METHODS IN HEMATOLOGY

MEASUREMENTS OF PLATELET FUNCTION

Edited by

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CHURCHILL LIVINGSTONE

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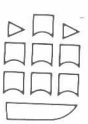

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Measurements of Platelet Function

METHODS IN HEMATOLOGY

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Preface

Platelets have essential roles in hemostasis and the vascular responses to injury, both acute and chronic. In particular, there is a growing body of evidence implicating platelets in the genesis of arteriosclerotic vascular disease and its thrombotic complications.

There have been great advances in recent years regarding our understanding of the structure, biochemistry, physiology and pharmacology of platelets. It is less clear what precise role platelets play in various pathologic processes and what functional measurements may be of diagnostic and prognostic importance. Equally inconclusive is the question of what therapeutic potential platelet-active drugs may have. Despite expanding investigative activity, advances have been limited by many reports of conflicting observations, presumably explained, at least in part, by differences in methodology. This collection of papers is intended to help solve this problem by providing recommended methodology for state-of-the-art testing of platelet function. Adoption of these techniques by those working in the field will permit the investigators to focus more clearly on the biology rather than the methodology.

In this volume seasoned, recognized authorities describe the rationale, detailed procedures, interpretation and trouble shooting regarding the various measurements of platelet function. These experts have been asked to select on the basis of their experience, the most specific, reliable and quantitative procedures. The book is intentionally not encyclopedic, and some may honestly disagree with various aspects of the approaches described. However, we believe that the authors have done their work well, and that this volume makes a unique and needed contribution.

We have not included a chapter on the use of monoclonal antibodies because their application to the study of platelets is relatively new. However, reports which have already appeared provide evidence of the impact their use will have on the study of platelet physiology, biochemistry and pathology. The advantages of these antibodies include the restriction of their specificity to one antigenic determinant or epitope and their availability in virtually unlimited quantities. McKever and his associates exploited these attributes in a study which clearly demonstrates the potential of this new tool.[1] These workers produced a monoclonal antibody to normal platelets which did not react with platelets from a patient with Glanzmann's thrombasthenia. They used this antibody in an immunoaffinity column to isolate the glycoprotein IIb–IIIa complex from Triton X-100 solubilized platelet membranes. The antibody was also used to quantitate the number of IIa–IIIb complexes on the normal platelet surface. From this information they were able to unequivocally demonstrate that Glanzmann's thrombasthenia platelets lacked the IIa–IIIb complex and to show a partial deficiency

in heterozygotes. In an extension of this approach, Montgomery and coworkers[2] have developed a simple test for diagnosing the Bernard–Soulier syndrome and Glanzmann's thrombasthenia. They utilized radiolabeled monoclonal antibodies to glycoprotein Ib and to the IIb–IIIa complex. Whole blood was incubated with the antibodies and the platelets pelleted by centrifugation through oil. Platelets from the Bernard–Soulier syndrome bound only the antibody to the IIb–IIIa complex whereas the reverse was true of platelets from Glanzmann's thrombasthenia. Heiler and associates[3] have shown that monoclonal antibodies to von Willebrand factor can profoundly alter platelet function *in vivo* as well as *in vitro*. Different antibodies had different effects stressing the functional relevance of specific von Willebrand factor epitopes. These few examples indicate that monoclonal antibodies should greatly expand our understanding of platelet structure-function relationships in the next decade.

We wish to express appreciation to the authors and editors for their cooperation in the preparation of this monograph.

La Jolla, California, 1983

L.A.H.
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J. G. White

INTRODUCTION

Morphological studies have played an important role in the development of basic knowledge concerning blood platelets.[7] The pathways travelled to this knowledge, however, have been filled with twists and turns and many other obstacles. Due to the marked sensitivity of platelets to chemicals, physical and environmental stimuli, it proved extremely difficult to separate them from other blood elements without causing irreparable damage or irreversible aggregation. As a result, *in vitro* studies were hampered for many years. Even when it proved possible to examine individual constituents of blood, the small size of the platelet made it virtually impossible to recognize as a separate cellular element. The problem was compounded by the absence of a nucleus and tendency of the platelet to spread into a thin film on contact with glass slides.

