugs such as anti-GPlIB/IIa antagonists. The test could potentially replace the in vivo bleeding time (Francis et al, 1999) and thus could be an excellent primary screening tool for assessing samples from patients with potential platelet dysfunction (Mammen et al, 1998). If the screening test PFA-100 result proves to be normal then it is highly probable that the patient has no primary haemostatic defect and other platelet tests would not be required. If the PFA-100 test is abnormal, although the degree of abnormality on each type of cartridge gives some information regarding the severity of platelet defect, further platelet tests including aggregometry and VWF testing will be required for...
diagnosis and administration of the appropriate treatment. The disadvantage of the test is its lack of flexibility and lack of detailed information that each test gives. Nevertheless, the test could replace the bleeding time as a primary screening tool and also has the potential of being utilized as a point-of-care test system as it is very simple to use. As well as being a primary screening tool, the instrument may also prove to be useful within surgical departments for predicting both peri- and post-operative bleeding in a similar manner to the TEG device. Future developments in the instrument may include different types of cartridge and the ability to perform the test with different flow rates.

Cone and plate(let) analyser (CPA)
Another test has recently been developed that is based upon the adhesion of platelets to the extracellular matrix (ECM) under flow conditions (Varon et al., 1997, 1998; Kenet et al., 1998). The system consists of a cone-and-plate device in which a blood sample is subjected to a plastic plate under arterial flow conditions for 2 min. Platelet adhesion and aggregation on the surface is monitored by an image analyser. Again, the interaction of platelets with the substrate is totally dependent upon the presence of Gp Ib/IIIa, Gp Ib and plasma VWF. The CPA is now a fully automated system and can be utilized to monitor for defects in primary haemostasis, anti-GpIb/IIIa therapy, hyper-reactive platelets and monitoring thrombocytopenic patients for bleeding risk, etc. (Kenet et al., 1998; Varon et al., 1997). The main advantages of this system are that it only uses a small volume of blood (150–250 μl), has a short test time (5 min), produces comprehensive data and exhibits a close resemblance to in vivo physiological conditions.

High shear filterometer
A very simple method for assessing high shear-dependent platelet function has been developed by O’Brien & Salmon (1987). This is a refinement of the earlier tests that utilized glass as a foreign surface. Briefly, anticoagulated blood is forced under controlled pressure through a fine glass fibre filter. The combination of shear forces and foreign surfaces activates the platelets, resulting in aggregate formation culminating in blockade of the filter. Platelets are counted prior to filtration and at various time-points to calculate the percentage of retained platelets in the filter. Again because of the high shear conditions, the test is highly dependent upon GpIb, VWF and GpIb/IIIa and could be utilized as a sensitive test for various types of platelet dysfunction.

PLATELET-MEDIATED THROMBIN GENERATION
It is well documented that blood coagulation occurs more efficiently on cell surfaces. In particular, the interaction between platelets and clotting occurs with many different proteins and at many different levels of the coagulation cascade. The exposure of negatively charged phospholipids during platelet activation (platelet factor 3) provides a surface on which both the tenase and prothrombinase complexes can generate thrombin. Thrombin, of course, converts fibrinogen to fibrin and is a potent activator of platelets. Recent intriguing data has also demonstrated that anti-GpIb/IIa antagonists (e.g. 7E3 and RGDS peptides) also inhibit thrombin generation (Reverter et al., 1996). As platelets from patients with Glanzmann’s thrombasthenia also exhibit reduced thrombin generation, Gp Iib/IIIa also appears to influence the conversion of prothrombin to thrombin. This is probably mediated by direct binding of thrombom on both resting and activated platelets (Byzova & Plow, 1997). Estimates suggest that platelets can accelerate thrombin generation by five to six orders of magnitude. Thus, the measurement of thrombin generation in the presence of platelets provides a simple assessment of an important physiological function of platelets. Although most assays have been either based upon either coagulation (Hardisty & Hutton, 1966) or use of thrombin-specific chromogenic substrates, a very simple instrument has been developed to measure this activity.

