

Preparation of Washed Platelet Suspensions From Human and Rodent Blood

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1. Introduction

Citrate is the preferred anticoagulant for blood collection, as EDTA damages platelets and heparin modifies their function (1). Citrate allows the rapid generation of platelet-rich plasma (PRP), with a high yield of platelets; however, this method has certain disadvantages. In particular, the PRP preparation has a limited stability (no longer than 2 h) and contains plasma proteins, including enzymes. In addition, human platelet-rich plasma (PRP) prepared from blood collected into trisodium citrate (3.8% w/v) has a depressed ionic calcium concentration, which can cause platelet aggregation and release of substances during centrifugation (2). To overcome these different problems, a centrifugation technique has been developed for the isolation and washing of platelets from human or rodent blood anticoagulated with acid-citrate-dextrose (ACD). The cells are resuspended in a physiological buffer under well-defined conditions, notably the presence of plasmatic ionic calcium concentrations (2 mM) and the absence of coagulation factors or other plasma components.

The method for isolation of human platelets by centrifugation and washing described by Cazenave et al. (3) is derived directly from the technique of Mustard et al. (4). Blood collected into ACD is used to prepare PRP, from which the platelets are isolated by successive centrifugation steps and resuspended in Tyrode's buffer, an iso-osmotic phosphate buffer at pH 7.35 containing glucose (0.1%, w/v), human serum albumin (HSA) (0.35%, w/v), calcium (2 mM), and magnesium (1 mM). Prostacyclin (PGI₂) is used to prevent transitory platelet activation during the preparation. Addition of apyrase (adenosine 5'-triphosphate diphosphohydrolase, EC 3.6.1.5) to the final suspending medium prevents the cells from becoming refractory to ADP and maintains their discoid shape (5). Suspensions of washed platelets prepared by this method are stable for 5–8 h at 37°C, compared with citrated PRP preparations, which are stable for no more than 2 h.

From: *Methods in Molecular Biology*, vol. 272:
Platelets and Megakaryocytes, Vol. 1: *Functional Assays*
Edited by: J. M. Gibbins and M. P. Mahaut-Smith © Humana Press Inc., Totowa, NJ

Suspending platelets in an artificial medium presents two main advantages: (1) plasma enzymes (e.g., thrombin generated during the collection and preparation process) are excluded and (2) it is possible to manipulate the inorganic ions, proteins, and other constituents in the suspension. When such a medium is employed, the pH should lie in the physiological range (7.2–7.4) to avoid affecting platelet functions (6). The solution should be iso-osmotic and contain glucose as a source of metabolic energy and physiological concentrations of divalent cations (7). In addition, the platelets can be labeled with radioisotopes (^{51}Cr , ^{111}In -oxine, ^{14}C - or ^3H -serotonin) and used either in vitro to study platelet secretion or accumulation on artificial and natural surfaces, or in vivo to follow platelet survival and detect sites of sequestration or thrombus formation in humans and animals.

2. Materials

2.1. Equipment

A Sorvall RC3BP centrifuge (Kendro Laboratory Products, Newton, CT) is used with an H6000A-HBB6 rotor and 15- or 50-mL conical-bottom polypropylene centrifuge tubes. Wide-bore plastic Pasteur pipets (Pastettes, Biolyon, Dardilly, France) are employed to transfer anticoagulated blood, PRP, and washed platelet suspensions, which must be kept in plastic tubes (polypropylene, ref. 430291, Corning Inc., Corning, NY) or siliconized glass containers to prevent platelet activation and blood coagulation. Human blood is collected with a 16-gauge needle mounted on a short length (10–20 cm) of plastic tubing (ref. HC-15R, Nissho Nipro Europe N.V., Zaventem, Belgium). Rat and mouse blood are collected with 18-gauge and 25-gauge needles, respectively. Platelets are counted in an automatic hematology analyzer for human blood (Sysmex K-1000, Merck, Darmstadt, Germany) or rodent blood (Coulter, Miami, FL).

2.2. Reagents for Blood Collection and Washed Platelet Preparation

All chemicals from commercial sources (Prolabo, Paris, France; Merck, Darmstadt, Germany; Sigma-Aldrich-Fluka, Saint-Quentin Fallavier, France) should be of analytical grade.

1. Acid-citrate-dextrose (ACD) anticoagulant solution (*see Note 1*): Prepare this anticoagulant by dissolving 25 g of trisodium citrate dihydrate, 14 g of citric acid monohydrate, and 20 g of anhydrous D(+)-glucose in a final volume of 1 L of distilled water. The final concentrations are, respectively, 85 mM, 66.6 mM, and 111 mM; the solution has an osmolarity of 450 mOsm/L and a pH of about 4.5. One volume of anticoagulant is required for six volumes of blood.
2. Stock solutions for Tyrode's buffer (*see Note 2*):
 - Stock I: 160 g 2.73 M NaCl, 4 g (53.6 mM) KCl, 20 g (238 mM) NaHCO_3 , and (1.16 g) 8.6 mM NaH_2PO_4 , H_2O made up to 1 L in distilled water and stored at 4°C.
 - Stock II: 20.33 g (0.1 M) $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ made up to 1 L.
 - Stock III: 21.9 g (0.1 M) $\text{CaCl}_2 \cdot 6\text{H}_2\text{O}$ made up to 1 L.
 - HEPES stock: 0.5 M (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]) sodium salt (119 g) made up to 1 L (*see Note 3*).

Human serum albumin (HSA) stock: 200 g/L pasteurized human serum albumin for intravenous injection (Etablissement Français du Sang-Alsace, Strasbourg, France) (purity > 98%).

