Laboratory techniques

Guidelines on platelet function testing

THE BRITISH SOCIETY FOR HAEMATOLOGY
BCSH HAEMOSTASIS AND THROMBOSIS TASK FORCE

Following a questionnaire from the Haemostasis and Thrombosis Task Force of the British Society for Haematology in 1985, there was obviously considerable variability and confusion as to how haematologists in the United Kingdom investigated platelet function. This document outlines a standardised approach which could be followed by most routine laboratories for the investigation of bleeding disorders. Platelet release studies are included for interest; these are not recommended for routine laboratories. Platelet function studies used specifically to investigate thrombotic disorders or an assumed hypercoagulable state will not be discussed in these guidelines. Tests which are primarily of a research nature or are in use only at a highly specialised referral unit are also not discussed. It is essential to have a working knowledge of platelet physiology so that the relevant platelet function tests can be performed in an orderly sequence and interpreted correctly. For this reason we have supplied some basic details on platelet biochemistry and structure.

The bleeding time

BACKGROUND
When investigating patients suspected of having a bleeding disorder, it is essential to obtain a detailed clinical history before embarking on tests of haemostatic function.

The peripheral platelet count, blood film examination, and the skin bleeding time are the first line basic laboratory tests of platelet function. If these tests are within normal limits it is unlikely that a clinically important platelet defect is responsible for excessive clinical bleeding.

A drug history is particularly important, and as fas as possible the use of drugs should be avoided when platelet function is assessed. This applies particularly to patients with congenital platelet disorders. In acquired bleeding states the drugs that patients are receiving may themselves be directly responsible for the haemostatic defect. When this is suspected platelet function should be assessed when the patient is both off and on the drug. Recent aspirin ingestion is of particular importance as a single dose may exert its effect for up to 10 days. Other drugs which affect the bleeding time include non-steroidal anti-inflammatory agents, ticlopidine, heparin, penicillin (in high doses) and the antibiotics carbenicillin and ticarcillin (table 1).

The bleeding time is arguably the most useful test of platelet function in that it provides clinically relevant information about the contribution of platelets to primary haemostasis.

How long it takes for bleeding to stop after a skin incision is largely influenced by rapid accumulation of metabolically active platelets at the site of the wound and the formation of a haemostatically effective platelet plug. The bleeding time reflects this process and if it is performed in a standardised manner is sensitive to changes in platelet function and platelet number.

Several attempts have been made to improve the sensitivity and reproducibility of the bleeding time since its introduction in 1910 by Duke. In the original method the ear lobe was punctured by a needle. Later, between 1935–41, Ivy described a method which consisted of three puncture wounds in the forearm. Some improvement in sensitivity was achieved by the application of a sphygmomanometer cuff, inflated to
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<table>
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<th>Drugs which affect platelet function</th>
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<td>Membrane stabilising agents</td>
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<td>α-antagonists</td>
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<td></td>
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<td>Local anaesthetics (procaine)</td>
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<td>Antihistamines</td>
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<td>Aspirin and proprietary preparations containing acetylsalicylic acid</td>
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<td>Antibiotics</td>
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<td>Penicillins</td>
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<td>4</td>
<td>Agents which increase cyclic adenosine monophosphatase concentrations</td>
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<tr>
<td></td>
<td>Dipyridamole</td>
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<td>Aminophylline</td>
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<td></td>
<td>Prostanoids</td>
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<tr>
<td>5</td>
<td>Others</td>
</tr>
<tr>
<td></td>
<td>Heparin</td>
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<td></td>
<td>Dextran</td>
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<td></td>
<td>Ethanol</td>
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<td>Phenothiazine</td>
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<td>Clofibrate</td>
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<td>Papaverine</td>
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<td>6</td>
<td>Foodstuffs</td>
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<tr>
<td></td>
<td>Alcohol</td>
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<tr>
<td></td>
<td>Garlic</td>
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<tr>
<td></td>
<td>Certain Chinese foods</td>
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</table>

40 mm Hg pressure, at a site proximal to the puncture wounds.

