Prolonged Holding of Whole Blood at 22°C Does Not Increase Activation in Platelet Concentrates

Abstract

Objectives: Platelets prepared after holding of whole blood overnight at 22°C have a well-preserved metabolism. However, the possibility that such prolonged incubation with active granulocytes may increase platelet activation has not been fully tested. Methods: We investigated this possibility by flow cytometric analysis of membrane glycoproteins (GPs) Ib and IIb/IIIa and the activation markers CD62P and CD63 in platelet concentrates (PCs) prepared from whole blood that was held for either 6 h without cooling plates (n = 20) or for 24 h on cooling plates of 1,4-butanediol (n = 20). PCs were prepared by the platelet-rich plasma method and analyzed on the second storage day. Results: Platelet yield and aggregation response to ristocetin, collagen and epinephrine + ADP were similar in both types of PCs, as was the mean fluorescence intensity for GPs Ib and IIb/IIIa. PCs prepared by the overnight-hold method did not differ from those obtained 6 h after collection in the percentage of platelets expressing CD62P (12.3±6.2% vs. 14.1±4.0%; p>0.1) or CD63 (9.8±6.4% vs. 8.8±3.6%; p>0.1). Conclusion: Prolonged holding of whole blood at 22°C prior to component preparation does not increase the level of platelet activation.

Introduction

Rapid cooling of whole blood to 20–24°C followed by storage at room temperature for up to 24 h prior to component preparation has become a standard practice in most European countries [1, 2]. This ‘overnight-hold method’ solves the logistic problems associated with collection by distant mobile teams and allows the preparation of platelets from nearly all blood donations.

Previous studies have shown that the overnight-hold method does not significantly affect platelet yield and that platelets prepared using this method have a well-preserved metabolism, as assessed by recovery after the osmotic shock, 14C serotonin uptake and plasma pH and lactate dehydrogenase measurements [3–5]. However, little attention has been paid to the possibility that prolonged incubation of platelets with metabolically active granulocytes, as in whole blood stored at room temperature, results in higher levels of platelet activation. Polymorphonuclear granulocytes are more numerous in whole blood than in platelet concentrates (PCs), in which mononuclear leukocytes predominate. Granulocytes may become activated in citrated blood by complement fractions, ill-defined lipids or contact with foreign surfaces [6, 7]. Upon stimulation, granulocytes can activate platelets through the release of cathepsin G, other proteinases, superoxide and platelet-aggregating factor (PAF) [8].

Platelet activation in stored platelets, as measured by P-selectin expression, diminishes platelet recovery after transfusion [9, 10]. Levels of platelet activation increase with the
storage time of PCs, but these seem to be mostly determined by the activation produced during blood collection and processing [11–13]. If the overnight-hold method produces higher levels of platelet activation, this may become apparent at any time during platelet storage.

We have tested this hypothesis by flow cytometric assays of the activation-dependent markers P-selectin (CD62P) and 53-kD lysosomal glycoprotein (GP) (CD63) and the GPs Ib and IIb/IIIa on the surface of platelets stored for 2 days, after having been prepared by either the overnight-hold method or 6 h after blood collection.

Materials and Methods

Blood Collection and Processing

Whole blood was collected from healthy donors who had not taken any medication during the previous 7 days. Four hundred and fifty milliliters of venous blood were drawn in a triple-bag system with 63 ml citrate phosphate dextrose as anticoagulant (PL-1240; Fenwal Laboratories, Deerfield, Ill., USA). Immediately after collection, blood units were either placed on cooling plates of solid 1,4-butanediol (NPBI, Amsterdam, The Netherlands) and stored at room temperature for 24 h or held at room temperature without cooling plates for 6 h before component preparation. In both cases, PCs were prepared by the platelet-rich plasma (PRP) method. In brief, whole blood was centrifuged in a Sorvall RC-3B centrifuge (DuPont, Wilmington, Del., USA) at 1,000 g for 7 min at 22°C. The PRP was pressed into a satellite bag that was centrifuged at 5,000 g for 5 min at 22°C. All but 55 g of the supernatant platelet-poor plasma was removed, and the platelets were resuspended after a 1-hour rest period. PCs were stored in continuous horizontal agitation at 22°C (Helmer Laboratories, Fort Wayne, Ind., USA). On the second storage day, aliquots of each PC were obtained for platelet function and flow cytometry studies.

