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Flow Cytometric Evaluation of Platelet Activation in Blood Collected Into EDTA vs. Diatube-H, a Sodium Citrate Solution Supplemented With Theophylline, Adenosine, and Dipyridamole

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With platelet activation, there is modulation of platelet surface molecule expression. In flow cytometric analyses of in vivo platelet activation, results are often confounded by activation induced in vitro by the preparative procedures. It is particularly important therefore to prevent or retard platelet activation as soon as possible after withdrawal of the blood sample. Taking blood into paraformaldehyde, or fixing the cells with paraformaldehyde as soon as possible after withdrawal, has been employed to prevent platelet activation in vitro, but paraformaldehyde-fixed platelets cannot be further used in functional studies. We investigated the efficacy of Diatube-H, a commercially available combination of platelet antagonists (theophylline, adenosine, and dipyridamole), in preventing or retarding platelet activation in vitro, along with its effects on modulation of platelet membrane glycoproteins (GP) and adhesion molecules. In contrast to blood taken into EDTA, blood taken into Diatube-H vacutainer tubes could be stored at room temperature for up to 4 hr prior to paraformaldehyde fixation without significant in vitro platelet activation, as measured by CD62P, CD63 and modulation of GPIb and GPIIbIIIa surface expression. Hence, paraformaldehyde fixation could be deferred for several hours, permitting transport of samples from distant sites. Studies of thrombin-induced platelet activation indicated that platelets taken into Diatube-H remained functional i.e. were able to be activated. Expression of the CD29, CD49b and CD31 adhesion molecules on the platelet surface was unaffected by storage in Diatube-H. The results suggest that Diatube-H may be a useful reagent for flow cytometric studies of platelets when the samples cannot be processed immediately. © 1995 Wiley-Liss, Inc.

Key words: platelets, platelet activation, flow cytometry, theophylline, dipyridamole, adenosine

INTRODUCTION

Investigation of platelets may be confounded by the ease with which platelets become activated. Platelet activation occurs rapidly, often at, or soon after, the time of blood withdrawal from a peripheral vein [1]. Agitation, centrifugation, washing and passage through plastic tubing, are some of the factors that lead to in vitro platelet activation [2]. The degree of platelet activation is dependent on the anticoagulant into which the blood is drawn [3,4]. Consequently, in platelet studies, a variety of anticoagulants and fixatives have been employed to minimize platelet activation in vitro associated with obtaining the blood sample [5]. There remains considerable controversy as to the optimal method of preventing in vitro platelet activation, but many investigators utilize paraformaldehyde fixation [6,7]. Theophylline and dipyridamole have been shown to inhibit phosphodiesterase, resulting in an increase of platelet cyclic AMP [8] and a decrease in platelet responsiveness [9]. Adenosine also is an inhibitor

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of platelet aggregation, preventing thrombin-induced platelet aggregation and rise in intracellular calcium [10]. Diatube-H is a commercially available combination of agents recognized to prevent platelet activation, and has been recommended mainly for monitoring plasma heparin levels and for assay of functional platelet activation markers, such as platelet factor 4 and β-thromboglobulin. The main contents of the Diatube-H tube are citrate, theophylline, adenosine, and dipyridamole.

This study compared the efficacy of Diatube-H with EDTA anticoagulant and with paraformaldehyde fixation, in inhibiting platelet activation. Flow cytometric analysis was employed to examine changes in platelet surface expression of membrane glycoproteins (GP) associated with platelet activation and adhesion in blood taken and stored in the different anticoagulants. Platelet surface expression of the selectin CD62P (GMP-140) was used as the principal marker of platelet activation. CD62P from the platelet α-granules is expressed on the platelet surface after activation of the cell [11]. Expression of CD63, a lysosomal antigen expressed on the platelet surface after platelet activation (12) was also examined. Platelet membrane GPIIbIIIa (CD41a) and GPIb (CD42b) were quantitated; surface expression of platelet GPIb and GPIIbIIIa may be downregulated or upregulated respectively after platelet stimulation [13,14]. Adhesion molecules examined included the integrins CD29 (platelet GPIIa), CD49b (platelet GPIa) and GPIIbIIIa, the selectin CD62P and CD31 (PECAM; platelet GPIIa′), a member of the immunoglobulin superfamily [15]. CD36, the thrombospondin receptor, is modulated in platelet activation and is involved in platelet adhesion [16].