Recently, further modifications of the bleeding time have resulted in a test that is both sensitive and reproducible. In 1958 Borchgrevink and Waaler recommended the use of incisions of predetermined length and depth rather than puncture wounds. Additional refinements were described by Mielke in 1969 and more recently a disposable spring loaded device (Simplate II, General Diagnostics) has been developed which produces incisions 5 mm long and 1 mm deep and gives reliable and reproducible results.3

METHODOLOGY
A recent survey of United Kingdom laboratories studied striking differences in reported bleeding times of normal subjects.4 These differences were even apparent among laboratories using template bleeding times. These observations emphasise the necessity for strict laboratory control, and it is therefore strongly recommended that for any given method individual laboratories should establish their own range of normal values. The result may be influenced by the choice of operator and this also should be borne in mind when establishing the normal range.

For the reasons outlined above, the template bleeding time is the method of choice.5 After a sphygmomanometer cuff, inflated to 40 mm Hg pressure, has been applied, incisions are made over the lateral aspect of the anterior surface of the forearm, care being taken to avoid superficial veins. After 30 seconds the blood issuing from the wound is gently blotted with filter paper. The wound edge is not touched. Blotting is continued at 30 second intervals until the blood no longer stains the filter paper. This represents the bleeding time end point.

Duplicate incisions are recommended unless it can be shown that reliable results can be achieved by a single incision.

FACTORS INFLUENCING THE BLEEDING TIME
The bleeding time is influenced by several factors including age, sex, skin temperature, haematocrit, venostasis and the site and direction of the incision. The most important variables are those which relate to methodological detail.

The bleeding time is longer when the incision is made on the lateral, as opposed to the medial aspect of the forearm, and a transverse incision produces a longer bleeding time than does one which is performed longitudinally. Many workers recommend a longitudinal technique as scarring is a potential problem with transverse incisions. The use of a “butterfly” dressing to approximate the wound edges may also minimise the risk of scarring.

When performed without venostasis pronounced variation of the bleeding time can occur. The use of a sphygmomanometer cuff, inflated to 40 mm Hg pressure, produces longer bleeding times and reduces the degree of variability.

Several workers have shown that there is a significant difference in bleeding times between males and females, with longer times being recorded in females. Although cold is known to prolong the bleeding time it is probably important only when the skin temperature falls below 25°C.

The platelet count also influences the bleeding time; as this falls below 80–100 × 109/l the bleeding time becomes progressively prolonged. In most patients with thrombocytopenia a bleeding time is therefore unnecessary. In a proportion, however, the bleeding time may be of clinical value in that it provides information about platelet reactivity. An example is disseminated intravascular coagulation where an acquired storage pool disorder may accompany thrombocytopenia. On these occasions the bleeding time will be disproportionately prolonged relative to the platelet count.

In normal neonates and children the bleeding time may be longer than that of adults. Although the establishment of a normal range in these groups is highly desirable, this may be precluded on ethical grounds. For departments where this applies, useful experience can be obtained by performing the technique in a standardised manner but results should be interpreted with appropriate caution.

If the bleeding time suggests a platelet functional
disorder, or when there is a high degree of clinical suspicion despite a normal bleeding time, further tests should be planned in a systematic way. Drugs and certain dietary practices (table 1) are the commonest cause of platelet dysfunction and ideally patients should refrain from taking drugs with known antiplatelet effects for seven to 10 days before blood sampling for more specific function investigation. If there is any doubt about a particular drug the bleeding time should be repeated if possible two weeks after stopping that drug. Causes of a prolonged bleeding time are summarised in table 2. In most instances these relate to changed platelet reactivity. Special attention should be paid to the possibility of von Willebrand's disease and its many variants.