Platelet and WBC counts were done by a Technicon H1 System (Bayer Diagnostics, Tarrytown, NY, USA). Platelet yield was calculated as the percentage of total platelet content in the whole-blood unit that was recovered in the PC.

Platelet Aggregation Studies

Platelet aggregation studies were performed at 37°C in a Chrono-Log Aggro-Meter (Chronolog corporation, Broomall, Pa., USA) after adjusting the platelet count to 200,000 with autologous plasma. The response to the following agonists was assessed: ristocetin 0.8 mg/ml; collagen 5 μg/ml, and epinephrine 10 μM. Flow Cytometry Analysis

To avoid activation during further manipulations, samples of PCs (5 ml) were directly drawn into tubes containing 600 μl of 8% formaldehyde and maintained at 4°C. Thereafter, volume corresponding to 2×10^6 platelets was placed in separate tubes and washed twice with PBS, pH 7.2, containing 1% bovine serum albumin and 1% human AB plasma. After washing, platelet samples were incubated for 1 h at 22°C with primary antibodies anti-CD42, anti-CD41, anti-CD62P or anti-CD63. Anti-CD42 was purchased from Immunotech (AP1; Marseille, France), anti-CD41 was kindly provided by Dr. J. Kunicki (AP2; Blood Research Institute, Blood Center of Southeastern Wisconsin, Milwaukee, Wisc., USA), RUU 2.17 (anti-CD62P) and RUU 2.28 (anti-CD63) were generously donated by Dr. K. Nieuwenhuis (University Hospital of Utrecht, The Netherlands). After primary incubation, platelets were washed twice in 0.1 M PBS, pH 7.2, containing 1% bovine serum albumin and 1% human AB plasma and incubated for 30 min at 22°C with the secondary antibody (FITC-labeled goat anti-mouse IgG; Dako, Glostrup, Denmark). The excess of the secondary antibody was removed by washing with PBS.

Flow cytometric analysis of the labeled samples was performed in a FACScan (Beckton-Dikinson, Mountain View, Calif., USA) with the Lysis II program. Platelets were selected using FSC and SSC gates (size and complexity) and reading in FL1. We determined background fluorescence by using a nonspecific mouse IgG1 control as primary antibody. For mAbs anti-CD41 and anti-CD42, the binding was expressed as the mean fluorescence intensity. Because resting platelets do not bind anti-CD62P or anti-CD63, binding was expressed as the percentage of positive cells, using a threshold set above background fluorescence.

Statistical Methods

Means ± SD were used for reporting results. Comparisons between groups were done by the Mann-Whitney U test. All p values were two sided. Computations were done with the Biomedical Data Package.

Results

Forty PCs were analyzed: 20 prepared from whole blood processed by the overnight-hold method and 20 from blood processed 6 h after collection. There were no significant differences in hematocrit, WBC and platelet counts between both kinds of whole-blood units. The platelet yields in PCs prepared from these blood units were, respectively, 45.8±7.7% and 47.9±13%; p >0.1.

Results of platelet aggregation studies are shown in Table 1. As seen, platelets prepared by the overnight-hold method and those obtained 6 h after blood collection did not

| Table 1. Comparison of maximal aggregation of platelets prepared 6 h after collection or storage at 22°C for 24 h |
|---------------------------------|--------|--------|-------------------------------|
|                                 | ristocetin 0.8 mg/ml | collagen 5 μg/ml | epinephrine 10 μM + ADP 4 μM |
| Platelets prepared after 6 h (n = 20) | 79.4±12.2 | 66.5±23.1 | 83.5±12.8 |
| Overnight-hold method (n = 20) | 81.8±11.2 | 75.7±24.4 | 86.5±14.9 |
| p value | >0.1 | >0.1 | >0.1 |

Values are means ± SD.
Table 2. Mean fluorescence intensity for platelet membrane GPIb (CD42) and GPIIb-IIIa (CD41) in platelets prepared 6 h after collection or storage at 22°C for 24 h

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<tr>
<th></th>
<th>Anti-CD42</th>
<th>Anti-CD41</th>
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<tbody>
<tr>
<td>Platelets prepared after 6 h (n = 20)</td>
<td>179±47</td>
<td>148±39</td>
</tr>
<tr>
<td>Overnight-hold method (n = 20)</td>
<td>193±48</td>
<td>148±41</td>
</tr>
<tr>
<td>p value</td>
<td>&gt;0.1</td>
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Values are means ± SD.

