Fixation and embedding of small volumes of platelets for transmission electron microscopy

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We frequently encounter small volumes of blood from children for biochemical and ultrastructural investigation, and the amount of blood platelets available for ultrastructural investigation is often very small. The problem is compounded when the platelet count is low because a cell pellet of adequate size, thickness, and hardness for embedding is not easily obtained.

Applying current methods to small volumes of platelets results in a cell pellet too thin and friable to dice and embed without loss or damage to the cells. To overcome this difficulty we developed the following method.

Material and methods

PREPARATION OF PLATELET-RICH PLASMA

Blood samples are obtained from patients and healthy donors by venepuncture and collected into tubes containing sodium heparin as an anticoagulant. The samples are mixed by gentle inversion, and a platelet-rich plasma (PRP) is prepared by centrifugation of the samples in the collection tube for 15 minutes at 900 rpm at room temperature.¹

PREFIXATION

Immediately after preparation one volume of PRP is added by plastic pipette to 5 volumes of prefix solution in a polyethylene 12 × 75 mm tube, the solution is gently swirled, and the sample is allowed to stand at room temperature for 30–45 minutes. The prefix solution consists of freshly prepared 0.1% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4.² All electron microscopy reagents are purchased from Ted Pella, Inc, Tustin, California, USA.

FIXATION

The PRP-prefix suspension is centrifuged at 3000 rpm for 10 minutes, and the supernatant is decanted from the platelet film which thinly covers the bottom of the tube. A room temperature fixation solution consisting of 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4, 420 mosm, is then poured

gently down the side of the tube to cover the film, and the platelets are fixed for 1–2 hours.

The fixation solution is decanted from the tube and the platelet film is covered with room temperature 0.1 M sodium cacodylate buffer, pH 7.4 for 5 minutes. The platelets are stored overnight at 4°C in a second rinse of the same buffer.*

POSTFIXATION, DEHYDRATION, AND EMBEDDING

Postfixation of the platelets is performed in 1% aqueous osmium tetroxide in 0.1 M sodium cacodylate buffer, pH 7.4 at 4°C for 1 hour. The platelet film is rinsed in two 15-minute changes of the same buffer and dehydrated in a graded ethanol series (50%, 70%, 80%, 95%, 100%) at 4°C for 2 minutes each. This is followed by two room temperature rinses of 100% ethanol for 5 minutes each. Each exchange of fluid is made by decanting one solution and introducing the next with a gentle pouring of the solution down the side of the tube.

The platelets in the same tube are infiltrated first with a 1:1 mixture of Spurr resin³ and ethanol, and secondly with a mixture of 2/3 Spurr and 1/3 ethanol for 20 minutes each. The platelets are then infiltrated overnight in 100% Spurr resin.

After infiltration the platelet film is dislodged from the tube bottom using a wooden applicator stick that has been shaved to a thin wedge and soaked in Spurr resin to soften. The film is dislodged into the tube of resin by gentle movements of the applicator stick towards the tube centre at the outer edge of the platelet film. Owing to the extreme thinness of the platelet film in small samples, it will dislodge into small fragments that suspend in the resin as the applicator stick is manoeuvred at the film edge. These fragments are easily brought to the mouth of the tube by a pouring action. The small fragments are removed from the tube by collecting onto an applicator stick a resin drop in which a platelet film fragment is suspended. The resin drop is then transferred to a size 3 Beem capsule or a flat embedding mould filled with a small portion of fresh Spurr resin. Additional Spurr resin is layered over the platelet film with a pipette, and the blocks are allowed to polymerise in an oven maintained at 70°C.

We routinely section these blocks on a Porter-Blum MT-2 ultramicrotome with glass knives. Ultrathin sections of silver interference colours are collected on to copper grids and stained routinely

*We have found that the platelets can be stored in this buffer at 4°C for up to two weeks without alteration of morphology when dehydration and embedding cannot follow fixation immediately.

Transmission electron micrograph of platelets from a healthy human donor. Platelets prepared by this method meet all criteria of good fixation and display a minimum of artifactual stimulation.

with uranyl acetate and Reynolds' lead citrate⁴ and viewed on an Hitachi HS-8 electron microscope at 50 kV.