**Tests of platelet adhesion**

The most widely adopted diagnostic adhesion test measures platelet retention after a single passage of whole blood through a glass bead column. Attempts to standardise this test have used glass beads of a constant size packed in a tube of fixed diameter and length (which are commercially available) with a steady infusion rate of non-anticoagulated whole blood directly from an arm vein, using an evacuated system. The percentage platelet retention in the column is calculated from a pre- and post-column infusion platelet count. Despite all these precautions the results are extremely variable and decreased glass bead retention of platelets is not only found in platelet adhesion defects. It has subsequently been shown that retention also depends on plasma von Willebrand factor (factor VIII:WF) activity, fibrinogen coating the glass bead surface, and on platelet aggregation which is promoted by local release of adenosine diphosphate (ADP) from haemolysed red cells in the column. These tests are therefore not generally recommended as a routine platelet function test.

**Platelet aggregation**

Studies of platelet aggregation are indicated in anyone suspected of having a platelet function defect, particularly in patients with a prolonged or borderline bleeding time test. These tests are routinely performed on platelet rich plasma. Recently techniques using whole blood have been developed, but because of their limitations for routine use and difficulties in interpretation they will not be further discussed here.

**TURBIDOMETRIC METHOD**

**Principle**

Blood is centrifuged gently to obtain platelet rich plasma, which is stirred in a cuvette at 37°C between a light source and a photocell. When an agonist is added, the platelets aggregate and absorb less light so that transmission increases and is detected by movement of a pen on the chart recorder. The addition of different agonists at a range of concentrations allows certain aggregation defects to be detected.

**Biochemical basis**

Reagents such as collagen, thrombin, and ADP bind to specific platelet membrane receptors, activating platelets and triggering a series of reactions which can culminate in shape change, granule release, and aggregation. Whether any or all of these responses occur depends on the normal function of the platelet, the concentrations of certain inhibitory substances, and the concentration of the agonist used.

There are thought to be three basic pathways of platelet aggregation all of which are interlinked: ADP causes a chain of events involving phosphatidyl inositol metabolism, leading to the exposure of fibrinogen binding sites on the membrane and aggregation. Low concentrations of collagen cause platelet aggregation through a pathway predominantly depen-
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This induces arachidonic acid mobilisation from the membrane, followed by conversion to thromboxane A₂, which is a potent stimulator of aggregation, causing ADP release and calcium flux. Higher concentrations of collagen will also activate platelets by prostaglandin independent mechanisms. Similar considerations apply to low and high concentrations of thrombin. Serotonin and adrenaline act synergistically with other reagents. The relation of in vitro platelet aggregation in plasma to physiological responses must be considered carefully as the low extracellular calcium ion concentrations generate artefacts such as the release reactions to ADP and adrenaline, which do not occur if blood is collected into the thrombin inhibitor hirudin, instead of citrate.

**Test samples**
Nine volumes of blood are added to one volume of trisodium citrate (0.109M) and citrated blood is then centrifuged at 170 g (800–1000 rpm in a bench centrifuge), for 10 minutes at room temperature to prepare platelet rich plasma. This must be removed and stored at room temperature in a capped tube. All handling should be kept to a minimum and be done with plastic pipettes and tubes.

The residual blood must then be centrifuged at 2700 g (about 3500 rpm) for 15 minutes and the resulting platelet poor plasma collected.

**Reagents**
1. ADP (Sigma Grade III): 10 mM solution in saline, stored in aliquots at −20°C.
2. Collagen (Hormon-Chemie, Munchen): 1 mg/ml stored at 4°C.
3. Adrenaline (Sigma): 1 mg/ml (5-5 mM) store at −20°C.
4. Arachidonate (Sigma): 20 mM solution in distilled water, store at −20°C.
5. Thrombin (Parke-Davis, Bovine): 50 u/ml solution in saline, at −20°C.
6. Endoperoxide Analogue U46619 (Upjohn Ltd); dissolve phial contents in a small quantity of ethanol and dilute to 25 μg/ml with saline, store at −20°C in aliquots.
7. Calcium Ionophore A23187, Ca²⁺ + Mg²⁺ salt (Sigma): 500 μg/ml (1 mM) in ethanol at −20°C.
8. Ristocetin (Lundbeck): 12.5 mg/ml in saline, store at −20°C.
9. Porcine FVIII Complex/Bovine Fibrinogen (Diagnostic Reagents Ltd), reconstitute according to manufacturer.