The Hemostatus device
Point-of-care measurements of the prothrombin time, activated partial thromboplastin time (APTT) and the platelet count have all been used to identify patients who are at risk of bleeding. A new point of care instrument (Hemostatus, Medtronic Blood management, Parker, USA) has been developed to assess platelet function by analysis of the effects of platelet-activating factor (PAF) on the kaolin-activated clotting time (Despotis et al., 1996, 1999). The test measures the effect of four different concentrations of PAF on the shortening of the clotting time. The Hemostatus test accurately identifies a bleeding tendency, especially when platelet counts are below 70 × 10^9/l. The test is insensitive to aspirin and GpIIb function, but is sensitive to abnormalities in Gp Iib/IIIa function. Thus, the test may be useful for monitoring GpIIb/IIIa inhibitors and identifying patients with Glanzmann’s thrombasthenia (Coiffic et al., 1999).

INSTRUMENTS THAT MEASURE THE PHYSICAL PROPERTIES OF THE CLOT
The balance in the dynamics of clot formation, retraction and lysis reflect the ability of the haemostatic plug to perform haemostasis. Thus, by measuring various properties of the clot during haemostasis one can potentially detect a number of acquired and congenital platelet abnormalities. The process of clot retraction may be measured either in whole blood or PRP by simply incubating in the presence of calcium in glass tubes and either calculating differences in volumes pre- and post clotting or more simply by visual assessment of the forming clots with time against suitable controls. Abnormal clot retraction may reflect deficiencies in platelet number, platelet glycoproteins, signalling or fibrinogen levels. A number of instruments have also been developed that can accurately measure various physical properties of the clot with time. In this section two commercial instruments are discussed: the TEG and Hemodyne devices.

Thromboelastography (TEG)
Thromboelastography (TEG) was originally developed by Hartert (1948). It remained largely a research tool until the
1980s when there was a resurgence of interest in instruments that monitor haemostasis as a whole dynamic process. The end result of haemostasis or the clot exhibits distinct physical properties that can be measured and thus facilitate the identification of patients with either normal, bleeding or thrombotic tendencies. The TEG device (Haemometer) gives a global assessment of haemostasis by monitoring the interaction of platelets within the fibrin mesh of the clot during clot formation and lysis. The TEG simply measures the clot’s physical property during time by use of a cylindrical cup that holds the blood at 37°C and is oscillated to and fro with a rotation cycle of 10 s. A pin is suspended into the blood by a torsion wire. Thus, as the clot forms, the torque of the rotating cup begins to be transmitted to the immersed pin. The degree of pin rotation is converted to an electrical signal via a transducer and monitored via a chart recorder. The strength of clot formation will increase the magnitude of the output. As the clot begins to lyse, the bonds between the cup and the pin are broken and the signal decreases. The instrument gives classical profiles and parameters for both normal samples and those with a range of haemostatic abnormalities. The test has been successfully utilized as a point-of-care test within surgical departments to detect abnormalities in haemostasis, thus allowing rationalization of the use of clotting factor concentrates and platelet transfusion therapy (Mallet & Cox, 1992; Caprini et al., 1995). The TEG test was shown to be a significantly better predictor of bleeding (87% accuracy) in post-operative haemorrhage than the activated clotting time (30%) or coagulation profile (51%) (Spiess, 1995). The instrument has recently been upgraded to the TEG coagulation analyser 5000 series, is ergonomically designed, easy to use and maintain and controlled by updated control and data management software. The new software enables the operator to perform a completely automated analysis of blood complete with platelet function assessment and a functional fibrinogen assay. The instrument is capable of not only reducing bleeding risk and unnecessary blood component transfusions, but may also be useful for monitoring anti-platelet drug therapy. However, some data suggests that the instrument is insensitive to samples from patients who have taken aspirin. Nevertheless, this instrument has shown its capability as a useful point-of-care instrument with surgical departments and reduces both the cost and frequency of post-operative transfusions.

