Indium (\(^{111}\)In)-labelled human platelets: optimal method

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Summary

1. A detailed laboratory method is described for the labelling of human platelets with \(^{111}\)In indium oxine. The 45 min method is simple, requires only 26 ml of blood and is suitable for routine clinical use.

2. After the labelling and resuspension of the platelets in plasma, aggregation responses to both adenosine diphosphate and collagen were similar to those of normal platelet-rich plasma. Less than 5% of the \(^{111}\)In indium oxine was released by secretory functions of platelets.

3. Labelling efficiencies of 90.1 ± 4.29% (n = 28) were achieved in 60 s by normal concentrations of plasma-free platelet suspensions.

4. Platelet survival in vivo in healthy volunteer subjects follows a linear function with a survival time of 8.44 ± 0.18 days.

Key words: blood platelets, indium, thrombocytes.

Introduction

There are several modifications of the original method of labelling cells with \(^{111}\)In indium oxine (Thakur, Welch, Joist & Coleman, 1976) which are applicable to platelets (Davies, Heaton, Siegel, Mathias, Joist, Sherman & Welch, 1978; Goodwin, Bushberg, Doherty, Lipton, Conley, Diamanti & Moores, 1978). Platelets can undergo adhesion, aggregation and release reactions, and are difficult to isolate in a purified form without loss of function. The methods of isolation designed for neutrophils or lymphocytes, where the functional nature to be preserved is phagocytic or antigenic (Lavender, Goldman, Arnott & Thakur, 1977; Thakur, Coleman & Welch, 1977; Segal, Deteix, Garcia, Tooth, Zanelli & Allison, 1978), may not be suitable therefore for platelets.

Previous workers have shown that it is necessary to remove plasma in order to label platelets efficiently. Scheffel, McIntyre, Evatt, Dvornicky, Natarajan, Bolling & Murphy (1977), using concentrated rabbit platelets in plasma and a labelling time of 30 min, achieved 27–67% labelling efficiency. This was confirmed by Wistow, Grossman, McAfee, Henderson & Roskopf (1978) and represents a marked improvement over \(^{51}\)Cr labelling efficiencies (Baldini, Costea & Dameshek, 1960). In our experience (Wilkinson, Hawker & Hawker, 1978) canine and human platelets cannot be labelled efficiently in plasma, and Scheffel, Tsan & McIntyre (1979) could achieve 55.5% labelling only after 90 min labelling time for human platelets in plasma. The low labelling efficiency in plasma is due to temporary sequestration of the complex by proteins (McAfee & Thakur, 1976) and in particular by lipoproteins (Hawker, Hawker & Wilkinson, 1978; Hwang, 1978). In a plasma-free medium, labelling efficiencies of greater than 90% may be achieved in 60 s (Hawker et al., 1978).

Thakur et al. (1976) reported that the platelet aggregation responses after labelling were similar to those of washed platelets before labelling. However, a comparison between aggregation responses of labelled cells with those of platelet-rich plasma would, of course, enable the optimal method to be devised (Hawker & Hawker, 1979).

The method described here is a laboratory method in which retention of normal platelet function, in vitro and in vivo, is demonstrated.
Materials and methods

Chemicals

Unless otherwise stated AnalR chemicals were obtained from British Drug Houses, Poole, Dorset, U.K. Anticoagulants and buffers were sterilized by 0.2 μm filtration.

Acid/citrate. This was 100 ml of aqueous solution of 2.5 g of trisodium citrate 2H₂O and 1.49 g of citric acid H₂O (Doery, Hirsch & Mustard, 1973). This anticoagulant, used in the proportion of 3 ml to 17 ml of blood, results in pH 6.5.

Calcium-free, Tyrode solution (the buffer: 296 mosmol/kg). One litre of aqueous solution of 8 g of sodium chloride, 0.2 g of potassium chloride, 1.0 g of sodium bicarbonate and 25 000 units of sodium heparinate (Sigma, Poole, Dorset, U.K.), adjusted to pH 6.5 with HCl (1 mol/l) and sterilized by 0.2 μm filtration, was measured into glass ampoules along with prostaglandin E₁ (300 ng/ml) and stored frozen at −20°C.

Siliconized glassware. Clean glassware was immersed in a 0.04% solution of dimethyldichlorosilane in acetone, allowed to dry in air, washed in a dilute solution of Decon 90 and thoroughly rinsed in distilled water. After drying, Pasteur pipettes were plugged, packaged and autoclaved. Where siliconized glassware was impractical, polystyrene vessels were used, e.g. sampling for aggregation studies utilized conical bottomed autoanalyser cups (Sterilin, Teddington, Middlesex, U.K.; code 108).

Preparation of [¹¹¹]In indium oxine complex

¹¹¹In (approximately 50 ng of In) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K., where it is made up by the addition of HCl (0.04 mol/l) to [¹¹¹]In indium chloride. Both the volume and final molarity of the acid are variable (1.0 ml ± 10%; HCl: 0.04–0.036 mol/l).