3. Tyrode's albumin buffer (0.35% albumin) (see **Note 4**): 5 mL stock I, 1 mL stock II, 2 mL stock III, 1 mL HEPES stock, 1.75 mL HSA stock, and 0.1 g anhydrous D(+)glucose in a final volume of 100 mL of distilled water. Adjust the pH to 7.35 with HCl and the osmolarity to 295 mOsm/L with 30% NaCl or distilled water.
4. Tyrode's buffer: 50 mL of stock I and 950 mL of distilled water, pH 7.35 with HCl.
5. Washing salines for platelet preparation
First wash: Tyrode's albumin buffer containing 10 U/mL heparin (Roche, Neuilly-sur-Seine, France).
Second wash: Tyrode's albumin buffer.
Final suspension: Tyrode's albumin buffer containing 2 μ L/mL apyrase (preparation described below).

6. Apyrase preparation: Apyrase (adenosine 5'-triphosphate diphosphohydrolase, EC 3.6.1.5) is an ADP scavenger that is added to the final suspension at 0.02 U/mL, a concentration sufficient to prevent the desensitization of platelet ADP receptors during storage at 37°C. The enzyme can be purchased (e.g., type VII apyrase, Sigma). However, we use apyrase prepared by a modification of the method of Molnar and Lorand (8) in a two-stage procedure at 4°C:

Stage I: Peel, wash, and slice 10 kg of potatoes (preferably white potatoes, at least 6 mo old), homogenize in a Waring blender with 1 L of distilled water, and then mix for 30 min. Centrifuge the suspension at 900g for 10 min, filter the supernatant fluid through multiple layers of cheesecloth, and measure the volume of the effluent.

Stage II: Add 0.1 M CaCl_2 to the supernatant to bring the solution to a final CaCl_2 concentration of 0.025 M, stir for 15 min, allow to settle for 60 min, and centrifuge for 20 min at 3500g. Resuspend the precipitate in 1 M CaCl_2 (1/10 of the original volume of effluent), stir for 60 min, and centrifuge for 20 min at 3500g. Dialyze the supernatant using a regenerated cellulose membrane tubing, diameter depending on the volume to be used (molecular weight cut-off (MWCO): 10,000 Daltons, Spectra/Por, American Pacific, CA) for 24 h against 0.1 M KCl (20 L for 10 kg of potatoes) and then centrifuge for 20 min at 3500g. Add to the supernatant fluid 30.4 g of $(\text{NH}_4)_2\text{SO}_4$ for every 100 mL of fluid; after dissolving (10 min), stir for 40 min and centrifuge for 15 min at 3500g. Discard the precipitate and add again to the supernatant 30.4 g of $(\text{NH}_4)_2\text{SO}_4$ to 100 mL of fluid; after dissolving, stir for 40 min and centrifuge for 15 min at 3500g. Discard the supernatant and dissolve the precipitate in a minimum of distilled water (precipitate obtained from 10 kg of potatoes is dissolved in 150 mL of distilled water) and dialyze with same tubing (MWCO: 10,000) against several changes of 0.154 M (0.9% w/v) NaCl.

Determine the protein concentration with the bicinchoninic acid (BCA) protein assay system (Pierce, Rockford, IL) and dilute to 3 mg/mL with 0.154 M NaCl. Store the concentrate (brown solution) in small aliquots (2 mL) at -20°C. Before use, centrifuge the concentrate for 5 min at 12,000g, dilute the supernatant 5- to 10-fold in modified Tyrode's buffer, aliquot in 1-mL quantities and store at -20°C (see **Note 5**). This apyrase stock is for use at a final activity of 0.02 U/mL (or higher, at 0.9 U/mL, if P2X₁ receptors are being studied). The optimal concentration of diluted apyrase to prevent ADP receptor desensitization should be determined empirically, since the amount required will vary with different enzyme preparations (see **Note 5**). The nucleotidase activity of the apyrase preparation can be determined by measuring the rate at which it degrades ADP and ATP. Incubate 2 μ L of

apyrase (0.3 mg/mL) with 20 μ L of 1 mM ADP or ATP in 1 mL of Tyrode's albumin buffer for various times (0, 1, 3, 4, and 5 min) at 37°C. At each time point, stop the reaction by adding 100 μ L of ice-cold 6 *N* perchloric acid (HClO₄). Centrifuge the samples at 12,000g for 5 min at 4°C and extract the non-degraded nucleotides from the supernatant in 2 mL of freon/trioctylamine (1/1), mix the samples for 10 min and centrifuge as before. The nucleotides (AMP, ADP, ATP) are separated by HPLC on an anionic exchange column (P10SAX-25QS, Interchim, Montluçon, France). The specific ADPase or ATPase activity is about 10–100 U/mg of apyrase, depending on the preparation (*see Note 5*).

7. Heparin, 5000 U/mL: Standard heparin (Sanofi-Synthélabo, Toulouse, France) is a solution ready to use at 5000 U/mL and can be stored at 4°C for several weeks. Heparin (2 μ L of the stock solution) is added in the first step of the platelet-washing procedure at a final concentration of 10 U/mL in the platelet suspension (*see Note 6*).
8. Prostacyclin (PGI₂), 1 mM: Prostacyclin (sodium salt, ref P-6188, Sigma) is prepared as a 1 mM stock solution in ice-cold buffer (50 mM Tris-HCl, pH 8.8) and stored in 100- μ L aliquots at –20°C. PGI₂ is used at each step of the platelet washing procedure (*see Subheading 3.*) at a final concentration of 0.5 μ M (0.5 μ L of the stock solution for 1 mL of platelet suspension) (*see Note 7*).