**Platelet contractile force (Hemodyne instrument)**

A technique originally reported by Carr & Zekert (1991) has been developed into a commercial instrument by Hemodyne. A number of studies suggest that the so-called platelet contractile force (PCF) is a clinically useful and sensitive measure of global platelet function. Within the platelet cytoskeleton, actin accounts for approximately 34% of the dry weight of the platelet. During platelet activation, contractile forces within the cytoskeleton mediate both shape change and pseudopodia formation. The platelets then adhere to the fibrin mesh via surface receptors (e.g. Gp IIb/IIIa) on the pseudopodia. Once the clot is fully formed, the platelets begin a process of clot retraction placing the entire clot under stress, resulting in the collapse of the clot and reduction in size. The Hemodyne instrument facilitates a relatively fast (<15 min) direct measurement of these platelet contractile forces (PCF) within only 800 μl of citrated whole blood. The PCF test is performed by placing a small sample of either whole blood or PRP within a sample cup between two parallel plates. Upon addition of thrombin or other agonists, the platelets within the sample attach to the plates and extend their pseudopodia along polymerizing strands of fibrin. Once the fibrin/platelet network is sufficiently formed, the platelet contractile forces begin to transmit outwards to the plates and are detected by a sensitive transducer. The electrical output is thus proportional to the amount of force generated. Two different key properties of the blood are measured simultaneously: (1) the PCF is a sensitive indicator of global platelet function and measures the force exerted by activated platelets on the fibrin network, and (2) the elastic modulus (EM) or clot rigidity is a measure of the physical structure of the fibrin/cellular network (Carr, 1995). The PCF has been shown to be decreased in thrombocytopenia, in various acquired and congenital platelet abnormalities (e.g. uraemia, during cardiopulmonary bypass (CPB) and Glanzmann’s thrombasthenia) (Greilich et al., 1995) and is inhibited by Gp IIb/IIIa inhibitors (Carr et al., 1995). Clot EM is influenced by fibrinogen concentration and is measurable in the absence of platelets. The EM parameter can also be sensitive for clotting factor deficiencies, anticoagulant effects and in myeloma. Overall, low PCF values appear to predict a bleeding risk while high PCF/EM values may predict a thrombotic tendency (e.g. in coronary artery disease) (Greilich et al., 1994). Thus, the test provides a means of testing the whole spectrum of platelet function. Given the speed and simplicity of the instrument the Hemodyne may be useful not only within the haemostasis laboratory, but may also be a useful point-of-care instrument within surgical departments, intensive care units and cardiology departments.

**Advances in the measurement of platelet activation**

Numerous tests have been developed in order to measure platelet hyper-reactivity of circulating platelets or to show that platelets have been activated in vivo by prothrombotic activity. The difficulty of these methods is to prevent artefactual ex vivo platelet activation by careful handling and blood processing. One of the earliest measurements for detecting activation was the detection of platelet aggregates within blood as described by Wu & Hoak (1974) and then modified by Bowry et al. (1985). Other methods involve measuring threshold aggregation responses to ADP/arachidonate or testing for the ability of patient’s platelets to spontaneously aggregate.

**Soluble activation markers**

Another method of assessing platelet activation is to measure platelet-release products within platelet-poor plasma. The problem with these methods is that some proteins/substances are not specific to platelets. The most useful tests are
the radioimmunoassays (RIAs) and enzyme-linked immuno-
osorbent assays (ELISAs) for platelet-specific proteins such
as PF4 and βTG. These proteins are released from the alpha
granules upon platelet activation and aggregation and
provide a simple means of assessing platelet activation \textit{ex vivo}.
However, careful venepuncture and blood handling are
essential to avoid artefactually high levels. Blood samples
are usually collected into tubes containing inhibitors of
platelet activation (e.g. theophylline and PGE$_1$). The ratio of
the two proteins often indicates if there has been a problem
with artefactually induced activation. Other platelet activa-
tion assays have included thromboxane B$_2$ measurement (a
metabolite of thromboxane A$_2$) within both plasma and
urine, and uptake and release of radiolabelled serotonin
from the dense granules. All of these assays have a number
of disadvantages including false elevation of levels \textit{ex vivo},
insensitivity, use of radioactive materials and expense in kit
form. More recently it has been demonstrated that soluble
P-selectin is also derived from activated platelets and can be
measured as a marker of platelet activation in platelet-poor
plasma (Fijnheer \textit{et al}, 1997). P-selectin or CD62p is an
alpha granular membrane protein that is expressed only on
the surface of activated platelets and can be detected by flow
cytometry (see below). The soluble protein is derived by
enzymatic cleavage of the membrane-bound form. \textit{In vivo}
studies have demonstrated that degranulated platelets
rapidly lose their surface P-selectin and continue to circu-
late and function (Michelson \textit{et al}, 1996). Although soluble
P-selectin has been shown to be specific for platelet
activation in normals, it is possible that some may also be
derived from endothelial activation/damage (Fijnheer \textit{et al},
1997).