However, crude optical systems, despite limited resolution, eventually permitted identification of the platelet and a few of its anatomical features.[3] For example, the clear fluid appearance of cytoplasm spread on glass and tendency of organelles to cluster in cell centers led to the terms 'hyalomere' and 'granulomere', respectively. By the late nineteenth and early twentieth centuries workers had not only recognized anatomical features of the platelet, but had established its involvement in hemostatic processes and blood coagulation. Many of the basic features of the platelet response after vascular injury *in vivo* or stimulation *in vitro* were accurately depicted. Shape change, pseudopod extension, adhesion, aggregation, acceleration of clotting, clot retraction and even secretion were recognized with reasonable clarity, despite limitations in cell preparation techniques and microscopic methods.

However, understanding of platelet structure and structural physiology might have stabilized at a primitive level, but for the development of the electron microscope.[22] While the early applications of ultrastructural methods added little to the knowledge gained from light microscopic studies, the stage was set for a revolutionary new approach to platelet functional anatomy and pathology. Yet, improvements in the resolving power of microscopes was not all that was needed. Rapid developments in electron optics and high vacuum technology quickly overcame the limitations in early instruments. The central problem rested with methods available for studying cells *in vitro* and fixing them for study in the electron microscope. Changes in the procedures necessary to isolate platelets from whole blood in an undamaged state, stimulate the cells under conditions resembling those found *in vivo* and preserve cells and aggregates in a pristine state were essential for progress in the field.

METHODS

Fortuitously, methods for isolating viable platelets, procedures for carrying out studies of their physiology and biochemistry and techniques for preserving fine details of ultrastructural anatomy were developed at about the same time that members of the blood research community began to appreciate a greater importance for platelets in hemostasis and vascular pathology. Glutaraldehyde was one of several dialdehyde fixatives introduced in the early 1960s.[8] Fixation of blood cells in various combinations of glutaraldehyde and osmium tetroxide separately or together was soon found to be superior, at least in most situations, to preservation in osmic acid alone. Initially, dual fixation with glutaraldehyde and osmic acid was carried out in the same manner as when osmium had been used alone.[19] Platelets were separated from whole blood collected in EDTA anticoagulant by centrifugation in a refrigerated centrifuge at 4°C. The isolated cells were kept chilled and sedimented in the cold a second time to obtain pellets. The supernatant plasma was discarded and chilled glutaraldehyde was layered over the pellet. After 15–30 minutes the pellet was broken up into small pieces and fixed for an additional 60 minutes at low temperature. Following this, the aldehyde fixative was discarded, the cell fragments washed in cold-buffer or distilled water several times, then combined with chilled osmic acid for about 1–2 hours. The fixed sample was again washed and dehydrated in a graded series of acetone. After exposure to pure acetone, the fixed samples were infiltrated with liquid plastic, usually methacrylate or vestopal-W. After polymerization, sections were cut from plastic blocks with glass knives on hand-driven microtomes. The sections were usually stained with a lead salt to enhance contrast and evaluated in the electron microscope.

Photographs of platelets prepared in this manner revealed morphological details which had not been apparent when osmic acid was used as the only fixative.[15,16] In particular, initial exposuure to glutaraldehyde prior to incubation with osmic acid preserved a new platelet organelle which had not been recognized previously.[23] The structure was smaller than the more numerous α granules, and appeared to consist of an electron dense core surrounded by a clear space separating the opaque substance from its enclosing membrane. Some initial studies suggested that glutaraldehyde acted as a Schiff base to stabilize the structure and as a mordant to attract osmic acid.[11] Based on these observations, the term osmiophilic dense body was proposed as the eponym for the new organelle. Subsequent studies, however, demonstrated that this hypothesis for preservation of dense bodies was incorrect because the organelles were inherently electron opaque.[14] Yet, the discovery had an important influence on the near universal adoption of dual fixation in glutaraldehyde and osmic acid to preserve platelet fine structure.

Though the improved ultrastructural preservation achieved with dual fixation represented a significant advance, many morphological features remained obscure or were completely missed because of inherent errors in the fixation procedure. Most of these difficulties were recognized and resolved in subsequent years, and form the basis of our current approach to platelet fixation. A few are emphasized here because they are important for obtaining optimal preservation based on our current understanding and use of fixation chemistry.

