

## 39 Platelet Function Testing

Platelets contribute to hemostasis in two main ways:

- They adhere to subendothelial microfibrils and collagen fibres in the blood vessel wall, after which they change shape, undergo a specific release reaction, and then aggregate to form a primary hemostatic plug.
- As a result of these events, particularly during release and aggregation, procoagulant activities are generated, chiefly involving the platelet membrane phospholipids so that blood coagulation is initiated at the areas where platelet aggregation has occurred.

Quantitative or qualitative platelet defects may result in a significant bleeding tendency, mainly due to failure of platelet plug formation, but also to a lesser extent to the sub-optimal activation of blood coagulation.

Defective platelet function, or thrombocytopenia, may present clinically with a variety of symptoms strongly suggestive of primary hemostatic failure (e.g. bruises or ecchymoses, epistaxis, gastrointestinal bleeding, or menorrhagia). Platelet defects usually give rise to fairly mild bleeding disorders. Patients may present only with excessive bleeding after surgery or dental extraction.

Platelet defects include storage pool disease, Glanzmann's thrombasthenia, and Bernard-Soulier syndrome.

In the investigation of patients suspected of having a bleeding disorder, it is important to collect a detailed clinical history. Examination of a blood film, determination of the blood platelet count, and determination of the skin bleeding time (described in Section 11) are important initial tests. If these tests are within normal limits, a clinically important platelet defect as the cause of the bleeding under investigation is unlikely.

A drug history is important. A number of drugs can influence results of platelet function tests including bleeding time. For example, recent Aspirin ingestion can exert an effect for up to 10 days.

For a list of drugs that can interfere with platelet function, see the British Society for Haematology's guidelines on platelet function testing referenced below.

Another simple test that can give an indication that an abnormality may be present is the clot retraction method, described below.

## 39.1 Clot Retraction

The retraction of the clot in clotted whole blood can give an indication of platelet number and function. When the clot retracts, serum is expressed, and the degree of clot retraction can be measured.

### METHOD

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- 1 Collect 1ml of blood into a glass test tube (75 mm × 10 mm) and place at 37°C.
  - 2 Examine the tube visually until a firm clot is present. Leave undisturbed at 37°C for another hour.
  - 3 Measure the distance from the base of the tube to the meniscus. Carefully remove the clot with a thin wooden stick (e.g. a cocktail stick), leaving the serum that has been expressed from the clot in the tube.
  - 4 Measure the distance from the base of the tube to the meniscus of the serum.
  - 5 Divide the serum distance by the total distance and multiply by 100 to give a percentage.
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### INTERPRETATION

Normally, more than 40% serum is expressed. A decreased expression is present in some platelet defects, notably Glanzmann's thrombasthenia.

### NOTES

- The tubes and wooden stick must be absolutely clean to keep the clot from adhering to the tube.
- The clot must be removed carefully and gently to avoid squeezing and therefore more serum being expressed.

### REFERENCES

Chanarin I (ed.). *Laboratory Haematology: An Account of Laboratory Techniques*. Edinburgh: Churchill Livingstone, 1989.

Guidelines on platelet function testing. The British Society for Haematology BCSH Haemostasis and Thrombosis Task Force. *J Clin Pathol* 1988; 41: 1322–1330.

## 39.2 Measurement of Platelet Aggregation

### PRINCIPLE

The optical density of platelet-rich plasma falls as platelets form aggregates. The amount, and to some extent the rate, of fall is largely dependent on platelet reactivity, provided that all other variables (e.g. platelet count, mixing speed, and temperature) are controlled.

The optical density changes are monitored, wherein the optical density of platelet-rich plasma reflects the degree of platelet aggregation induced by one of a variety of agonists.

The optical density is monitored using an aggregometer connected to a chart recorder so results may be recorded graphically.

A number of reviews of current practice in different centres (Jennings et al. 2008, Cattaneo et al. 2009) and guidelines (Bolton-Maggs et al. 2006) have been published.

### PRECAUTIONS PRIOR TO STUDYING PLATELET AGGREGATION

Unless their effect on platelet aggregation is being specifically investigated, Aspirin-containing compounds should be excluded for at least 10 days prior to testing, as Aspirin interferes with the release reaction.

Ingestion of other drugs known to influence platelet function should also be avoided for at least the time required for their elimination from the circulation. These include certain antihistamines, antibiotics, and anti-depressants. A check should be made of any drugs being prescribed before performing platelet function testing.