3. Methods

3.1. Preparation of Washed Human Platelets

3.1.1. Blood Collection (*see Note 8*)

Collect blood from a forearm vein, using a wide-bore (16-gauge) needle mounted on a short length (10–20 cm) of plastic tubing, directly into a conical 50-mL centrifuge tube containing 1 volume of ACD anticoagulant for 6 volumes of blood (final pH 6.5 and citrate concentration 22 mM). After discarding the first few milliliters, which are contaminated with tissue factor (TF) and containing trace amounts of thrombin, allow the blood to flow down the tube wall in order to minimize air contact or bubble formation. Immediately after collection, close the tubes, mix the blood gently with the anticoagulant, and place the tubes in a water bath at 37°C for a maximum storage period of 15 min.

3.1.2. Washed Platelet Preparation

1. Prewarm the Sorvall centrifuge to 37°C and centrifuge the anticoagulated blood at 250g for a period of time depending on the quantity of blood (*see Table 1*) to obtain platelet-rich plasma (PRP) (*see Fig. 1*).
2. Carefully collect the PRP with a 10-mL syringe and transfer to a new conical 50-mL centrifuge tube.
3. After 10 min incubation at 37°C, centrifuge the PRP at 2200g for a period of time depending on the quantity of plasma (*see Table 1*).
4. Discard the supernatant consisting of platelet-poor plasma (PPP) using a Pasteur pipet connected to a vacuum pump, taking care to remove all traces of plasma from the tube walls or near the platelet pellet to avoid generation of thrombin during the subsequent washing steps (*see Note 9*).

Table 1
Centrifugation Time and Relative Centrifugal Force (RCF) Used for Platelet Isolation and the Different Washing Steps

	Volume of fluid (mL)		RCF (g)	RPM	Time (min)	Brake
	15-mL tube	50-mL tube				
Human blood	15	50	250	926	16	no
	10	40	250	926	15	no
	6	25	250	926	13	no
Human PRP	10	40	2200	2749	16	yes
	9	35	2200	2749	15	yes
	8	30	2200	2749	14	yes
	6.5	25	2200	2749	13	yes
	5	20	2200	2749	12	yes
	4.5	15	2200	2749	10	yes
Washing steps	10	40	1900	2254	8	yes
	8	30	1900	2254	8	yes

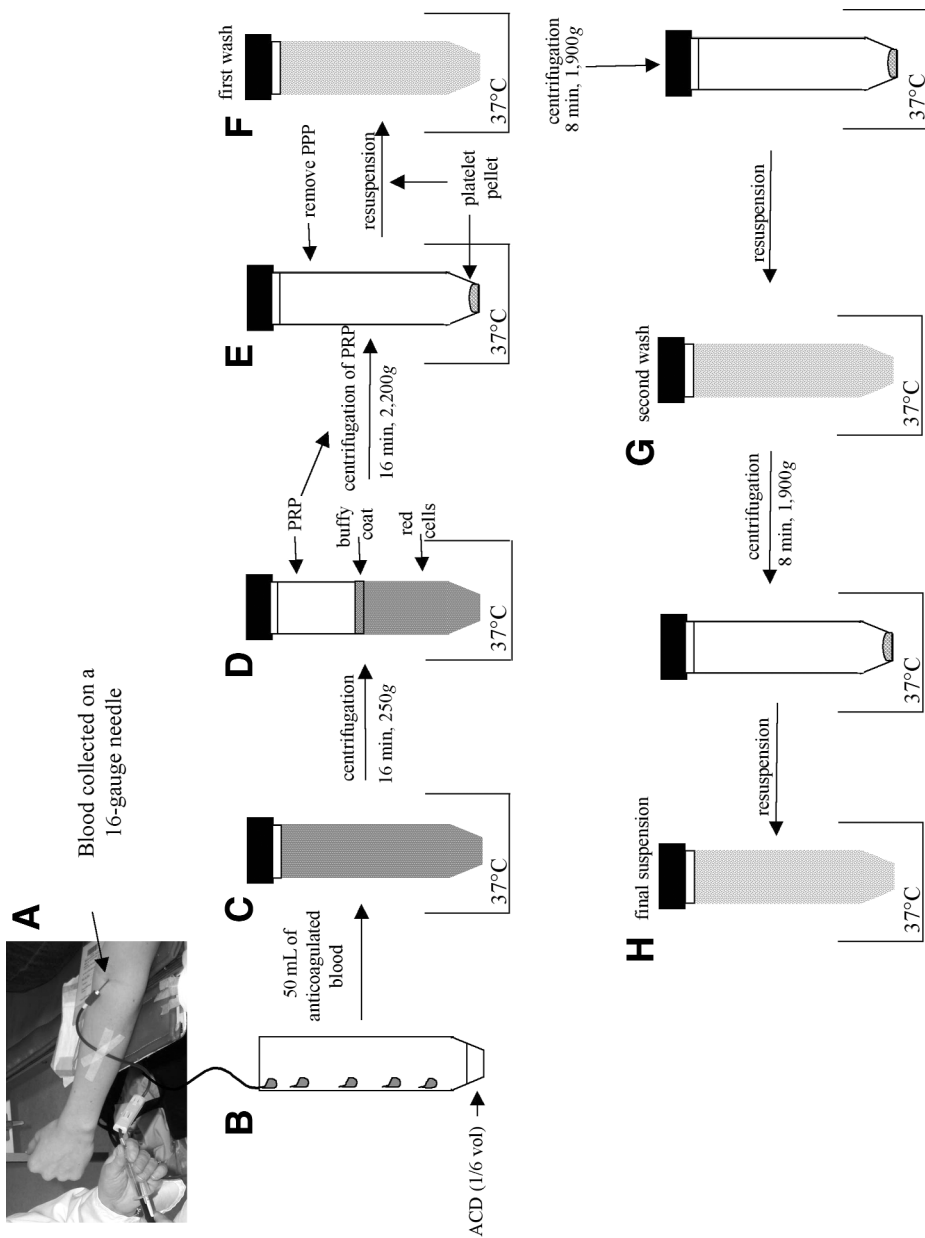
Centrifugation times depend on the volume of fluid; the time and relative centrifugal force (g) are given here for the Sorvall RC3BP centrifuge with an H600A-HBB6 rotor.