Differ in their maximal aggregation response to ristocetin, collagen or epinephrine + ADP.

By flow cytometric analysis, platelets obtained by the two methods show no significant differences in surface density of both GPIb and GPIIb/IIIa (table 2). Figure 1 presents the percentage of platelets expressing the activation markers CD62P and CD63. Platelets prepared by the overnight-hold method did not differ from those obtained 6 h after collection in the expression of CD62P (12.3±6.2% vs. 14.1±4.0%; p>0.1) or CD63 (9.8±6.4% vs. 8.8±3.6%; p>0.1).

Discussion

Despite using a sensitive method and what seems to be the most appropriate timing, our study failed to find that platelets prepared following the overnight-hold method were more activated than those obtained 6 h after blood collection. Platelet function was also well preserved, as assessed by aggregation in response to collagen, ristocetin and ADP + epinephrine.

With the advent of modern plastic containers and conservation methods for PCs, which maintain pH levels at or near 7.0 up to the end of the storage period, platelet activation that occurs during blood processing and PC storage has become an important determinant of posttransfusion recovery [12]. Platelet activation can be measured by flow cytometric assaying of surface P-selectin, CD63 (a 53-kD GP from the lysosomal granules) and GPs Ib and IIb/IIa, which has proved to be a simple and sensitive method [11]. Expression of P-selectin, CD63 and GP IIb/IIa increases during the storage of PCs [12, 13], whereas membrane GP Ib decreases with storage time [12, 14]. Surface expression of these activation markers correlates with poor posttransfusion recoveries and with other assays of platelet activation, such as β-thromboglobulin release [11]. Therefore, their measurement by flow cytometry provides a sensitive and meaningful way to investigate whether the overnight-hold method produces a significant increase in platelet activation.

In addition, we have chosen to study PCs obtained by the PRP method on the assumption that any degree of platelet activation that may have occurred during the blood holding at room temperature would be greatly amplified by the high-speed centrifugation of PRP. This centrifugation forms pellets of platelets on the bottom of the container, where they are in close cell-to-cell contact, a condition that favors the activation of resting platelets by products released from previously activated cells. In fact, platelets prepared from PRP are usually more activated than those derived fromuffy coats [15]. While activation during storage is a cumulative phenomenon that increases with storage time [11], the effect of the platelet preparation method disappears after the
second day [15]. Therefore, it seems that the second storage day after preparing PCs from PRP would be the most appropriate time to notice any supplementary activation due to the overnight-hold method.

Concern about platelet activation during prolonged storage of whole blood at room temperature arises from the realization that, under such conditions, platelets are incubated with a large number of metabolically active granulocytes. If these latter cells become stimulated, they can activate platelets through the release of reactive oxygen species, proteinases and PAF [8]. Failure to find higher levels of platelet activation in PCs obtained after the overnight-hold method indicates that granulocytes either do not become activated in such conditions or fail to activate platelets. Granulocyte-mediated platelet activation requires close cell-to-cell contact since proteinases released by granulocytes are quickly inactivated by plasma inhibitors [16], and PAF remains mostly attached to the granulocyte membrane [17]. Furthermore, reactive oxygen species have a very short life and may be rapidly degraded by the RBC superoxide dismutase and catalase. In whole blood, RBCs predominate over granulocytes and platelets in a proportion of about 1250:1 and 25:1, respectively. Therefore, it is plausible that RBCs may play a protective role in granulocyte-dependent platelet activation in blood components by hampering the physical contact between platelets and granulocytes.

In conclusion, the present study shows that the overnight-hold method does not increase the level of platelet activation, as measured by the surface expression of P-selectin, 53-kD lysosomal GP and GPs Ib and IIB/IIIa in PCs.

Acknowledgement

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References