Because chylomicrons can interfere with the measurement of platelet aggregation, studies should not be carried out shortly after a fatty meal.

Many other “normal” dietary constituents, including alcohol, onions, garlic, peppers, and ginger, may also inhibit platelet aggregation. Bear this in mind when evaluating results.

### PREPARATION OF PLATELET-RICH AND PLATELET-POOR PLASMA

Venous blood is collected with minimal venous occlusion into a 1/10th volume of tri-sodium citrate (0.109M) in a polypropylene or siliconized glass tube. Approximately 20 ml of blood is needed for a full aggregation study.

Chilling activates platelets, and so the blood is processed at 20°C–25°C. Platelet-rich plasma (PRP) is prepared by centrifugation for 10 to 15 minutes at 200 g.

The PRP is carefully removed, avoiding contamination with red cells or the buffy coat (aggregation will be diminished if either are present), into a polypropylene tube and held at 20°C–25°C until tested. The precise temperature of storage is not critical, but it can influence the results, and so the same temperature should be adhered to for all PRP samples.

The remaining blood is centrifuged at 2000 g for 20 minutes, and the platelet-poor plasma (PPP) is removed and stored at 4°C.

A platelet count is performed on the PRP: the number of platelets will influence the aggregation responses obtained, but within the range in which most PRP counts fall ( $200\text{--}600 \times 10^9/\text{l}$ ), such effects are minimal. Indeed, there is evidence that diluting the PRP with PPP from the same patient can inhibit the responses obtained, probably because the PPP may contain substances released from platelets as a result of the additional trauma caused by the higher speed centrifugation (Cattaneo et al. 2007, Linnemann et al. 2008). For these reasons, PRP with a platelet count in the above range should not normally be adjusted.

For exceedingly high PRP counts ( $>1000 \times 10^9/\text{l}$ ), the aggregation response may be affected. Thus it may be advantageous to adjust the platelet count to a more suitable level. This may be done by diluting the PRP in the patient's PPP. Similarly, low platelet counts ( $<200 \times 10^9/\text{l}$ ) may show diminished aggregation response. Concentrating platelets virtually always induces a functional change and is not recommended. However, a normal control diluted (in PPP) to the same PRP count may be run for comparison.

Platelet aggregation is pH-dependent, and so the PRP should be maintained within the range 7.7–8.0. Satisfactory control can be achieved by storing the PRP in full, tightly stoppered tubes and by completing the tests within two hours of blood collection.

## **AGGREGATING AGENTS**

Reagents such as ADP and collagen bind to specific receptors in the platelet membrane, activating the platelet and triggering a series of reactions. This leads to the platelet undergoing shape change, contraction, mobilization, and release of granular constituents, and finally to aggregation of platelets.

Several different but interlinked pathways of platelet activation occur, depending on the type and concentration of agonist employed, leading to platelet aggregation. In the reaction tube, the change from a uniform platelet suspension to aggregates leads to a reduction in light absorbance and an increase in light transmitted through the platelet suspensions. This is detected by a recorder in combination with a platelet aggregometer.

The five aggregating agents listed below should be sufficient to allow the various functional platelet disorders to be identified.

### *Adenosine-5- diphosphate (ADP)*

A stock solution of 1mM/l of the disodium salt is prepared in Owren's buffered saline (OBS) and stored in small amounts at -40°C. This is stable for at least three months. Once thawed, the solution should be used within three hours or discarded.

For use, further dilutions are prepared in OBS. The pattern of response to ADP depends on its final concentration.

- At 2  $\mu\text{mol/l}$ , clearly defined primary and secondary waves can be seen: the first represents the direct agonist-induced effect and the latter is due to release of endogenous ADP and generation of thromboxone  $\text{A}_2$  ( $\text{TXA}_2$ ), which itself aggregates platelets.
- Below 2  $\mu\text{mol/l}$ , progressively fewer normal subjects show a secondary response, and the primary wave usually reverses as the ADP is enzymatically degraded.
- Above 3  $\mu\text{mol/l}$ , the primary phase is usually so intense that the distinction between it and the secondary phase is masked.

ADP induces a change in shape of the platelets from a disc to a spiky sphere. This initially causes a slight increase in the optical density of the platelet suspension, which can be seen only if primary aggregation is impaired.