5. Gently resuspend the platelet pellet in Tyrode's albumin solution containing 10 U/mL heparin and 0.5 μ M PGI₂ (see **Note 10**). A wash volume of 10 mL is normally required for the platelet pellet from 50–100 mL of blood.
6. After 10 min incubation at 37°C, add 0.5 μ M PGI₂ to the first wash and centrifuge at 1900g for 8 min (for 30–40 mL of platelet suspension).
7. Resuspend the platelet pellet in Tyrode's albumin solution containing PGI₂ (0.5 μ M).
8. Remove 120 μ L of platelet suspension with a pipet and count the platelets in an automatic analyzer. After 10 min incubation at 37°C, add 0.5 μ M PGI₂ to the second wash and centrifuge at 1900g for 8 min (for 30–40 mL of suspension).
9. Resuspend the platelet pellet in Tyrode's albumin buffer containing 0.02 U/mL apyrase and adjust the cell count in the final suspension to 300,000/ μ L with the same buffer (see **Note 11**).

3.2. Preparation of Washed Rodent Platelets

3.2.1. Blood Collection

Local and national regulations for animal care, anesthesia, and removal of blood should be followed at all times. In our laboratory, rats weighing 250–300 g are anaesthetised by injection of a mixture (1/4: v/v) of 2% xylazine and 10% ketamine (1 mL/kg of body weight). Mice 8 wk old and weighing about 20 g are anesthetized by injection of a mixture of 0.2% xylazine base and 1% ketamine in physiological saline (50 μ L/10 g of body weight). Blood is then collected from the abdominal aorta using an 18-gauge (rat) or 25-gauge needle (mouse) mounted on a syringe containing 1 volume of ACD for 6 volumes of blood. The quantity of blood obtained from a rat weighing 200 g is about 10 mL, while the volume drawn from a mouse weighing 20 g is about 1 mL.



3.2.2. Washed Platelet Preparation

1. Rodent blood is centrifuged at a higher force (2300g) than human blood to obtain PRP. Centrifugation time depends on the volume (10 s per mL).
2. Collect the PRP and centrifuge at 2200g for a period of time depending on the volume of plasma: in a 15-mL centrifuge tube, 4 min for 1 mL of PRP, 5 min for 2 mL, 6 min for 3 mL, 8 min for 4 mL, and 10 min for 5 mL.
3. Discard the supernatant PPP using a Pasteur pipet connected to a vacuum pump, taking care to remove all traces of plasma from the tube walls or near the platelet pellet to avoid generation of thrombin during the subsequent washing steps.
4. Resuspend the platelets in Tyrode's albumin buffer containing heparin (10 U/mL) and PGI₂ (0.5 μ M). After incubation for 10 min at 37°C, add PGI₂ (0.5 μ M) and centrifuge at 1900g for a period of time depending on the suspension volume: in a 15-mL centrifuge tube, 3 min for 2–3 mL, 4 min for 4–5 mL, 5 min for 6–7 mL, 6 min for 8–9 mL, and 7 min for 10 mL.
5. Resuspend the platelets in Tyrode's albumin buffer containing 0.5 μ M PGI₂. After incubation for 10 min at 37°C, add 0.5 μ M PGI₂ and centrifuge as in **step 4**.
6. The platelets are finally resuspended at approx 100,000 to 300,000/ μ L in Tyrode's albumin buffer containing 0.02 U/mL apyrase. A blood volume of 10 mL from one rat yields 10 mL of washed platelets at 300,000/ μ L. A mouse blood volume of 5 mL (pool from five mice with 1 mL/mouse) gives 30 mL of washed platelets at 100,000/ μ L. In this case, working below 5 mL of blood does not give a good yield.

3.3. Results and Discussion of Platelet Responses to Agonists

3.3.1. Human Platelets

Use of washed human platelets separated from their plasma environment and in the absence of anticoagulants is essential for the study of intrinsic platelet properties. The results reported by Mustard et al. (4) and Cazenave et al. (3) demonstrate that human