To the supplied vial, 200 μl of sodium hydroxide solution (0.2 mol/l) followed by 400 μl of acetic acid/sodium acetate buffer (0.3 mol/l, pH 5.4) was added to bring the solution to pH 5.4. 8-Hydroxyquinoline (100 μl of a 1 mg/ml solution in absolute ethanol) was added and mixed. After standing for 5 min the mixture was extracted three times with 1.5 ml of dichloromethane (Merck, Darmstadt, Germany) by using a glass syringe. The dichloro-

methane was evaporated to dryness in a current of warm air and the residue dissolved in 100 μl of redistilled ethanol, with a further 50 μl used to wash out the vessel. An extraction efficiency of $81.64 \pm 4.58\%$ ($n = 10$) was observed. The ethanolic extract was then transferred to a sterile glass microvial (Camlab, Cambridge, U.K.). A 5 μl aliquot contained approximately 3.34 μg of oxine and 1.34 ng of [¹¹¹]In, assuming an 80% extraction efficiency.

A preparation of [¹¹¹]In indium oxine in physiological medium is now available from The Radiochemical Centre.

Optimal method (Fig. 1)

Blood (26 ml) was taken by clean venepuncture with a syringe and 18 gauge needle. Of this volume 17 ml was mixed with 3.0 ml of acid/citrate in a sterile Universal container (Sterilin 128/B) and the remaining blood transferred to a sterile round-bottomed tube (Sterilin 142 AS) containing 1.0 ml of trisodium citrate (38 g/l). After mixing by inversion, both tubes were immediately centrifuged at 200 g for 10 min to generate platelet-rich plasma. A sample of citrated platelet-rich plasma was retained for aggregation studies before the contents were centrifuged at 1000 g for 10 min to obtain citrated platelet-poor plasma.

![Diagram](image-url)
**Indium-labelled human platelets**

Two 100 μl samples of the resuspended platelets were taken for aerobic and anaerobic bacteriological culture. A further 100 μl was used as a standard and 0.5 ml retained for aggregation responses. The remaining platelet suspension was weighed and re-injected into the donor.

**Aggregation responses**

The aggregation response to adenosine diphosphate and to collagen were studied in a Payton aggregometer with 0.25 ml of platelet suspension. Labelled platelets resuspended in citrated plasma were compared with the original citrated platelet-rich plasma. Aggregation responses to adenosine diphosphate cannot be carried out in acid/citrated plasma because of the lowered pH. Adenosine diphosphate was obtained as the trilithium salt (Sigma) and made up as a stock solution in sodium chloride solution (154 mmol/l:saline) at 10⁻¹⁴ mol/l. A final concentration of 1.2 μmol/l was used.

Soluble collagen was obtained from Hormon-Chemie (Munchen, Germany) at a concentration of 1 mg/ml and diluted 1:20 in saline before use. A final concentration of 2 μg/ml was used.

**Release of [¹¹¹In]indium oxine**

Platelets labelled with [¹¹¹In]indium oxine and resuspended in citrated plasma were aggregated with adenosine diphosphate (1 μmol/l or 10 μmol/l), collagen (1 μg or 2 μg/ml) or thrombin (100 m-units/ml).

Labelled platelets in buffer were incubated for 1½ h with trypsin (250 μg/ml), EDTA (4 mmol/l) or were lysed by dilution with water.

**Survival studies in vivo**

Human volunteer subjects (Hospital Ethical Committee Approval and consent of the subjects were given) were injected with autologous platelets that were functional to adenosine diphosphate. Approximately 100 μCi of radioactivity was administered; 5 ml blood samples were taken 1 h post-injection and then daily for up to 10 days. The samples were counted for radioactivity, which was expressed as a percentage of the 1 h sample. Elution in the 1 h sample and subsequent samples were measured after separation of plasma.

A linear regression analysis was carried out,
relating percentage of the initial sample remaining in circulation with time. The correlation coefficient was also calculated.

The extrapolated line of best fit crosses the ordinate at a point which represents increased loss of platelets between the 1 h and subsequent samples. The survival can be calculated by extrapolation of the line to zero per cent. The slope of the regression line is a measure of the mean loss per day, from which survival times can also be derived (Wilkinson, Hawker & Hawker, 1978).

Results

Labelling efficiencies were 90-11 ± 4.29% (n = 28). Lowered labelling efficiency could be related to poor venepuncture technique, inadequate washing of the platelets to remove plasma or loss of function caused by excessive manipulation.

Platelet survival in vivo

Platelets re-injected into four healthy subjects showed a reproducible survival of 8.44 ± 0.18 days. Fig. 3 shows a typical survival curve. The linear loss of platelets (r = 0.993, n = 8, P < 0.001) can be explained by platelet destruction based on senescence and the presence of label in all ages of platelets. The $^{111}$In contained in the plasma of the samples did not exceed 5%.