METHODS

Collection of Blood Samples

Using a 21-gauge needle, blood from ten normal healthy laboratory volunteers (five males, five females; aged 21–41), was drawn into 7-ml EDTA vacutainer tubes (Becton Dickinson, Rutherford, NJ) or into 5-ml Diatube-H vacutainer tubes (Diagnostica Stago, Asnières, France) and maintained at room temperature (22°C) until sample preparation.

Preparation and Staining of Platelets, Using a Whole Blood Method

EDTA or Diatube-H blood samples were fixed, at the times indicated in Results, in equal volume with 1% (w/v) paraformaldehyde, pH 7.4, at room temperature (22°C) for 20 min. For staining with fluorochrome-labeled antibody, 5μl of fixed blood were incubated in the dark for 30 min at 4°C with 25 μl of FACSFlow fluid (Becton Dickinson, San Jose, CA) containing the monoclonal antibody at saturating concentration. Fluorescein isothiocyanate (FITC)-conjugated monoclonal antibodies to CD41a and CD42b (AMAC, Westbrook, ME) and phycoerythrin (PE)-conjugated anti-CD62P (Becton-Dickinson) were used in dual-color analyses. After incubation, 750 μl of filtered FACSFlow fluid was added to each tube and the samples were acquired, within 4 hr after staining, using a FACScan flow cytometer (Becton Dickinson), equipped with a 15-mW argon ion laser. Gating on GPIb- or GPIIbIIIa-positive single platelets, 10,000 events were acquired on each sample and were analyzed using LYSYS II software (Becton-Dickinson).

Preparation and Staining of Purified Platelets

Platelets were isolated from whole blood collected into either Diatube-H or EDTA by centrifugation at 150*g* for 15 min at 22°C. The platelet-rich plasma (PRP) was transferred to 5-ml polypropylene tubes and centrifuged at 500g for 10 min at 22°C and plasma removed; 5 ml of saline was then added to the platelet pellet without disturbing it, then immediately aspirated; the pellet was then gently resuspended in 2 ml phosphate-buffered saline (PBS), pH 7.4, to a concentration of 1×10^9/ml. This method was applied in order to remove the platelet antagonist solution without inducing platelet activation. Immediately following platelet resuspension in PBS, a 0.5-ml aliquot was fixed with an equal volume of 1% paraformaldehyde for 10 min at 22°C, centrifuged and resuspended in 0.5 ml of FACSFlow fluid. This sample was used to assess the baseline level of activation of the platelets (time 0). To an additional aliquot of 0.5 ml platelet suspension, 0.5 units of human α-thrombin (Thrombin (Human) FIBRINDEX Ortho Diagnostic Systems, Johnson & Johnson Co., Raritan, NJ) was added for 10 min at 22°C prior to paraformaldehyde fixation. Platelets to which thrombin had not been added were incubated in parallel for 10 min, in order to determine the level of spontaneous platelet activation. This procedure was followed with platelets left untreated and unagitated at 22°C before staining and fixation at the times indicated in Results.

Fixed platelets (50 μl) were incubated with monoclonal antibodies (e.g., anti-CD41a-FITC, -CD42b-FITC and -CD62P-PE) for 30 min in the dark at 4°C, then washed by centrifugation and resuspended in 0.5 ml FACSFlow fluid. Platelets stained with FITC- or PE-isotypic mouse IgG were used as controls for nonspecific staining in both the whole blood and purified platelet assays. Acquisition and analysis was performed using the FACScan flow cytometer and Lysis II software. Antibody binding sites were determined for GPIb and GPIIb–IIIa using Simply Cellular beads (Flow Cytometry Standards Corporation, San Juan, Puerto Rico) as previously described [17,18]. Purified platelets were also stained with the following monoclonal antibodies to platelet activation or adhesion molecules: anti-CD9-PE, -CD29-FITC, -CD31-PE, -CD49b-PE (Serotec, Oxford, UK), -CD36-FITC, -CD63-FITC (AMAC, Westbrook, ME).

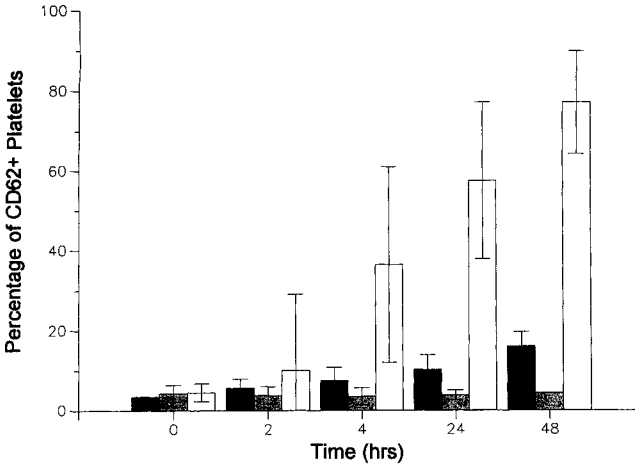

Fig. 1. Comparison of the percentage of CD62P positive platelets in whole blood taken into either Diatube-H or into EDTA stored for various times (median ± SD). The shaded bars represent data of platelets from EDTA blood fixed with paraformaldehyde immediately; platelets in blood taken into Diatube-H (dark bars) were fixed after the indicated times, as were those in EDTA blood shown by the open bars. N = 9.