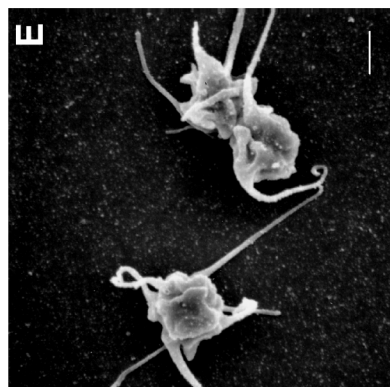
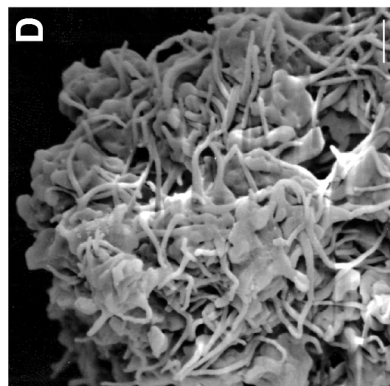
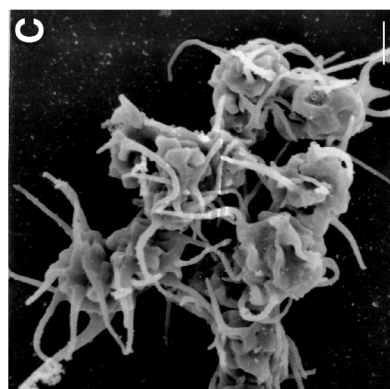
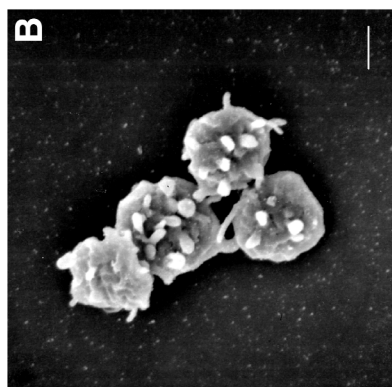
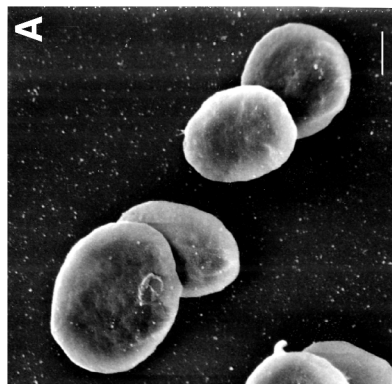
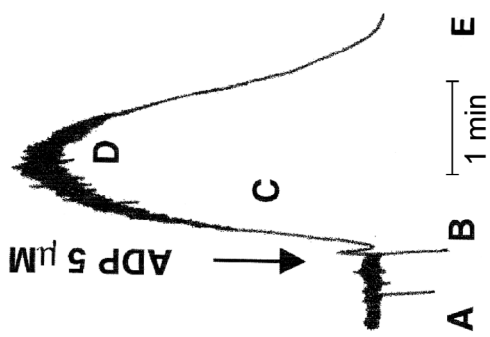
Fig. 1. (see opposite page) Preparation of washed human platelets. (A) Blood is collected from a forearm vein using a 16-gauge needle (B) into a conical 50-mL tube containing 1 volume of ACD for 6 volumes of blood. (C) Each 50 mL of anticoagulated blood is centrifuged at 37°C for 16 min at 250g (see **Table 1** for centrifugation details of other volumes). (D) The upper phase (PRP) is carefully collected while the lower phase (red cells) and the interface (buffy coat containing mainly leukocytes and a few platelets) are discarded. (E) The pooled PRP (30 mL obtained from 100 mL of anticoagulated blood) is centrifuged for 16 min at 2200g and the PPP is removed. (F) The platelet pellet is gently resuspended in the first washing solution (Tyrode's albumin buffer containing 10 U/mL heparin and 5 μ M PGI₂). After 10 min, a further 5 μ M PGI₂ is added and the platelets are centrifuged at 1900g for 8 min. (G) The platelet pellet is resuspended in the second washing solution (Tyrode's albumin buffer with 5 μ M PGI₂). (H) After a second centrifugation, the platelets are adjusted to 300,000/ μ L in the final suspending medium (Tyrode's albumin buffer containing 0.02 U/mL apyrase without PGI₂) and stored at 37°C.

platelets isolated by a centrifugation technique and resuspended in Tyrode's albumin buffer containing apyrase retain their physiological properties and their ability to respond to agents inducing aggregation and release equally as well as platelets in citrated PRP (5). The platelet suspensions are stable for 5–8 h at 37°C. Scanning electron microscopy (**Fig. 2A**) confirms the discoid shape of the cells and the absence of pseudopods, while transmission electron microscopy (**Fig. 3**) shows the presence of normal dense and α -granules (9).

In Tyrode's albumin containing calcium (2 mM) and magnesium (1 mM), the platelets aggregate in response to ADP (5 μ M), but only in the presence of added human fibrinogen (0.25 g/L) (*see Note 12*). The platelets then spontaneously disaggregate rather than show a second wave of aggregation, which is observed in ADP-stimulated citrated PRP and is due to granule secretion and thromboxane A₂ formation (**Fig. 2**, **Fig. 4A1**; percentage 5HT release from the dense granules is shown beside each figure) (7). It is well known that the lack of a secondary aggregation phase in washed platelets is due to the presence of millimolar concentrations of calcium. If magnesium is omitted from the Tyrode's albumin, only a slight modification is observed (a more rapid disaggregation) (**Fig. 4A2**). In contrast, if calcium is omitted from the external medium, a second wave of aggregation occurs (**Fig. 4A3**) (7). This second wave of aggregation requires thromboxane A₂ synthesis, as it is inhibited by aspirin (either in washed platelets or in citrated PRP; data not shown). When external calcium is further lowered by chelation with EDTA or EGTA, ADP fails to induce primary or secondary aggregation, although shape change is still observed (7). This effect is due to the absolute requirement of external calcium for fibrinogen binding to the IIb/IIIa receptor (10). Sufficient calcium is present in the experiments conducted in nominally Ca²⁺-free Tyrode's albumin for fibrinogen binding; however, the chelators lower Ca²⁺ to levels that do not support fibrinogen binding and thus aggregation.

In the absence of added external fibrinogen, the "weak" agonist ADP only induces shape change whereas thrombin does not require added fibrinogen to induce full aggregation and granule secretion (**Fig. 4B1,2**). After addition of collagen, arachidonic acid, or ionophore A23187, the platelets aggregate and secrete their granule contents, while in response to PAF they aggregate without secretion (not shown). Adrenaline per se does not induce shape change or aggregation of washed platelets in the presence or absence of fibrinogen (**Fig. 4B3**), but potentiates the effects of traces of any other aggregating agent (not shown) (11). Addition of ristocetin (0.05–0.1 g/mL) in the pres-

Fig. 2. (*see facing page*) Morphological changes of washed platelets during ADP-induced aggregation. An aggregation response was obtained by stimulating platelets with 5 μ M ADP (arrow). The platelets were fixed at different time points and their surface features were visualized by scanning electron microscopy (SEM). (A) Discoid cells in the resting state. (B) Formation of early pseudopods (7 s). (C) Full shape change and first platelet-platelet interactions (20 s). (D) Large platelet aggregates (45 s). (E) Isolated platelets after disaggregation (3 min). Bars = 1 μ m.