Results

Platelets prepared by this method showed excellent morphological preservation and minimal damage or artifactual stimulation (Figure). Platelets of healthy donors appeared as flat, disc-like structures in cross-section and circular in shape when sectioned through the equatorial plane. Cytoplasmic outlines were regular, and only a few short pseudopods were observed. Cytoplasmic granules and organelles were evenly distributed in the cytoplasm. Microtubules and mitochondria were well preserved so that the platelets in every way met the criteria for good fixation.

Discussion

Platelet ultrastructure is known to be altered by procedural manipulations.⁵ In this study, care was taken to minimise alterations in morphology by controlling temperature, fixative, pH, buffer type, anticoagulant, and method of platelet separation.

Sodium heparin was used as an anticoagulant because it is our standard anticoagulant,¹ and we have not observed any alterations in platelet structure or biochemistry attributed to its use.

Preparation of the PRP and the fixation procedures were carried out as soon as possible after

venepuncture to avoid time-induced changes in morphology. Our use of centrifugation to prepare and fix the PRP is similar to that of Mattson *et al.*² who have obtained excellent results with centrifugation methods. Activation of the platelets by glass was avoided by the use of plastic containers.

We elected to maintain the platelets at room temperature during preparation and fixation in order to guard against cold-induced morphological changes⁶ and because Mattson *et al.*² and White⁷ have found successful preservation of platelet morphology at room temperature.

Glutaraldehyde was the fixative of choice in this procedure because it has been shown to stop platelet cell activity within seconds.⁸ The percentages of glutaraldehyde in sodium cacodylate buffer used in this procedure are well documented in the literature for the successful fixation of platelets.^{2,8} Our experience has shown that a pH of 7.4 is optimum for good platelet preservation (Sawatzke, unpublished observation).

Although Mattson *et al.*² recommend a 10-fold volume of prefixative to PRP, we have demonstrated that one volume of PRP to 5 volumes of prefixative is adequate when small amounts of PRP (less than 1 ml) are used. The thin film of platelets which results from centrifugation of small volumes of PRP is easily penetrated by all the reagents used in this procedure, and we have obtained excellent results with as little as 30 minutes prefixation and 1 hour fixation.

The procedure described is simple to perform and

results in excellent morphological preservation of small volumes of platelets. A key advantage of the procedure is the insurance against sample loss by completing all procedural steps before embedding in the same tube. The cells are manipulated minimally and the entire platelet film can be embedded.

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Letters to the Editor

Correlation between automated cytochemical staining (Hemalog-D) and visual microscopy monocyte counts

The Hemalog-D differential cell counter measures individual cell size, cell peroxidase and esterase activities, and cell staining with alcian blue. From these results, differential white blood cell counts are obtained on blood samples.¹⁻³ A series of alarm signals indicates the presence of cells which the machine is unable to classify, for example, monocytes with low esterase activity. If such alarm signals are activated, then it is necessary to examine a Romanowsky-stained film from that particular blood sample. In the absence of alarm signals, the correlation between differential counts by routine microscopy and the Hemalog-D machine is satisfactory, except with regard to the monocyte counts.^{4,5} We have tried to

define more accurately those Hemalog-D differential counts that do not require visual checks.

Differential white blood cell counts were obtained from examination of 200 cells counted in wedge-shaped blood films stained with May-Grünwald-Giemsa and were compared with the corresponding differential counts read from the Hemalog-D.

As recommended,⁶ all results with total leucocyte counts greater than $20 \times 10^9/l$ or less than $5 \times 10^9/l$; large unstained cell (LUC) values greater than 2.5%; high peroxidase (HPX) values greater than 2.5%; positive low peroxidase (LPX) and positive low rate (LR) were excluded from this study. Only those differential counts were included which gave a remainder signal but no other alarm signals on the Hemalog-D. The remainder signal was considered to be abnormal when it

exceeded ± 5 .

As our results show, with a positive remainder less than 5, a poor monocyte count correlation ($r = 0.412$; $n = 76$) was obtained. In 91 cases with a remainder greater than 5, the monocyte count was greater by visual microscopy than by Hemalog-D (Table 1). The correlation was poor ($r = 0.182$) but could be improved if the remainder was added to the Hemalog-D monocyte count (Table 2). With a negative remainder less than -5 there was reasonable correlation ($r = 0.613$), but with a remainder between -5 and -10 and with less than 10 monocytes by visual microscopy, the Hemalog-D monocyte count was greater than the visual monocyte count (Table 1). The results could be reconciled if the remainder was subtracted from the Hemalog-D monocyte count (Table 2). With a remainder greater than -5, and with