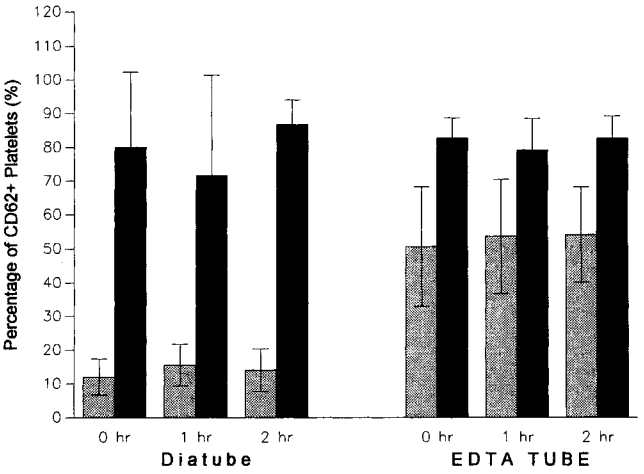

Fig. 2. Platelet activation following thrombin stimulation. Isolated platelets were incubated with thrombin (solid bars) or with saline (shaded bars) for 10 min and fixed with paraformaldehyde. They were stained with anti-CD62P either immediately, or after 1 or 2 hr of storage at 22°C in either Diatube-H or EDTA. N = 10.

Analysis of Data

Results are expressed either as percentage platelets expressing the antigen (e.g. for CD62P), as antibody binding sites (e.g. for CD41a, CD42b), or as mean channel fluorescence (MCF). All data are expressed as median ± SD. The Wilcoxon test was used for statistical analysis, and significance was set at $P < 0.05$.

RESULTS

Activation of Platelets in Whole Blood Taken Into EDTA or Diatube-H

Figure 1 shows that, at time 0, few platelets in whole blood taken into either EDTA or Diatube-H expressed the P-selectin CD62P (GMP-140). Platelets in blood taken into EDTA and fixed with paraformaldehyde immediately, did not become further activated, despite time delays of up to 48 hr before staining with monoclonal antibody. Platelets taken into Diatube-H and not paraformaldehyde-fixed until time of staining showed a progressive increase in activation, i.e., 5.7 ± 2.2% expressed CD62P when stained and fixed at 2 hr, 7.5 ± 3.3% at 4 hr, 10.2 ± 3.1% at 24 hr and 16.3 ± 2.9% at 48 hr after blood was drawn. In contrast, in blood taken into EDTA and not paraformaldehyde fixed until time of staining, there was a marked progressive increase in platelet activation, with 79 ± 15% expressing CD62P after 48 hr.

Since there is often a delay before clinical samples reach the laboratory where they can be treated with paraformaldehyde, blood taken into either EDTA or Diatube-H were allowed to sit at room temperature for 4 hr before paraformaldehyde fixation. The platelets were then fixed, stained and data acquired by flow cytometry at three different times: immediately and at 24 and 48 hr. The percentage of CD62P positive Diatube-H platelets remained unchanged at all three time intervals (7.3 ± 2.1%, 7.0 ± 1.7% and 6.6% ± 1.4%). The platelets in the blood taken into EDTA were, however, as expected, strongly activated when left for 4 hr before fixation, but this did not change significantly once the cells were paraformaldehyde treated (67.6 ± 18.2%, 76.4 ± 15%, 56.1 ± 16.8%).

Hence, although immediate paraformaldehyde fixation is optimal, if this is not possible and blood is not to be processed immediately, Diatube-H appears to be better at preventing platelet activation, at least up to within four hours of blood withdrawal. Once fixed and stained, acquisition may be delayed for at least 48 hr without in vitro platelet activation.