All subjects, volunteers or patients, had a linear platelet survival pattern. The difference between 100% circulating and the calculated value of a (Fig. 3) is a measure of any increase in loss of platelets in the first 24 h (Wilkinson et al., 1979) and for the four healthy subjects was 3.1 ± 1.2%.

$^{111}$InIndium oxine is not excreted (Goodwin et al., 1978) and accumulates upon platelet sequestration in the spleen. One additional healthy subject, under stress, had a reduced platelet survival time of 7.47 days and two patients studied with arterial disease had platelet survival times of 6.7 and 7.6 days, the latter determined whilst the patient was undergoing anticoagulant therapy. In the four healthy subjects, the recovery of circulating labelled platelets in the 1 h post-injection sample was calculated from the standards as 69.3 ± 2.97%.

Aggregation responses

After labelling of platelets, resuspension in citrated plasma and incubation at 37°C for 15 min, aggregation responses were routinely assessed with

adensine diphosphate (0.8 μmol/l and 1.2 μmol/l). This period of time was found optimal for restoration of full responsiveness. Responses of 87.81 ± 10.6% (n = 38) of the control platelet-rich plasma were obtained and on many occasions maximal responses were observed. The response to collagen (2.0 μg) was always normal.

Release of $^{111}$InIndium oxine

The amount of $^{111}$InIndium oxine released by platelet aggregation was proportional to the expected granule release (Joist, Baker, Thakur & Welch, 1978). There was no difference in release of $^{111}$InIndium oxine in response to 1 μmol of adenosine diphosphate/l, which causes no granular secretion, when compared with an EDTA control. Adenosine diphosphate at 10 μmol/l released dense granules and 1.7% of the radioactivity was released. Collagen (2 μg) and thrombin (100 munits) caused both dense granule and α-granule release and an associated increase in the loss of radioactivity (4.9%). Trypsin, which is reported to cause secretion of dense granules (Holmes, Salganicoff & Fukami, 1977), released 2% of the $^{111}$InIndium oxine. Rupture of the platelet membranes by hypotonic shock released 70% of radioactivity (Hawker & Hawker, 1980).
Sterility

No bacterial growth was observed in any of the 80 specimens sent through routine bacteriological screening in the hospital laboratory.

Discussion

Methods of separating and washing human platelets have successfully maintained platelet function when kept simple and rapid, and if the buffers and temperatures used closely resemble physiological conditions.

The system described maintains platelets in a plasma-free state for only 60 s, the minimum time necessary for the efficient uptake of $^{111}$In-labelled indium oxine. At all other times the platelets are suspended in at least 37.5% autologous plasma. The use of acid/citrate as anticoagulant lowered the pH of the platelet-rich plasma to approximately 6.5 and made the platelets less likely to aggregate spontaneously (Han & Ardlie, 1974). The use of inhibitors of aggregation greatly assists the dispersion of centrifuged platelets (Shio & Ramwell, 1972) but their inhibitory effect must be removed if a function study is to be checked after preparation. Thus a period of 15 min incubation at 37°C was found necessary for the platelets to return to a normal functional state. This may be due to several factors, such as prostaglandin E; elution, recalcification of platelets after their period in reduced calcium concentrations, or a return to a physiological pH when resuspended in citrated plasma.

The method utilizes, apart from anticoagulants, only one buffer in a small volume. This makes the procedure suitable for a routine pharmaceutical preparation. The technique takes approximately 45 min from venepuncture to the return of labelled platelets to the patient.

When platelet counts are within the normal range only 26 ml of blood is required to give sufficient platelets for an efficient label. The removal of plasma is an important stage of the labelling technique and only by careful washing of the undisturbed platelet button (Fig. 2), before resuspension in the buffer, can the high labelling efficiency be achieved. Poor labelling efficiency was directly related to reduction or loss of aggregation responses and therefore to poor technique. It should be emphasized that 'no special equipment is needed but practice is essential for the success of the method' (Goodwin, 1978).

The survival of $^{111}$In-labelled human platelets is comparable with that seen with $^{51}$Cr. However, the $^{111}$Inindium oxine method has several advantages: a higher labelling efficiency, short times ex vivo, lack of platelet damage, as judged by aggregation responses, and the higher energy gamma emissions which make localization of platelets with the gamma camera possible. The reproducibly linear platelet loss with time is not always observed with $^{51}$Cr-labelled platelets (Panel on Diagnostic Application of Radioisotopes in Hematology: International Committee for Standardization in Hematology, 1977), and exponential or a mean between-linear and exponential functions have been ascribed to the platelet loss (Scheffel et al., 1977).

The technique described can be used for both platelet-survival studies using as little as 75 μCi or 150 μCi for localization studies (e.g. in diagnosis of renal transplantation complications visualization of kidney for 4–5 days is allowed; Smith, Chandler, Hawker, Hawker & Barnes, 1979).

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References