**Equipment**
(i) Optical aggregometer—the method described is for an aggregometer, which does not have an autobaseline facility, fitted with cuvettes for 250–500 ul.
(ii) Chart recorder.
(iii) Glass cuvettes.
(iv) Stir bars.
(v) Equipment for platelet counting.

**Preparation of samples and reagents**
Platelet rich plasma is diluted with autologous platelet poor plasma to give a final platelet count of 200 × 10⁹/l.
ADP—prepare 100, 50, 25, 10, and 5 μM solutions in saline on ice.
Collagen—prepare 40 and 10 μg/ml solutions in buffer (Hormon-Chemie) on ice.
Ristocetin—thaw stock and keep on ice. Dilute as necessary in saline.
Adrenaline—prepare 100, 50, and 10 μM solutions in saline on ice.
Arachidonate—dilute stock solution with an equal volume of distilled water on ice.
Thrombin—dilute in saline to 1 and 5 μ/ml on ice.
Calcium Ionophore A23187—dilute in saline to give 5 and 100 μg/ml solutions, store on ice.
Porcine Factor VIII—dilute to 20 u/ml at room temperature.

**Method**
1. Allow aggregometer to warm at 37°C and set stirrer speed to 900 rpm.
2. Set chart recorder to 10 mv and 3 cm/minute.
3. Platelet poor plasma (300 μl) is placed in one cuvette and 270 μl platelet rich plasma with a stir bar in another; these are used to calibrate the aggregometer signal on the chart recorder to 10% and 95% settings, using the output and zero controls, respectively (see manufacturer's instructions).
4. Undiluted platelet rich plasma (270 μl) is placed in a cuvette in the aggregometer and stirred for 15 minutes to check for spontaneous aggregation (pen deflection > 20% of chart).

**Table 3 Agonists for platelet aggregation studies**

<table>
<thead>
<tr>
<th>Agents routinely used in screening tests</th>
<th>Final concentrations usually used</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>0.5–10.0 μM</td>
</tr>
<tr>
<td>Collagen</td>
<td>1.0–4.0 μg/ml</td>
</tr>
<tr>
<td>*Ristocetin</td>
<td>0.5–1.2 mg/ml</td>
</tr>
</tbody>
</table>

*Agents causing platelet agglutination rather than true aggregation.
†This varies according to species and tissue source of collagen. The manufacturer's instructions should be followed.
5 If spontaneous aggregation is present dilute the platelet rich plasma in platelet poor plasma and repeat the test until a dilution is found where spontaneous aggregation disappears. If this point is found at or above a platelet count of 200 $10^9/L$, aggregation tests may proceed.

6 Place 270 μl dilute platelet rich plasma in a cuvette and warm until a steady baseline is obtained.

7 Add 30 μl of aggregating reagent (agonist) and record response.

8 Continue with other agonists at the final concentration range indicated (table 3). The three agonists listed in table 3 should be initially used routinely over the final concentration ranges stated. Only if defective aggregation responses are obtained should one proceed with the agents listed.

Expression and reporting of results
Subjective assessment of aggregation responses by a trained eye is usually sufficient for clinical interpretations. Typical traces obtained with some commonly used aggregating agents are shown in the figure. Quantitation of the aggregation response may be made by measuring the lag phase (d) (with collagen only) the angle (x) of the initial aggregation slope, and the maximum height (y) of the response after a standard time (usually three minutes). Alternatively, threshold concentrations may be determined by starting with a very low dose of an aggregating agent and progressively adding higher concentrations of the agonist until a response is obtained. Similarly, an EC$_{50}$ (50% of effective concentration) value can be obtained by finding the dose of aggregating reagent which causes 50% of the maximum response; this is usually found by drawing a dose-response curve for a number of concentrations of agonist.