Anticoagulant

EDTA is a useful anticoagulant. It keeps blood in a fluid state by chelating the calcium necessary for the coagulation process. Unfortunately, it also affects platelet

membranes. Platelets lose the discoid form characteristic of their appearance in circulating blood and become irregularly convoluted resembling spiny spheres.[24] As a result, the morphology of circulating platelets is not well preserved in samples prepared for electron microscopy from blood collected in EDTA anticoagulant. This problem was resolved quite simply by employing one of several different citrate anticoagulant solutions (Fig. 1.1). Our choice has been citrate-citric acid dextrose, pH 6.5 (Table 1.1). Other formulae for citrate anticoagulants are probably just as effective, but this variation has worked well for us. Blood for our studies is usually collected from

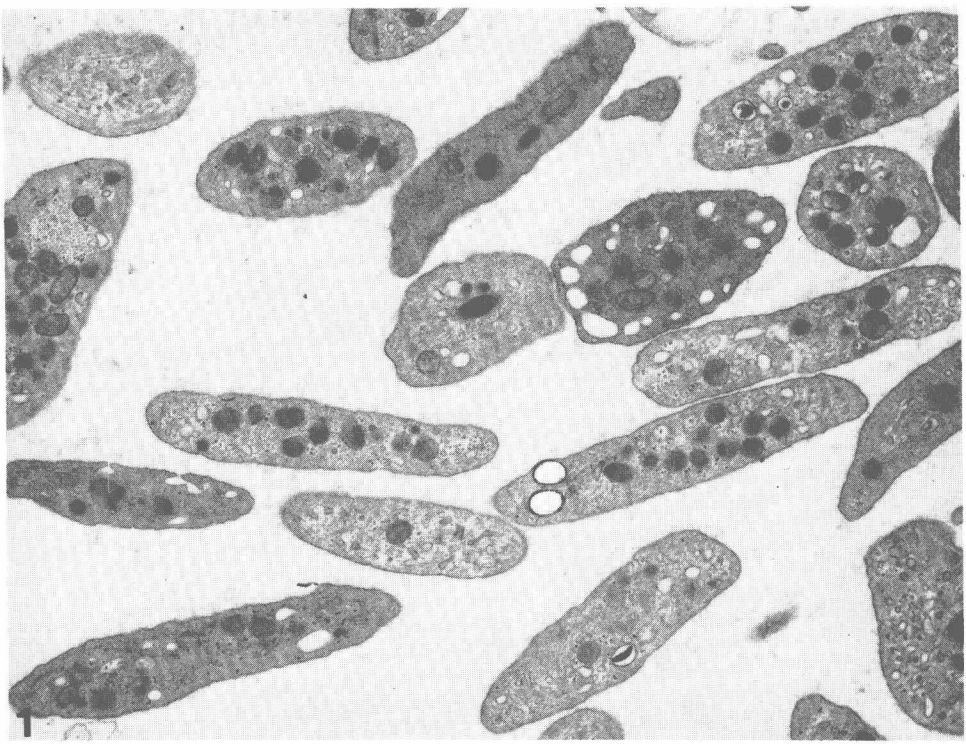

Fig. 1.1 Discoid platelets. The cells in this example are from platelet-rich plasma separated at room temperature from whole blood collected in citrate-citric acid dextrose anticoagulant and fixed in glutaraldehyde and osmic acid. Under these conditions the discoid shape characteristic of circulating blood cells is well maintained. Mag. × . 13,000.

Table 1.1 Anticoagulant

1. 0.1 mol/l citric acid 10.508 g to 500 ml with distilled H_2O
2. 0.1 mol/l sodium citrate $2H_2O$ 14.706 g to 500 ml with distilled H_2O

To make the reagent:
35 ml 0.1 mol/l citric acid
465 ml 0.1 mol/l sodium citrate
12.25 grams dextrose (0.1 mol/l)
Add 5+ drops 10N NaOH (0.1 mol/l) to pH 6.5
Refrigerate

an anticubital vein distended by application of a tourniquet to the upper arm. A 'butterfly' infusion set with 30 cm (12 in) of loose plastic tubing between the hub and a 19-gauge needle is routinely employed in adults and children. Venous blood is drawn into 35 ml plastic syringes and then mixed immediately in a ratio of 9 parts blood to 1 part anticoagulant. In some cases the first few milliliters of blood are drawn into a 5 or 10 ml plastic syringe and discarded before collecting the blood to be used for study in the larger volume plastic syringes. Occasionally 50 ml syringes are used, but in general, the 35 ml syringes are preferred because they are less unwieldy and blood can be mixed with anticoagulant sooner. We have not found it necessary to insert large gauge needles to collect venous blood by free flow as suggested by other workers. On occasion heparin anticoagulant is used in place of citrate, but for electron microscopy we prefer the citrate-citric acid dextrose. In some cases EDTA or EGTA is desirable to maximally reduce levels of calcium in plasma without disturbing platelet disc form. This can be achieved by adding EDTA or EGTA to citrate platelet-rich plasma (Fig. 1.2). Discoid form in the presence of the chelating agents will be preserved under these conditions for about 30 minutes.