### *Adrenaline (epinephrine)*

A stock solution of 1mM/l of the bitartrate salt is prepared in OBS. It should be stored and used as for ADP. With adrenaline, the concentrations used and the patterns of response are similar to those of ADP. However, in the absence of a secondary wave, the primary wave does not reverse, nor is it ever so intense that the secondary wave is masked.

### *Collagen*

A very stable suspension of equine tendon collagen fibrils (1 mg/ml), available from Hormon-Chemie, Munich, Germany, is widely used. A number of other materials are equally suitable. It is stored at 4°C and must be well mixed immediately prior to dilution in the buffer packaged with it. It should be used at final concentration of 0.5–2.0  $\mu\text{g/ml}$  in PRP, and diluted suspensions are stable for one week at 4°C.

With collagen, no primary wave occurs. The response is usually defined by the duration of the lag phase prior to the onset of aggregation and by the intensity of the latter. A slight increase in the optical density caused by the shape change precedes aggregation.

Collagen from a number of different sources is in use. Both the type of collagen and the species from which the preparation is prepared (e.g. equine or bovine) can have an important effect on the results obtained. Indeed, more than a

hundred-fold range of concentrations are required, depending on the source material. It is therefore important to select a suitable source and to establish a local reference range for this material, which should be re-evaluated if the source is changed. For a review, see Jennings et al. (2008).

### **Ristocetin**

At a final ristocetin concentration of 1 mg/ml in PRP, distinct primary and secondary waves are usually discernable, but above this the direct effect is so intense that the two phases merge.

The primary wave is a measure of the amount of von Willebrand factor present in the plasma, whereas the second wave is due to release of endogenous substances.

### **Arachidonic acid**

Sodium arachidonate (99% purity) is dissolved in OBS to a concentration of 10mM/l. Small aliquots are placed in darkened glass vials that are flushed with nitrogen to prevent oxidation, then tightly capped and stored frozen below -20°C.

Aggregation is generally monophasic and preceded by a short lag phase.

## **REAGENTS**

*Note: These concentrations are appropriate if one part is added to nine parts of PRP.*

- ADP  
Make a 1 in 10 dilution = 100  $\mu$ M (i.e. 0.1 ml 1000  $\mu$ M solution + 0.9 ml OBS)  
From this, make two working strengths:
  - 20  $\mu$ M (i.e. 0.2 ml 100  $\mu$ M + 0.8 ml OBS)
  - 30  $\mu$ M (i.e. 0.3 ml 100  $\mu$ M + 0.7 ml OBS)In cases where hyperaggregability is being tested, lower concentration may be needed (e.g. 10  $\mu$ M, 5  $\mu$ M).
- Adrenaline (epinephrine)  
Dilute as for ADP
- Collagen  
Mix well and dilute in OBS:
  - 1 in 500 (i.e. 0.1 ml stock + 4.9 ml OBS) = 20  $\mu$ g/ml
  - 1 in 100 (i.e. 0.5 ml 1.50 dilution + 0.5 ml OBS) = 10  $\mu$ g/ml
- Ristocetin  
This is used at up to four concentrations depending on the results obtained: 15 mg/ml, 12.5 mg/ml, 7.5 mg/ml, and 5 mg/ml
- Arachidonic acid  
10mM/l

## METHOD

Because of platelet refractoriness to aggregation caused by centrifugation, aggregation studies must not be started within 30 minutes of preparing the PRP. In particular, the response to ADP is reduced during the first 20 to 30 minutes after preparation of PRP. However, testing should be completed within two hours.

- 1 Switch on aggregometer to warm to 37°C, and set stirring speed to 900 rpm. Some aggregometers have two channels, so it is convenient to use half of the width of the chart paper for each channel. With a 10 mV deflection, the PRP settings used are 0.5 and 5.5 mV for channels 1 and 2 respectively (represents 0% aggregation), and the corresponding blank values (which are set using PPP) would be 4.5 and 9.5 mV (represents 100% aggregation).
- 2 Place 0.45 ml PRP in a glass cuvette (dispose of after use) containing a siliconized "file" for mixing.
- 3 Place in holder for channel 1 and set transmission to 0% (0.5 mV). Replace with a cuvette containing 0.5 ml PPP, and set transmission to 100% (4.5 mV). Repeat this procedure until no further adjustment is required.
- 4 Repeat for channel 2, using the 5.5 and 9.5 mV settings.
- 5 Allow PRP to warm to 37°C for two minutes. Add 0.05 ml of agonist to bottom of cuvette and monitor optical density change for three minutes.