Technical comments
1 The volumes of platelet rich plasma used will depend on the aggregometer and cuvettes used. The smaller the cuvette, the more responses that can be obtained with a given volume of platelet rich plasma, but the poorer the optical quality (due to a shorter lightpath) and the more likely the influence of factors such as debris or air bubbles.

2 Care should be taken to exclude red cells and granulocytes from platelet rich plasma as these will interfere with the light transmittance and cause reduced response heights which can be mistaken for abnormal aggregation. In diseases such as thalassaemia, where there may be red cell fragments and membranes, these may be removed by further centrifugation of platelet rich plasma at 150 g for two minutes, or after settling has occurred.

3 If cryoglobulins are present they may cause changes in transmittance like spontaneous aggregation. Warming the platelet rich plasma to 37°C for five
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minutes allows aggregation to be performed in the normal way.
4 Lipaemic plasma may cause problems in adjusting the aggregometer and the responses may be compressed owing to the small difference in transmitted light between platelet rich plasma and platelet poor plasma. Care should be taken in the interpretation of results from such samples.
5 The aggregometer must be adjusted with platelet rich plasma at the platelet count that will be used in the test, as this will affect optical density—for example, when changing from spontaneous aggregation to responses to agonists.
6 Aggregation should be performed within two hours. If longer delays cannot be avoided it is best to store the platelet rich plasma under 5% carbon dioxide, or if this is not available, to leave the smallest possible air gap over the platelet rich plasma and cap the tube.
7 Lumi-aggregometers are also available; these measure ATP release simultaneously with aggregation. This is possible using a firefly extract known as luciferase, which emits energy in the form of luminescence when ATP is available as a substrate; the latter can be detected if a luminescence detector is fitted to the aggregometer.
8 Ristocetin concentrations above 1·4 mg/ml may cause non-specific protein precipitation which can be mistaken for agglutination. Absent aggregation induced by ristocetin may occur in patients of negro racial origin and is not associated with a bleeding diathesis.

Interpretation

Patterns of typical abnormal aggregaion responses are shown in table 4.

For a more detailed discussion of aggregation agonist responses and congenital and acquired defects of platelet function, the following reviews are useful.15 9 10

<table>
<thead>
<tr>
<th>Disorder</th>
<th>ADP</th>
<th>Collagen</th>
<th>Ristocetin (1-2 mg/ml)</th>
<th>Ristocetin (0-5 mg/ml)</th>
<th>Arachidonic acid</th>
<th>Endoperoxide analogue</th>
<th>A23187</th>
<th>Porcine VIII</th>
<th>Confirmatory tests</th>
</tr>
</thead>
<tbody>
<tr>
<td>vW disease (type I and II A)</td>
<td>N</td>
<td>N</td>
<td>A/R/N</td>
<td>A</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Factor VIII studies</td>
</tr>
<tr>
<td>vW disease (type II B)</td>
<td>N</td>
<td>N</td>
<td>A</td>
<td>H</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>Factor VIII studies</td>
</tr>
<tr>
<td>Bernard-Soulier syndrome</td>
<td>N</td>
<td>N</td>
<td>A</td>
<td>A</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>A</td>
<td>Not required</td>
</tr>
<tr>
<td>Glanzmann’s disorder</td>
<td>P/N</td>
<td>A</td>
<td>N</td>
<td>A</td>
<td>A</td>
<td>—</td>
<td>P</td>
<td>P</td>
<td>Not required</td>
</tr>
<tr>
<td>Storage pool disease</td>
<td>P/N</td>
<td>R/N</td>
<td>P/N</td>
<td>A</td>
<td>R/N</td>
<td>R/N</td>
<td>R/N</td>
<td>P</td>
<td>5-HT release studies</td>
</tr>
<tr>
<td>Membrane receptor defect</td>
<td>R/N</td>
<td>R</td>
<td>R/N</td>
<td>A</td>
<td>N</td>
<td>N</td>
<td>N</td>
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</tr>
<tr>
<td>Cyclo-oxygenase deficiency</td>
<td>R/N</td>
<td>R</td>
<td>N</td>
<td>A</td>
<td>R</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td></td>
</tr>
</tbody>
</table>

N: Normal response; A: Absent response; R: Reduced response; H: Heightened increased response; P: Primary wave only.