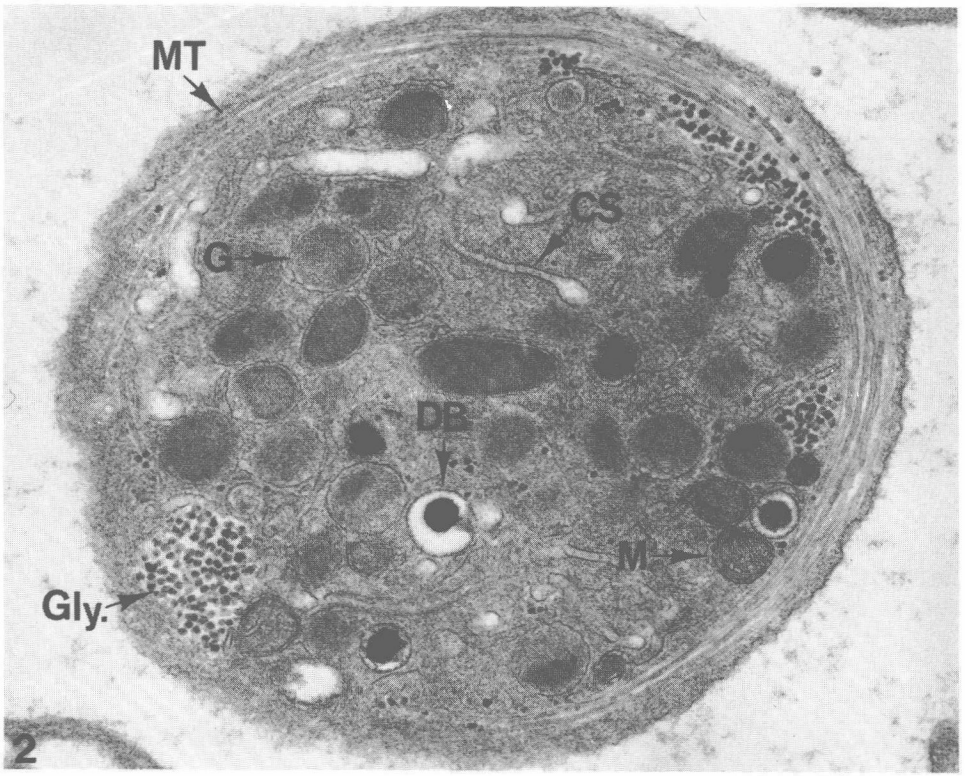

Fig. 1.2 Effects of EDTA. The platelet is from a sample of C-PRP combined with EDTA at 10 mg/ml for 30 minutes at room temperature before fixation in glutaraldehyde and osmic acid. The discoid form of the cell is well preserved and supported by a circumferential bundle of microtubules (MT). Organelles in the platelet cytoplasm, including granules (G), dense bodies (DB) and mitochondria (M), are randomly dispersed. Glycogen (Gly.) occurs as single particles or in masses. Channels of the surface-connected open canalicular system (CS) appear narrow and elongated, rather than tortuous and dilated, as a result of exposure to EDTA. Mag. × 38,000.

Temperature

The importance of temperature for preserving the morphology of discoid platelets was demonstrated several years ago.[1,20] Samples of citrate platelet-rich plasma (C-PRP) rapidly lose their discoid form on chilling. After a few minutes in an icebath at 2–4°C all platelets are irregular in form with multiple pseudopods. The shape change was shown to be associated with disappearance of the circumferential bundle of microtubules which acts as a cytoskeletal system to support the lentiform appearance of unstimulated platelets. Rewarming the chilled platelets restores their discoid form and the recovery is associated with reassembly of the annular band of tubules. However, the cells are somewhat deformed by the cycle of chilling and rewarming. Therefore, to avoid the effects of cold, platelets should be collected and separated from whole blood at room temperature and maintained at 23°C (or 37°C). Initial fixation in glutaraldehyde should be carried out in this same temperature range.

pH

Blood collected in the citrate anticoagulant described will have a pH of about 7.2–7.3. The pH will be maintained during separation of C-PRP if the procedure is carried out soon enough after blood collection. Within an hour after obtaining blood the pH of C-PRP will rise spontaneously, and the alkaline conditions will favor discocyte to echinocyte transformation, just as in the case of erythrocytes. The pH rise can be avoided by exposing the air above the C-PRP to a stream of 95 per cent O_2 5 per cent CO_2 gas and then sealing the tube with a cork.[10] However, maintenance under these conditions for prolonged periods will cause a fall in pH. The initial fall from 7.3 to 6.5 will tend to make platelets more discoid. However, at pHs below 6, platelets undergo disk to sphere transformation in the same manner as erythrocytes. Therefore, it is best to avoid wide swings in pH if optimal morphology is a major goal.