Activation of Platelets Isolated From Whole Blood Taken Into EDTA or Diatube-H

In order to determine the effect of the anticoagulants on platelet function, platelet activation response to thrombin stimulation was studied in purified platelets isolated from blood taken into Diatube-H or into EDTA. Tests were done in parallel with untreated and thrombin-treated platelets, handled similarly in every other way. As shown in Figure 2, thrombin treatment resulted in increased numbers of platelets expressing CD62P ($P < 0.005$). CD62P mean channel fluorescence, reflecting antigenic density on individual platelets, was also increased after thrombin

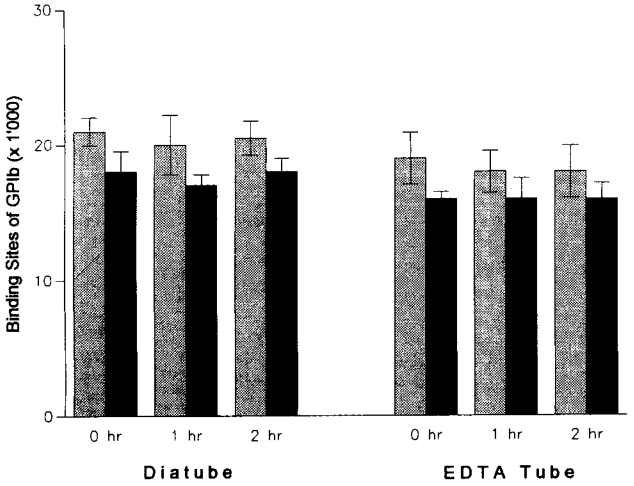

Fig. 3. Platelet GPIb expression before (light bars) and after (dark bars) thrombin stimulation in blood stored in Diatube-H or EDTA for 0, 1, and 2 hr. N = 10.

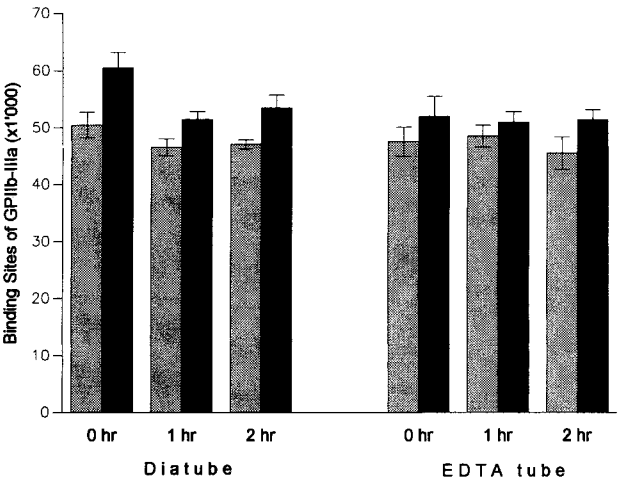

Fig. 4. Platelet GPIIbIIIa expression before and after thrombin stimulation (light and dark bars respectively) in blood stored in Diatube-H or EDTA for 0, 1, and 2 hr. N = 10.

stimulation (Table I). Although purified platelets derived from either EDTA or Diatube-H samples could be activated with thrombin to express CD62P, the difference from non-thrombin-treated platelets was more pronounced in the platelets derived from blood taken into Diatube-H, since the spontaneous (non-thrombin-treated) activation in EDTA stored platelets was much higher ($51 \pm 11\%$ vs $11 \pm 3\%$). The increased spontaneous CD62P expression on platelets from blood taken into EDTA is likely the result of the washing and centrifugation procedures involved in the isolation of the platelets. By contrast, the platelets from blood taken into Diatube-H were protected from spontaneous in vitro activation, as shown in Figure 1.

Platelet activation may be reflected by downregulation of platelet GPIb expression and upregulation of GPIIbIIIa expression. Figure 3 shows that anti-GPIb binding sites were slightly lower in untreated platelets derived from EDTA blood than from blood taken into Diatube-H, but this was not statistically significantly different except for blood taken into EDTA at time 0 ($P < 0.01$). With platelets derived from either source, thrombin stimulation resulted in a similar downregulation of GPIb expression ($P < 0.03$), and this was similar whether the platelets were fixed in paraformaldehyde immediately or at 1 or 2 hr after blood was withdrawn from the subject. Conversely, platelets taken into EDTA or Diatube-H showed an increase in GPIIbIIIa expression ($P < 0.03$) with thrombin stimulation (Fig. 4) and this was similar whether the sample was taken into Diatube-H or EDTA.