Repeat this procedure for each agonist.

## NOTES

- For ADP and adrenaline, start with the 20 µM concentration. This will be a final concentration in PRP of 2 µM.
- For collagen, use the 10 g/ml concentration. This will be a final concentration of 1 µg/ml.
- For ristocetin, use the 12.5 mg/ml concentration. When screening for type 2B VWD, also test the 7.5 mg/ml and 5 mg/ml concentrations. These will give a final concentration in PRP of 1.25 mg/ml, 0.75 mg/ml, and 0.5 mg/ml respectively. Use 15 mg/ml if there is no response to 12.5 mg/ml.

If the platelets hyperaggregate with 0.5 mg/ml ristocetin (indicating possible 2B VWD or platelet type VWD):

- Check for spontaneous aggregation by monitoring PRP under the same stirring conditions on the aggregometer without adding any agonist to stimulate aggregation.

- Wash a measured volume of PRP (e.g. 1 ml of both patient's and normal plasma × 3) in EDTA citrate saline washing buffer. Carefully resuspend patient's platelets in normal plasma and the normal platelets in patient's plasma. Retest with 0.5 mg/ml ristocetin.

Reactions should fit into one of the patterns below.

**Figure 39.1. Reaction patterns**

Patient PRP Patient PPP	Patient Platelets Normal PPP	Normal platelets Patient PPP
Aggregation	No response	Aggregation = Type 2B VWD
Aggregation	Spontaneous aggregation	No response = Platelet-type VWD*

\*For a review of platelet-type VWD, see Franchini et al. (2008).

### INTERPRETATION OF AGGREGATION PATTERNS

- If the platelets being tested do not give a normal response – i.e. two phases of aggregations covering over 50% of the scale, with the above concentrations covering over 50% of the scale with the above concentrations of reagents – then the concentration of the agonist is increased (within reason) until a satisfactory response is obtained.
- In cases where there is an obvious release defect, monitor the response to arachidonic acid.
- If no response is obtained with arachidonic acid, test with thromboxane A2 and/or calcium ionophore.
- When studying platelet aggregation as part of a hyperaggregability assessment, ADP and adrenaline are used at lower concentrations to obtain a dose response curve. Concentrations used are: 2 μM, 1 μM, 0.5 μM, 0.1 μM final concentration in PRP. A spontaneous aggregation is also performed before the rest of the agonists are tested:
  1. Place 0.5 ml PRP in cuvette and place in aggregometer.
  2. Monitor any change in optical density for 15 minutes.

If PRP volume is insufficient, the minimum volume that may be used in the standard cuvettes is 0.36 ml PRP + 40 μl agonist.

- PRP prepared in the same way from a healthy normal donor should be processed as a check on the reagents. This is especially important if abnormal patient results are obtained, as some agonists are labile, particularly once diluted to working concentrations. Results from healthy normal subjects tested in this way can be used to derive reference ranges to aid interpretation of patient results.

## CALCULATION OF RESULTS

Results are usually expressed in three ways:

1. Percentage fall in optical density measured three minutes after addition of agonist. This is the most convenient way, although it does not provide information on the shape of the aggregation curves.
2. Initial slope of the aggregation tracing. This indicates the rate of aggregation, but does not indicate whether secondary aggregation has occurred.
3. Determination of threshold agonist concentration, i.e. the amount of aggregating reagents required to just induce a second phase response. (This tends to be wasteful of PRP.)

## INTERPRETATION OF RESULTS

Great caution is required when interpreting platelet aggregation patterns. A number of technical factors may influence the results. Bear in mind that there are a number of important differences between aggregation determined by nephelometry and that occurring in the body.

Nevertheless, useful diagnostic information can be obtained, and some examples of aggregation patterns are shown in Figure 39.2.

**Figure 39.2. Platelet aggregation results in various disorders**

Disorder	ADP	Collagen	Ristocetin 1.25 mg/ ml	Ristocetin 0.5 mg/ ml	Arachadonic Acid	Adrenaline
<b>VWD type 1 and 2A</b>	N	N	A/R**	A	N	N
<b>VWD type 2B</b>	N	N	N	H	N	N
<b>Bernard-Soulier syndrome</b>	N	N	A	A	N	N
<b>Glanzmann's thrombasthenia</b>	A	A	N	A	A	A
<b>Storage pool disease</b>	P/N	R/N	P/N	A	R/N	P/N
<b>Cyclo-oxygenase defect*</b>	R/N	R	N	A	R	R/N

N = Normal; A = Absent; R = Reduced; H = Heightened response; P = Primary wave only  
\*Or Aspirin effect \*\*Can be normal in mild VWD type 1

## FURTHER INVESTIGATION OF PLATELET FUNCTION

If an abnormal aggregation pattern is observed in an individual, it is advisable to repeat the assessment on at least one further occasion to check for consistency of the abnormality.