Buffer

Many different solutions are used to buffer glutaraldehyde and osmic acid. Some are used to facilitate cytochemical procedures carried out after initial fixation. Others are employed because the ionic composition tends to favor retention of specific chemical constituents or structures in cells. It is for that reason that White's saline (Table 1.2) is used to buffer glutaraldehyde in most of our routine studies of platelet ultrastructural morphology.[21] Many years ago we found that a high concentration of calcium in the

Table 1.2 Stock solutions for White's saline

A.	NaCl	14.0 g
	KCl	0.75 g
	$MgSO_4$	0.55 g
	$Ca(NO_3)_2.4H_2O$	1.5 g
	Add distilled H_2O to 100 ml	
	Refrigerate	
B.	$NaHCO_3$	1.1 g
	$Na_2HPO_4.7H_20$	0.22 g
	KH_2PO_4 anhydrous	0.052 g
	Phenol red	0.01 g
	Add distilled H_2O to 100 ml	
	When dissolved at room temperature bubble CO_2 in for about 15 seconds, if necessary. Mix well and pH to 7.4	
	Refrigerate	

buffer preserved platelet dense bodies while phosphate ions tended to extract them during fixation. It is for this reason we were able to demonstrate that thin sections of normal platelets contained 1–2 dense bodies per cell, rather than the 1 dense body per 1300 platelets in thin sections reported earlier.[12] Many workers worry about the osmolarity contributed by glutaraldehyde to the buffer solution and reduce the concentrations of salts to adjust for it. This has been found to be unnecessary. Though glutaraldehyde does contribute significantly, it appears that the buffer strength and osmolarity in relation to that of plasma need be the only major concern. Thus, for other cells we often use Hanks' balanced salt solution, Ringer's lactate or other isotonic buffers as the vehicle for the fixative.

Conditions for fixation

At the time osmic acid was the only fixative in common use it was standard practice to sediment platelets to a pellet before combining with osmium. This set of conditions continued to be employed after dual fixation was introduced. However, studies carried out many years ago demonstrated that close cell contact caused by centrifugation activated platelets and resulted in shape change.[6] Therefore, in most cases, it is advisable to fix platelets in suspension. To accomplish this we simply mix the platelet sample with an equal volume of fixative solution, stir gently and allow to stand at 37°C or room temperature for 15 minutes before centrifugation to a pellet. A similar procedure is used for platelet aggregates prepared on a platelet aggregometer. Under these conditions glutaraldehyde appears to penetrate quite rapidly and to terminate both physical and biochemical reactions.

Initial concentration of glutaraldehyde

Early investigators demonstrated that excellent preparations of discoid platelets could be obtained for electron microscopy by collecting native blood directly into a fixative solution containing 6 per cent glutaraldehyde. We have found it easier to combine samples of C–PRP with an equal volume of 0.1 per cent glutaraldehyde in White's saline (Table 1.3) for a few minutes, centrifuge the sample to a pellet, discard the supernatant and replace it with 3 per cent glutaraldehyde in White's saline (Table 1.3).

Table 1.3 Fixatives

1. 0.1% glutaraldehyde in White's saline
   8.9 ml distilled H_2O
   0.5 ml White's A
   0.5 ml White's B
   0.1 ml 10% glutaraldehyde

2. 3% glutaroldehyde in White's saline
   6 ml distilled H_2O
   0.5 ml White's A
   0.5 ml White's B
   3 ml 10% glutaraldehyde

3. 1% OSO_4 Prepare and keep in a dark brown glass-stoppered bottle
   2.5 ml 4% OSO_4 aqueous
   2.0 ml stock buffer (Zetterquist's)
   0.68 ml stock salt solution
   2.0 ml 0.1N-HCl (0.1 mol/l)
   2.82 ml distilled H_2O
   Keeps one week in refrigerator