The effect of anticoagulant on other platelet activation markers was studied. As shown in Table I, with blood taken into Diatube-H or EDTA and processed immediately, there was a marked increase in platelet expression (mean channel fluorescence; MCF) of CD63 after thrombin stimulation; as for CD62P, there was greater spontaneous activation with untreated platelets derived from EDTA blood than from blood taken into Diatube-H, and fewer EDTA platelets than Diatube-H platelets expressed CD63 after thrombin stimulation but the difference was not statistically significant. As shown in Table I, the results were similar when the bloods were stored for four hours before processing.

Table II, shows the effect of thrombin stimulation on adhesion molecule expression on platelets from Diatube-H or EDTA blood processed immediately. Although all platelets from either source expressed CD49b at time 0 or at 4 hr, this was a low-intensity expression (MCF 6 ± 1) and did not change following exposure to thrombin. By contrast, CD29 expression decreased slightly and CD31 expression increased slightly after thrombin stimulation, but these changes were not statistically significant. Hence, there were no differences in adhesion marker expression related to anticoagulant and, under these conditions, thrombin stimulation did not significantly alter adhesion molecule expression. Results were similar for samples stored for 4 hr before processing. Tests for CD9 and CD36 expression yielded similar results (data not shown).

DISCUSSION

Diatube-H is a vacuum blood collection tube containing sodium citrate and citric acid anticoagulant, and the inhibitors of platelet aggregation, theophylline, adenosine, and dipyridamole. These inhibitors prevent the release of platelet factor 4 and β-thromboglobulin, early indicators of in vivo platelet activation [3]. The attributed

TABLE I. Percentage of Cells Expressing CD62P and CD63 Platelet Activation Markers Before and After Thrombin Stimulation, on Platelets Processed at Time 0 or After Storage for 4 Hr

	Diatube-H				EDTA			
	Time 0		4 hr		Time 0		4 hr	
	Untreated	Thrombin	Untreated	Thrombin	Untreated	Thrombin	Untreated	Thrombin
CD62P	14 ± 8	98 ± 1	7 ± 6	98 ± 1	75 ± 5	94 ± 3	65 ± 6	96 ± 1
CD63	6 ± 3	85 ± 4	6 ± 2	90 ± 3	17 ± 4	73 ± 7	19 ± 6	66 ± 8

TABLE II. Mean Channel Fluorescence of Labeled Monoclonal Antibodies Bound to Platelet Adhesion Molecules Before and After Thrombin Stimulation, at Platelets Processed at Time 0 or After Storage for 4 Hr

	Diatube-H				EDTA			
	Time 0		4 hr		Time 0		4 hr	
	Untreated	Thrombin	Untreated	Thrombin	Untreated	Thrombin	Untreated	Thrombin
CD49b	6 ± 1	6 ± 1	8 ± 1	9 ± 2	6 ± 1	6 ± 1	9 ± 2	10 ± 2
CD29	42 ± 6	28 ± 2	22 ± 3	37 ± 8	44 ± 4	35 ± 3	37 ± 8	36 ± 4
CD31	64 ± 18	88 ± 18	47 ± 11	54 ± 9	65 ± 15	78 ± 16	49 ± 10	54 ± 11

purpose of Diatube-H vacutainers is in obtaining samples for the monitoring of plasma heparin levels, testing platelet activation by platelet factor 4 and β-thromboglobulin assays, and tests for fibrinolysis [19].

We investigated the usefulness of Diatube-H in flow cytometric evaluation of platelet activation. Platelet surface glycoproteins, activation markers and adhesion molecules were quantitated and change in level of expression after thrombin-induced activation measured. Surface P-selectin (CD62P) expression was used as the primary measure of platelet activation [20]. The data indicate that, when using routine venipuncture procedures, if a "baseline" platelet activation up to 10% CD62P-positive platelets is considered acceptable, up to 4-hr sample storage time may be permitted prior to fixation and processing for samples taken into Diatube-H. If fixed with paraformaldehyde within 4 hr after the blood is drawn, the platelets remain inactivated for at least 48 hr. Similar observations were obtained with other markers of platelet activation. Adhesion molecules expressed on platelets were, in general, unaffected by storage in either Diatube-H or EDTA.

After storage in Diatube-H, platelets remain functional in that they are able to respond well (i.e., become activated) to thrombin stimulation. We harvest platelets from PRP for use in activation studies; therefore, the Diatube solution must be removed. Platelets from blood taken into EDTA were more easily spontaneously activated and have less functional reserve on exposure to thrombin (i.e., a lower differential increase in activation marker expression after thrombin treatment). The results indicate that washing out Diatube-H restores ability to activate the platelets. The data support the use of Diatube-H for short-term storage of platelets in flow cytometric studies of platelet activation and function when the samples cannot be processed immediately.

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