In the presence of abnormal aggregation, further investigation may be useful. These include the measurement of platelet nucleotide content and their release during platelet aggregation.

Quantitation of membrane glycoproteins can be performed for the unequivocal diagnosis of Bernard-Soulier syndrome and Glanzmann's thrombasthenia.

The platelet release mechanism can be assessed by the measurement of total platelet content of ADP and adenosine triphosphate (ATP) and the release of ATP or 5-hydroxytryptamine (or both) from dense granules.

### Figure 39.3. Technical factors that influence platelet function

Anticoagulant	1/10th volume of trisodium citrate.
Time	Start tests 30 minutes after preparation of PRP. Complete studies within two hours of blood collection.
Centrifugation	Should be sufficient to remove red cells and white cells, but not large platelets. Should be done at room temperature, not at 4°C.
Platelet count	Low counts $< 100 \times 10^9/l$ cause slow, weak responses. High counts $> 1000 \times 10^9/l$ may show reduced response.
pH	$< \text{pH } 7.7$ inhibits aggregation. $> \text{pH } 8.0$ enhances aggregation.
Mixing speed	$< 800$ rpm shows reduced aggregation. $> 1200$ rpm breaks up platelet clumps.
Hematocrit	$> 55\%$ shows progressively less aggregation, especially second-phase inhibition due to increased citrate concentration.
Temperature	$< 35^\circ\text{C}$ shows decreased aggregation with regular doses of all agonists, but increased response to low doses of ADP.
Lipemia	Increased chylomicrons cause reduced aggregation.
Dirty cuvette	May cause apparent spontaneous aggregation.
No stir bar	No response on addition of aggregating agent.
Air bubbles	Rapid, large oscillations of pen prior to aggregation. Also caused by low platelet count.

## DIFFERENCES BETWEEN *IN VIVO* AND *IN VITRO* CONDITIONS FOR PLATELET AGGREGATION

With *in vitro* blood tests:

- Blood is anticoagulated
- RBC and WBC are removed
- Vascular components are not involved
- Coagulation is not involved
- Platelet population is selected
- Products of platelet activation and release are retained
- Reagents used are unphysiological in composition and dose
- Platelets are unstable out of the body
- Drugs may show more or less marked effects than *in vivo*

## REFERENCES

- Bolton-Maggs PH, Chalmers EA, Collins PW, Harrison P, Kitchen S, Liesner RJ, Minford A, Mumford AD, Parapia LA, Perry DJ, Watson SP, Wilde JT, Williams MD; UKHCDO. A review of inherited platelet disorders with guidelines for their management on behalf of the UK HCDO. *Br J Haematol* 2006; 135:603–33.
- Cattaneo M, Hayward CP, Moffat KA, Pugliano MT, Liu Y, Michelson AD. Results of a worldwide survey on the assessment of platelet function by light transmission aggregometry: A report from the Platelet Physiology Subcommittee of the Scientific and Standardisation Committee of the ISTH. *Thromb Haemost* 2009; 7:1029.
- Cattaneo M, Lecchi A, Zighetti ML, Lussana F. Platelet aggregation studies: Autologous platelet-poor plasma inhibits platelet aggregation when added to platelet-rich plasma to normalize platelet count. *Haematologica* 2007; 92:694–7.
- Franchini M, Montagnana M, Lippi G. Clinical, laboratory and therapeutic aspects of platelet-type von Willebrand disease. *Int J Lab Hematol* 2008; 30:91–4.
- Jennings I, Woods TA, Kitchen S, Walker ID. Platelet function testing: practice among UK National External Quality Assessment Scheme for Blood Coagulation participants, 2006. *J Clin Pathol* 2008; 61:950–4.
- Linnemann B, Schwonberg J, Mani H, Prochnow S, Lindhoff-Last E. Standardization of light transmittance aggregometry for monitoring antiplatelet therapy: An adjustment for platelet count is not necessary. *Thromb Haemost* 2008; 6:677–83.