The release reaction and platelet adenine nucleotide measurement

The platelet release reaction is characterised by secretion of the constituents of the three types of platelet storage granule. These comprise dense bodies, \( \alpha \) granules, and lysosomes.

Dense bodies are the storage site for calcium, serotonin, and the non-metabolic pool of adenine nucleotides. As they are osmiophilic and absorb electrons when unstained they appear as dense intra-platelet inclusions on transmission electron microscopy.

Alpha granules contain several platelet specific proteins, such as platelet factor 4 and \( \beta \)-thromboglobulin, and certain coagulation factors. The stimulus to release \( \alpha \) granule contents is often less than that for dense body release and increased plasma concentrations of the aforementioned platelet specific proteins may be interpreted as evidence of platelet activation.

In the investigation of patients with bleeding disorders the release mechanism is usually assessed by determination of the total platelet content of ADP and adenosine triphosphate (ATP), and the release of ATP or 5-hydroxytryptamine or both, from the dense bodies.

There are two separate nucleotide pools within the platelet, with 60% being stored in the dense granules. The remainder constitute the metabolic pool of adenine nucleotides. There are pronounced differences in the relative concentrations of ADP and ATP within the two pools. In the metabolic pool the ATP:ADP ratio is 8:1; in the dense granules the ratio is 2:3. The nucleotide exchange between the pools is slow. Thus in storage pool defects where there is reduction or absence of dense bodies and their associated nucleotides the ratio of total platelet ATP to total platelet ADP increases.

There are various methods for determining total
platelet nucleotides. In routine clinical practice assays based on firefly bioluminescence techniques are increasingly being used. The firefly enzyme luciferase produces light proportional to the concentration of ATP and this is measured with a luminometer. Although ADP cannot be measured directly by this method, it is possible to perform an indirect assay by converting the available ADP to ATP.

Firefly bioluminescence techniques are also of value in assessing platelet release. This has been facilitated by the recent introduction of an aggregometer which also incorporates a luminometer. The system therefore permits simultaneous monitoring of platelet aggregation and platelet release of ATP.

High performance liquid chromatography is a more sensitive method for measuring platelet adenine nucleotides but it is not generally available in routine haematology departments.

5-hydroxytryptamine (5-HT) is readily taken up by platelets and stored in the dense bodies. Determination of the uptake and release of 5-HT by platelets is a convenient method of assessing platelet secretion. In this system thrombin or some other agonist is used to induce platelet secretion and the proportion of incorporated radioactivity released from the platelets determined. Additional information can also be obtained by measuring the proportion of radioactivity that "leaks" from unstimulated platelets. Normally this does not occur but it may be detectable in storage pool disorders.

Before investigating platelet release it is important for each laboratory to establish its own normal range with respect to the agonist and also to the concentration used. Thrombin induces the release of 70–90% total radioactivity while that induced by collagen is somewhat less—20–60%. When using collagen it is important to use the same reagent throughout as the result is influenced by the type of collagen used.

**ATP release: luciferin-luciferase method**

**PRINCIPLE**

When a platelet aggregating agent is added to platelet rich plasma in the presence of luciferin-luciferase, the ATP released during platelet aggregation will react with the luciferin in a light-emitting reaction:

\[
\text{ATP} + \text{luciferin} \rightarrow \text{Mg}^{2+} \quad \text{luciferase} \rightarrow \text{adenyl luciferin}
\]

The light released is measured and recorded by the lumigaggregometer.