\textbf{Flow cytometric analysis of platelets and activation markers}

Whole blood flow cytometry is a very powerful, but rela-
tively new, laboratory technique for assessing platelet acti-
vation and function (Michelson, 1996; Michelson & Furman,
1999). This technique requires a fair degree of expertise and
expensive equipment/reagents and was initially limited to
research laboratories. However, with the increasing popu-
larit of flow cytometry and availability of commercial
reagents (e.g. antibodies and dyes), the technique has
developed into a useful clinical tool for studying platelets.
Flow cytometry can be utilized to measure activated platelets,
platelet hypo- or hyper-reactivity, platelet–leucocyte aggre-
gates, platelet microparticles and platelet turnover. These
assays may be of particular use in identifying of patients
at risk from thrombosis who may benefit from anti-platelet
therapy. Specific flow cytometric assays have also been
developed to monitor anti-GpIIb/IIIa antagonist therapy,
diagnosing congenital deficiencies in platelet glycoproteins,
storage pool disease and heparin-induced thrombocytopenia.

The use of whole blood has several advantages over either
purified platelets or PRP. Firstly, platelets are analysed in the
presence of leucocytes and erythrocytes and only small
quantities of blood are required per tube (∼2–5 μl). Providing
the venepuncture technique is well standardized, the minimal
manipulation of the fresh samples results in very little artefactual \textit{in vitro} activation. It is also possible to
study platelets from patients with thrombocytopenia. Also,
both the \textit{in vivo} activation state and dose response to
classical agonists (e.g. thrombin, TRAP, ADP, etc.) can be
studied and the assays are extremely sensitive. For recom-
mendations on how to perform flow cytometric analysis of
platelets, refer to the recent articles by the European
Consensus group (Schmitz \textit{et al}, 1998) and Michelson
(1996).

The platelet activation markers that are commonly
measured include exposure of granule membrane markers
(e.g. CD62p, CD63, LAMP-1 and CD40L), activation-
dependent changes in the conformation of the GpIIb/IIIa
complex [e.g. PAC-1, ligand-induced binding site (LIBS) and
receptor-induced binding site (RIBS) antibodies], binding of
secreted proteins (e.g. TSP and multimerin) and exposure of
phosphatidyl serine (factor Vla or Vllla binding, annexin-V).
As discussed above, surface expressed CD62p (P-selectin) is
rapidly cleaved to release soluble P-selectin into the plasma
(Michelson \textit{et al}, 1996). Thus, the levels of CD62p on the
platelet surface may not be a true reflection of the \textit{in vivo}
activation state of platelets and should be complemented
by measurements of all other markers including soluble
P-selectin. A full spectrum of all activation markers should
be measured, particularly as it is unknown what the exact
time-frame of expression is for each marker. Activated
platelets have been demonstrated in a wide variety of patients
including acute coronary syndromes (Michelson, 1996;
Furman \textit{et al}, 1998; Ault \textit{et al}, 1999), stroke (Grau \textit{et al},
1998; Zeller \textit{et al}, 1999), anti-phospholipid syndrome
(Joseph \textit{et al}, 1998) and during angioplasty (Tschoepe
\textit{et al}, 1993). Platelet activation markers thus have the potential
to optimize anti-platelet therapy (e.g. anti-Gp IIb/IIIa drugs)
and act as a predictor of thrombotic events after invasive
techniques (e.g. angioplasty) (Tschoepe \textit{et al}, 1993).