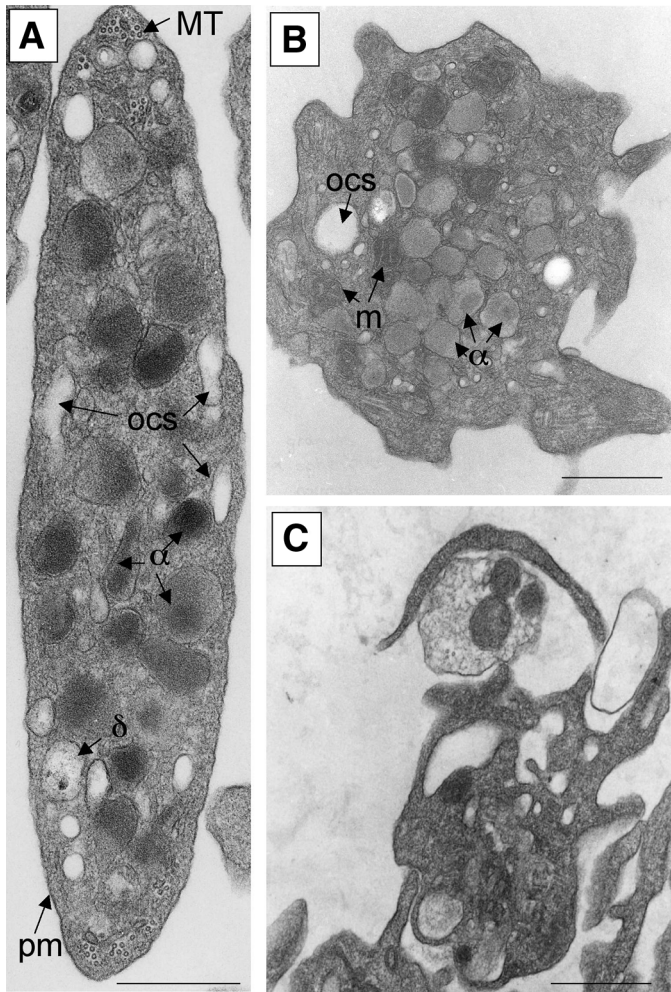


Fig. 3. Ultrastructure of washed human platelets visualized by transmission electron microscopy (TEM). (A) The characteristic discoid shape of a resting platelet in cross-section. (B) ADP-stimulated platelet showing a spherical shape and centralisation of granules. (C) Degenerated platelets after stimulation with thrombin. Abbreviations: Structures labeled by arrows are plasma membrane (pm), open canalicular system (OCS), microtubules (MT), alpha granules (α), dense granules (δ), mitochondrion (m). Bars = 500 nm.

ence of EDTA (10 mM) and platelet-poor plasma gives rise to a single wave of irreversible agglutination (not shown).

One important point is the requirement of apyrase, an ATP- and ADP-degrading enzyme, to the final platelet suspension in order to preserve platelet responsiveness to

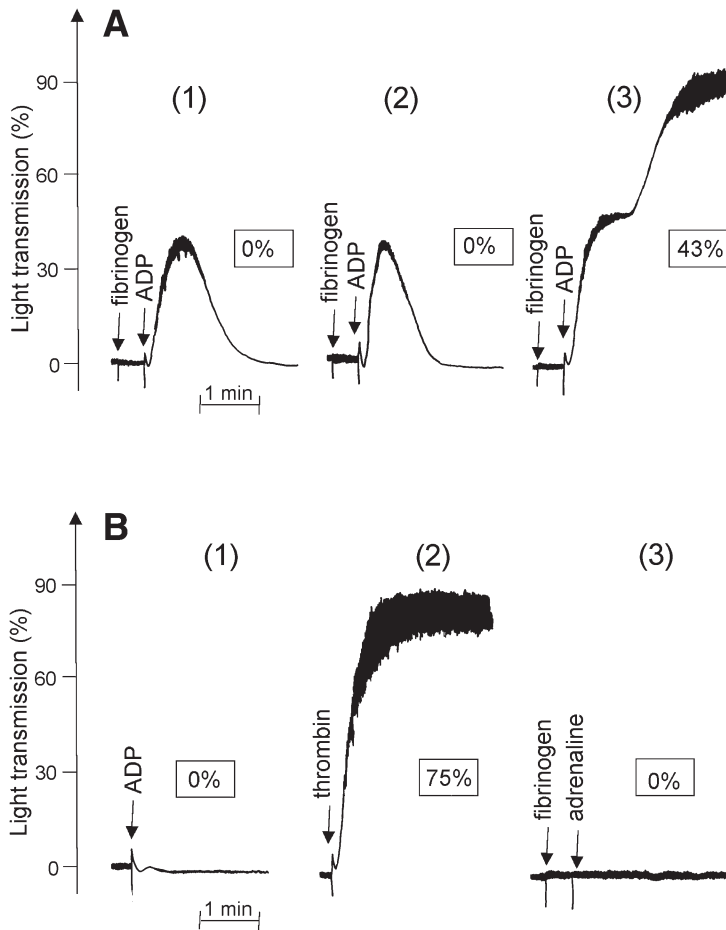


Fig. 4. Platelet responses to a weak agonist (ADP) and a strong agonist (thrombin). (A) Effect of calcium and/or magnesium on the aggregation induced by ADP ($5 \mu M$) in washed platelets that have also been labeled with tritiated serotonin to allow parallel studies of percentage serotonin release. The platelets were resuspended in modified Tyrode's albumin buffer containing apyrase and DiFP-treated fibrinogen (final concentration 0.25 mg/mL) was added before addition of the agonist (see **Note 12**). (1) Platelet suspension containing calcium (2 mM) and magnesium (1 mM); (2) suspension without magnesium; (3) suspension without added calcium. (B) Responses to various agonists in the absence of fibrinogen; (1) ADP ($5 \mu M$); (2) thrombin (1 U/mL), or (3) adrenaline ($100 \mu M$) in the presence of fibrinogen. The percentage release of radioactivity into the supernatant was measured 3 min after addition of the agonist and is indicated beside each aggregation curve.