**Reagents**

Luciferin-luciferase 40 mg/ml (Sigma)

ATP standard 100 μM (Sigma)

Arachidonic acid, sodium salt 15 μM (BioData)

Collagen 40 μg/ml (Hormon-Chemie, Munich)

**Method**

1. Take 18 ml blood into 2 ml 0.19 M sodium citrate in a plastic container, using a 19 gauge needle and minimum stasis.

2. Prepare platelet rich plasma as for platelet aggregation studies.

3. Prepare platelet poor plasma by centrifuging the remainder of the sample for 10 minutes at 2000 g.

4. Adjust platelet rich plasma count to 200 × 10^9/l with platelet poor plasma.

5. Using 500 μl platelet poor plasma and 450 μl platelet rich plasma, add the appropriate amount of freshly prepared luciferin-luciferase (25 μl for collagen, 50 μl for arachidonate) to the platelet rich plasma in a cuvette in the aggregometer and warm for one minute.

6. Add 50 μl of the appropriate agonist.

7. Observe the aggregation and record the luminescence in terms of peak height.

8. When the aggregation is complete add 20 μl (2 nmol) of ATP standard and record the peak height. Then further add 30 μl (3 nmol) of the ATP standard and again record the peak height.

9. Ideally the luminescence peak height should fall between that of the two standards, so if the luminescence peak is low use less standard, such as 10 μl and 15 μl.

10. Plot the standard concentrations as nmols against peak heights and read off the release peak obtained with each agonist.

**Calculation**

ATP release is expressed in terms of nmol/10^6 platelets.

\[
\text{ATP release} = \frac{\text{nmoles ATP released}}{\text{No of platelets} \times 10^6 \times 0.45}
\]

(0.45 is the volume of platelet rich plasma in the cuvette in ml).

**Interpretation**

Laboratories should establish their own normal ranges. Reduced ATP release indicates a platelet release defect.

**Radiolabelled 5-hydroxy tryptamine (serotonin) uptake and release**

**PRINCIPLE**

Serotonin is present in the dense granules of platelets and is released when the platelets aggregate. Platelets will also actively take up serotonin from solution.

Platelets are incubated with radiolabelled serotonin. The amount taken up by platelet dense bodies is measured as the difference between the total added radioactivity and the activity remaining in the incubation solution after the platelets are removed by centrifugation. Imipramine is added to prevent further uptake.
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The platelets are aggregated with collagen or thrombin, or both, and the amount of serotonin released measured. The amount of serotonin leaking out of the platelets is also determined.

Reagents
1 Impiramine hydrochloride: 31.5 mg in 5 ml distilled water, prepared fresh.
2 Thrombin 500 μl/ml.
3 Collagen 40 μg/ml: make up as for platelet aggregation.
4 Stock 14C-5-HT: prepare 0.5 ml volumes containing 1.258 μCi 14C-5-HT.
5 Streptokinase 25000 u/ml store at −80°C.

Procedure
1 Prepare platelet rich plasma from citrated blood, as for platelet aggregation studies. Adjust the platelet count to 200 × 10^9/l using platelet poor plasma. A control from a healthy donor should be studied concurrently.
2 Add 4.5 ml of platelet rich plasma to a 0.5 ml aliquot of 14C-5-HT. Mix gently and incubate at 37°C in a water bath. Start the stopwatch.
3 Remove 0.5 ml for measurement of total counts. Transfer duplicate 100 μl aliquots to scintillation phials for counting (phials 1 and 2).
4 At 30 minutes, 60 minutes, and 90 minutes, remove 0.5 ml for measurement of uptake. Centrifuge to prepare platelet free plasma (an Eppendorf centrifuge is suitable) and transfer 100 μl aliquots of supernatant to scintillation phials (phials 3 and 4, 5 and 6, 7 and 8).
5 At 90 minutes add 3 μl of imipramine solution (final concentration 2 × 10^{-5} M). At 120 minutes remove 0.5 ml. Centrifuge as above and transfer 100 μl aliquots for counting. This represents “leakage” (phials 9 and 10).
6 Remove 1 ml and incubate at room temperature on a rotary mixer, with 10 μl thrombin 500 μl/ml and 10 μl streptokinase 2500 U/ml, for about 10 minutes. Centrifuge and transfer 100 μl aliquots of supernatant for counting. This represents “release to thrombin” (phials 11 and 12).
7 Remove 300 μl and transfer to an aggregometer cuvette with stirrer bar. Add 30 μl collagen to give a final concentration of 4 μg/ml and incubate in an aggregometer for five minutes at 37°C. Centrifuge and transfer 100 μl aliquots for counting. This represents “release to collagen” (phials 13 and 14).