One of the problems with measuring the activation status
or reactivity of circulating platelets alone is that the clinical
picture is incomplete, as fully activated platelets within
thrombi and clots are unlikely to circulate. However, recent
data suggests that the measurement of platelet–leucocyte
complexes may be a more sensitive marker of \textit{in vivo} platelet
activation. By using a double-labelling technique it is possi-
bile to study aggregates of platelets with both neutrophils
and monocytes by flow cytometry (Li, N., \textit{et al}, 1997). When
platelets become activated they can bind to monocytes and
neutrophils by a number of different receptor–ligand pairs
or bridges, e.g. CD62p–PSGL1, CD36–TSP–CD36, Mac1–
Fg–GpIIb/IIIa and CD40L–CD40. Increased numbers of
circulating platelet-leucocyte complexes have been demon-
strated in patients with myocardial infarction (MI), unstable
and stable angina (Furman \textit{et al}, 1998), and during CPB.
Circulating monocyte–platelet complexes may be a rela-
tively stable and more sensitive marker than other activa-
tion-dependent markers, e.g. P-selectin. The platelets induce
tissue factor expression after binding to monocytes, perhaps
increasing the prothrombotic tendency.

Flow cytometric analysis of platelets can also be utilized to
count platelets accurately (see section on platelet counting),
diagnose various glycoprotein deficiencies (e.g. Bernard
Soulier syndrome and Glanzmann’s thrombasthenia) and to
study platelets from patients with defective aggregation, secretion, microparticle generation and procoagulant activity (e.g. Scott syndrome) (Weiss, 1994; Horstman & Ahn, 1999).

By using the dye Mepacrine in conjunction with platelet activation in vitro, it is possible to diagnose dense granule deficiency and storage pool disease (both storage and release defects) in a very simple and reproducible assay (Gordon et al, 1995; Wall et al, 1995). This assay is much simpler than either the state-of-the-art luminescence or HPLC assays that measure intracellular or released ADP levels (Summerfield et al 1981).

Nucleic acid-specific dyes can also be utilized to measure young platelets that contain residual mRNA. These calls are analogous to red cell reticulocytes and have thus been termed reticulated platelets. This assay is a simple non-invasive method of assessing platelet turnover or thrombopoiesis in different types of thrombocytopenia, is a simple predictor of bone marrow recovery after transplantation and can be utilized to monitor the response to therapy with growth factors such as thrombopoietin (Harrison et al, 1997). Interestingly, the assay may also be a predictor of thrombosis in patients with myeloproliferative syndromes such as ET (Rinder et al, 1998). Recent evidence suggests that the nucleic acid dyes may also be labelling the dense granules (Robinson et al, 1998). Although there is evidence to support that it is the youngest platelets being measured (Ault & Knowles, 1995; Dale et al, 1995), it is possible that the granular component of the larger young platelets is also contributing to the signal (Balduini et al, 1999). Assays can now be optimized in order to control for this problem (Matic et al, 1998).

Flow cytometric analysis is a powerful approach for studying many properties of platelets. Although primarily a research tool, many of the assays are now being shown to have enormous clinical potential. With concurrent advances in cell counting technology, including the addition of light scattering and fluorescent measurements within existing impedance counters, some of these platelet assays have the potential of being fully automated without the need of either a flow cytometer or specialized operators.

SUMMARY

There have been many recent potential useful additions to our existing portfolio of platelet function tests. In particular, the development of reliable, sophisticated, but simple to use, whole blood tests (e.g. CSA, PFA-100, Hemodyne, etc.) that mirror physiological conditions provides the ability to screen potential samples rapidly before our existing tests are utilized. These tests should provide us with a rapid, but reliable, means of assessing whether there is a primary haemostatic defect. The in vitro bleeding time may therefore be potentially replaced by simple in vitro tests. Many of the instruments (e.g. Ultegra, PFA-100, CSA, Hemodyne and TEG) have been or will be utilized as point-of-care instruments for assessing bleeding risk or monitoring anti-GpIIb/IIia or anti-platelet therapy. For the first time therefore, simple platelet function testing may not require the specialized pathology laboratory and will increase cost effectiveness. Flow cytometry now offers a powerful approach to studying a number of different platelet activation markers and will increasingly reveal the true clinical picture of platelet activation in various disorders. Clinically useful flow cytometric assays will also have the potential of being fully automated within existing cell counters. Also, many of the new tests will be able to predict whether certain types of patients exhibit a prothrombotic tendency. For the first time, we will be able to reliably and accurately assess both platelet hypofunction and hyperfunction. With the advent of the millennium, we can now look forward to a completely new approach to both the diagnosis and management of patients with the full spectrum of platelet disorders.

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**Keywords:** platelets, platelet function testing, aggregometry, bleeding time, flow cytometry.