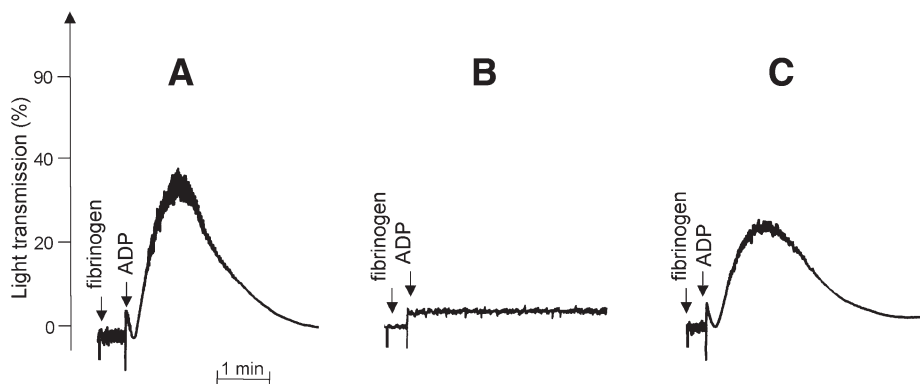


Fig. 5. Effect of apyrase on the platelet aggregation induced by ADP. Platelets were resuspended in Tyrode's albumin buffer and aggregation was induced by addition of ADP ($5 \mu\text{M}$) in the presence of fibrinogen (0.25 mg/mL). Platelets were resuspended in buffer (A) containing apyrase (0.02 U/mL), (B) without apyrase, or (C) initially without apyrase, to which 0.02 U/mL apyrase was added 30 min before ADP.

ADP (**Fig. 5A**). When apyrase is omitted, the platelets become refractory to ADP and are unable to change shape or aggregate (**Fig. 5B**). This is at least partly due to a leakage of nucleotides, which causes desensitization of one of the two G protein-coupled ADP receptors (P2Y_1). It is a transient phenomenon, as addition of apyrase to refractory platelets rapidly (15 min) restores a full ADP response (**Fig. 5C**) (12). Apyrase is also needed to study the P2X_1 receptor, a nonselective cation channel activated by ATP (13). To reduce P2X_1 receptor desensitization during isolation of platelets, apyrase is added at all steps of the washing procedure at the high concentration of 0.9 U/mL . In platelet suspensions prepared under these conditions, the specific P2X_1 agonist $\alpha, \beta\text{-MeATP}$ induces a transient rise in intracellular calcium and shape change (12) (see Chapter 15, vol. 2).

3.3.2. Rodent Platelets

In many respects, rodent platelets behave in a similar manner to human platelets. However, some differences can be observed in response to several agonists. For example, it is well known that PAF does not activate rat platelets because they lack the PAF receptor. Because rat platelets express both alpha and beta adrenergic receptors, adrenaline is inhibitory, while it amplifies human and mouse platelet responses. Other subtle differences make rodent platelets secrete low amounts of granule contents in response to ADP. Importantly, mouse and rat platelets do not aggregate in response to PAR1 -agonist peptides (14).

3.4. Limitations of the Method

Washed platelet suspensions have the distinct advantage that platelet function can be studied in the absence of an anticoagulant. Furthermore, provided that the necessary

precautions are taken to avoid thrombin generation and platelet activation during the preparation, the cells resuspended in the final medium containing apyrase retain their discoid shape and functional properties for storage periods of 5–8 h at 37°C. However, compared with gel filtration, the platelet yields from PRP are lower and more time is required to carry out the successive centrifugation and incubation steps. A further limitation of the method described in this chapter is the possibility that subpopulations of platelets may be selected during centrifugation, either when isolating PRP from whole blood or in subsequent steps of the washing procedure.

3.5. Related Preparative Methods

Platelets can also be isolated from human blood by density gradient centrifugation. In the albumin technique described by Walsh et al. (15), citrated PRP is layered onto a continuous albumin gradient at 300 mOsm in calcium-free Tyrode's buffer containing apyrase at pH 6.5. After centrifugation, the platelets are resuspended and the washing procedure is repeated in the absence of apyrase.

In order to avoid preparing PRP by centrifugation, and thus reduce the risk of selecting subgroups of the platelet population, Corash et al. (16) have developed a method whereby whole blood is centrifuged through an arabinogalactan (Stractan) gradient. The platelets are thus separated in a single step from the plasma and other cellular blood components and the Stractan is later removed on an iso-osmolar albumin gradient.

Gel filtration of PRP on Sepharose 2B is another alternative method (17,18). Using this technique, the platelets appear in the void volume, while the majority of plasma proteins are retained in the gel and eluted later. The separation is rapid and reproducible with minimal loss of platelets from the initial PRP, an advantage when only small blood volumes are available, but the platelet count in the resulting suspension is often low and the technique is not readily adapted to the preparation of sufficient quantities of platelets for experimental work. In addition, it does not enable the elimination of larger plasma components, e.g., immunoglobulins, factor V, and factor VIII-von Willebrand.