Calculation
Calculate:
(i) Mean of each replicate count.
(ii) Mean of each duplicate phial.
(iii) % Uptake = \( \frac{\text{Total counts} - \text{uptake counts}}{\text{Total counts}} \times 100 \)
Calculate uptake at T30, T60 and T90.
(iv) % Leakage = \( \frac{\text{Leakage counts} - \text{T90 uptake counts}}{\text{Total counts} - \text{T90 uptake counts}} \times 100 \)
Calculate release to collagen and to thrombin.

Interpretation of results
For each agonist, normal ranges should be established for 5-HT uptake and release. Using the above procedure, thrombin is a more potent stimulus to 5-HT release than collagen (70–90% release compared with 20–60%, respectively), but in respect of collagen the result is influenced by the type used. Reduced 5-HT release indicates a platelet release defect. Normally, the “leakage” of 5-HT from platelets is 0–8%. Increased values suggest a storage pool defect.

Conclusions
These guidelines outline the routine methodology which should be available for the initial investigation of a patient with a presumed haemorrhagic platelet defect. Platelet release studies are included for interest and are not recommended for routine laboratories.

Because of the importance of platelet aggregation by the standard turbidimetric technique, this has been described in greater practical detail as it is with this widely available laboratory test that a great deal of confusion has arisen. For further details the reader is referred to two more detailed articles by members of this Task Force. To help haematologists identify congenital disorders we enclose minimal diagnostic criteria (Appendix 1).

Appendix

CONGENITAL PLATELET DISORDERS: MINIMAL DIAGNOSTIC CRITERIA

A Glanzmann’s disease
   a Absent aggregation responses to ADP.
   b Primary agglutination response to ristocetin.

B Bernard-Soulier syndrome
   a Large platelets on peripheral blood film.
   b Absent ristocetin-induced platelet agglutination not corrected by normal plasma (or cryoprecipitate).

C Platelet release defects
   Impaired secondary aggregatory response to
ADP. In some instances these responses are normal. This group can be further categorised by the demonstration of additional features:

1 Storage pool disorders
   a Increased platelet ATP:ADP ratio with reduced platelet ADP concentration.

2 Defect of arachidonic acid peroxidation—for example, cyclo-oxygenase deficiency, thromboxane synthetase deficiency.
   a Impaired arachidonate-induced platelet aggregation with normal primary aggregatory responses to ADP.
   b Reduced arachidonate-induced platelet thromboxane production.

3 Presumed abnormalities of Ca++ mobilisation
   a Impaired aggregation responses to calcium ionophore (A23187).

D Gray platelet syndrome
   a Morphological abnormalities on peripheral blood film (typically, absence of azurophilic granules).
   b Evidence of reduced α granule platelet specific peptides (PF₄ and βTG).

E Familial platelet
   1 Giant platelet syndromes (excluding Bernard Soulier)
      a Large platelets on blood film
      b Exclusion of Bernard-Soulier syndrome.
   2 Congenital thrombocytopenia with normal platelet morphology.

F Platelet-type ("pseudo") von Willebrand's disease
   a Features suggesting type 11A von Willebrand's disease.

British Society for Haematology Guidelines 1988
b Enhanced agglutination with ristocetin or agglutination by cryoprecipitate in the absence of ristocetin.

References
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