The principal advantages of the centrifugation technique for the isolation of platelets are that washed platelet suspensions may be prepared in large quantities with the required platelet count and in the absence of red blood cells and plasma proteins, including high-molecular-weight constituents such as the multimeric von Willebrand factor. Use of an artificial buffer medium as the suspending fluid further allows adjustment of the pH, osmolality, and divalent cation concentrations to physiological levels.

4. Conclusion

This chapter describes a method for the isolation of non-activated platelets from human or rodent blood. Platelets that are resuspended in a well-defined medium under physiological conditions (pH, temperature, osmolality, ions, and proteins) can respond for several hours to platelet agonists, or may be used in different protocols such as radiolabeling, electron microscopy, fluorescent labeling, immunolabeling, secretion experiments, or reinjection. To reproduce this technique over time, it should be kept in mind that all stages of the procedure are important and any modification can result in erroneous results.

5. Notes

1. ACD solution has the advantage that it not only chelates the calcium in blood and thereby prevents coagulation but also lowers the pH of the blood to 6.5. Platelets do not aggregate at this pH (19).
2. To facilitate the dissolution of the different salts, it is better to add the NaCl, KCl, and NaH_2PO_4 together into 600 mL and the NaHCO_3 separately into 200 mL, which must be warmed to 80°C to obtain dissolution. When all salts have dissolved, mix the NaHCO_3 solution with the NaCl, KCl, and NaH_2PO_4 solution and bring the volume to 1 L. The stock solution can be stored for several weeks at 4°C but it must be filtered through a 0.22- μm filter under vacuum.
3. HEPES is used as the pH buffer in the Tyrode's salines. pH buffers such as Tris[tris(hydroxymethyl)aminomethane] should be avoided because, like other amines, they inhibit some platelet responses and potentiate others.
4. Before adding the different stock solutions, 50 mL of distilled water should be poured into the beaker to prevent the precipitation of calcium phosphate or calcium carbonate when adding stock I and stock III. All washing solutions are kept at 37°C throughout the preparation.
5. Diluted apyrase should not be frozen a second time but can be stored for several weeks at 4°C. Some commercially available preparations are not suitable because they contain impurities (e.g., potato lectin, a platelet-agglutinating agent) and 5'-nucleotidase activity, which hydrolyses AMP to adenosine. In the final suspending medium, use a concentration of apyrase capable of converting 0.25 μM ATP to AMP in 120 s at 37°C. Alternatively, choose an apyrase concentration that maintains platelet sensitivity to ADP, but does not have an appreciable inhibitory effect on ADP-induced aggregation (tested in the presence of fibrinogen) (3). If too much apyrase is used, ADP-induced aggregation will be diminished; if too little is used, the platelets will become refractory to ADP (desensitization of platelet ADP receptors). The ratio of the ADPase to the ATPase activity is highly variable, depends on the origin of the potatoes, and can change during storage.
6. Some heparin preparations cause platelet aggregation and make platelets stick to the walls of their container. Batches of heparin should therefore be screened before use to ensure that this does not occur.
7. Transient platelet activation during the preparation may be inhibited by using PGI_2 in the centrifugation and resuspension steps (3). Since the half-life of PGI_2 is short (a few minutes), it must be added to the washing solution just before centrifugation or platelet resuspension. The PGI_2 solution should be stored at 4°C immediately after thawing and should not be frozen again.
8. Institutional guidelines for collection and disposal of human blood should be followed at all times. As many drugs affect platelet responses (particularly aspirin and other nonsteroidal anti-inflammatory drugs, and thienopyridines like ticlopidine or clopidogrel), donors should be asked carefully about the medication they have taken during the previous two weeks. The manipulation of blood samples is also subject to strict hygiene controls and all donors are screened for the absence of viral infections (HCV, HBV, HIV).
9. It is particularly important to avoid generating traces of thrombin during the preparation of washed platelet suspensions (3). Therefore, blood should be drawn with a minimum of vessel trauma and a rapid blood flow, while attention should be paid to eliminating all traces of plasma from the centrifuge tube before resuspending the platelet pellet after centrifugation of PRP. Platelets stimulated by exposure to low concentrations of thrombin may have reduced granule contents or be more sensitive to ADP- or adrenaline-induced aggregation (2,3).

10. In order to stabilize the pH of the platelet suspension at about 7.3, the operator should breathe air (containing elevated CO₂ levels compared to atmospheric air) into the tube before closing it at each step of the washing procedure (6).
11. The washed platelet preparation is kept at 37°C in a closed tube under a 5% CO₂/95% air atmosphere and under these conditions is stable for 5–8 h (6).
12. Human fibrinogen 4% (w/v, Grade L, Kabi, Stockholm, Sweden). The lyophilized powder is dissolved in 0.9% NaCl (4 g in 100 mL) and treated with diisopropylfluorophosphate (DiFP, Sigma-Aldrich-Fluka) (3) to inactivate traces of contaminant plasma serine proteases. Aliquots (1 mL at about 10 mg/mL) are stored at –20°C and thawed and warmed to 37°C before use. Note that DiFP is highly toxic and should only be used under appropriate conditions (gloves, mask, fume hood).

Acknowledgments

The authors wish to thank Monique Freund for animal care and J. N. Mulvihill for reviewing the English of the manuscript. This work was supported by INSERM, EFS-Alsace, and ARMESA.

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Platelets and Megakaryocytes

Volume 1: Functional Assays

Gibbins, J.M.; Mahaut-Smith, M.P. (Eds.)

2004, XIX, 385 p., Hardcover

ISBN: 978-1-58829-101-1

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