Measurement of reticulated platelets by simple flow cytometry: An indirect thrombocytopoietic marker

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Abstract

Background: The aim of this study was to determine whether measurement of reticulated platelets (RP) by flow cytometry directly from whole blood, with no fixation or manipulation, is as useful a thrombocytopoietic marker as other more complex techniques.

Methods: RP percentage was prospectively assessed in thrombocytopenic patients (platelets <100×10⁹/L) and non-thrombocytopenic controls using a direct, whole-blood, dual-labelling flow cytometric method. Direct, whole-blood double coverage was achieved using a monoclonal antiglycoprotein (GP)-III antibody (CD61-PerCP®) for platelet identification and thiazol orange (Retic-count®) as platelet mRNA stain. After establishing thrombocytopoiesis etiology, patients were grouped according to whether their rate of thrombopoiesis was increased or decreased.

Results: RP were measured in 53 thrombocytopenic patients with several etiologies and in 53 non-thrombocytopenic controls. The mean RP in 14 thrombocytopenic patients with no increased thrombopoietic activity was 4.8% (95% CI: 3.2–6.4) and the RP absolute number was 1.98×10⁹/L (95% CI: 1.3–2.6). The mean RP in 17 thrombocytopenic patients with increased thrombopoietic activity was 29.4% (95% CI: 24.7–34.1) and the RP absolute number was 7.24×10⁹/L (95% CI: 4.9–9.5).

Conclusions: RP measurement by flow cytometry, directly from whole blood without manipulation, is a useful screening test to differentiate thrombocytopenia with high or low thrombopoietic activity.

Keywords: Reticulated platelets; Thrombocytopenia; Idiopathic thrombocytopoietic purpura; Flow cytometry

1. Introduction

Reticulated platelets (RP), by analogy to reticulocytes, are the youngest circulating platelet population and they contain abundant amounts of mRNA. RP were first described in 1969 [1] by direct visualization from peripheral blood. In 1990, Kienast and Schmitz [2] reported the first measurement of RP by flow cytometry that showed clinical utility in thrombocytopenic disorders. Since that first description, two substrates have been reported for RP determination: platelet-rich plasma (PRP) and whole blood. Several dyes for mRNA stains, including thiazole orange (TO) and auramine O, have been used and different incubation times have been described. Nevertheless, none of these techniques has been standardized. All reports have suggested that flow cytometric analysis of RP is useful for the differential diagnosis of thrombocytopoietic disorders. Recently, simple protocols using whole blood for RP determination have been reported [3,4] with results as good as those from more complex techniques using PRP.

The purpose of this prospective study was to evaluate whether RP measured directly from whole blood by flow cytometry, and avoiding sample manipulation, is as useful an
indirect thrombocytopenic peripheral marker as other more complex techniques.

2. Materials and methods

2.1. Subjects

We prospectively investigated in- and outpatients with thrombocytopenia at Parc Taulí Hospital in Sabadell who met the inclusion criterion, i.e., thrombocytopenia <100×10⁹/L, confirmed after peripheral blood film review, and who could be included in one defined etiologic group. The exclusion criterion was pseud thrombocytopenia after peripheral blood film review. Also excluded were patients who could not be included in one defined etiologic group.

Controls and thrombocytopenic etiologic groups were defined as follows:

Group A. Thrombocytopenia with normal or decreased thrombopoietic activity.

A1. Central thrombocytopenia when there was diminished or defective platelet production. Diagnoses included in this group were: acute or chronic lymphoproliferative disorders, multiple myeloma, acute leukemia, aplastic anemia, myelodysplastic disorders, and medullar infiltration by solid tumors, based on blood results, bone marrow findings, and cytogenetic analysis. Post-chemotherapy thrombocytopenia was defined as thrombocytopenia after chemotherapy treatment that persisted for more than 7 days after RP determination.

A2. Thrombocytopenia by abnormal distribution when there were splenomegaly and hypersplenism analytical data. Diagnoses included in this group were liver cirrhosis and other diseases with portal venous system hypertension.

Group B. Thrombocytopenia with increased thrombopoietic activity.

B1. Thrombocytopenia due to enhanced peripheral platelet destruction by an immunological mechanism. This included acute idiopathic thrombocytopenic purpura (ITP), a diagnosis that was made on the basis of clinical history, physical examination, complete blood count, and clinical course after treatment. All patients with ITP fulfilled the criteria advised by the American Society of Hematology Practice Guidelines [5].

B2. Thrombocytopenia due to enhanced peripheral platelet destruction by no immunological mechanism. Diagnoses included were: disseminated intravascular coagulation (DIC), idiopathic thrombotic thrombocytopenic purpura, and hemolytic-uremic syndrome, all defined by classical clinical symptoms and confirmed in blood studies.

Group C. Chronic thrombocytopenia for more than 6 months.

C1. Chronic thrombocytopenia with no clear etiology. Thrombocytopenia for more than 6 months with neither significant bleeding symptoms nor autoimmunity data, if there is no definite diagnosis.

C2. Chronic autoimmune thrombocytopenia. Thrombocytopenia for more than 6 months with some autoimmune marker (antiplatelet antibodies, antinuclear antibodies, anticardiolipin antibodies, or lupus anticoagulant).

Group D. Control groups.

D1. Normal control group: those with a normal platelet number in blood sample.

D2. Thrombocytosis control group: those with platelets above 450×10⁹/L.

2.2. Methods

RP were identified following the previously described technique by Robinson et al. [3], without sample manipulation, avoiding fixation and blood centrifugation. Briefly, blood was collected in hematological tubes (Vacutainer type) containing ethylene diamine tetra acetic acid (EDTA)-2K. All blood samples were kept at room temperature until analysis was performed, less than 6 h after collection. Some 5 μl of whole blood was incubated for 15 min in the dark at room temperature with 5 μl of PerCP®-labelled antiglycoprotein III monoclonal antibody (CD61-PerCP® Becton Dickinson SA) and 30 μl of phosphate-buffered saline (PBS). A control tube was used for each sample with 5 μl of isotypic mouse control (IgG1-mouse PerCP® Becton Dickinson SA). After incubation, 1 ml thiazole orange (TO; Retic-count®, Becton Dickinson SA) 1/10 solution in Isoton II® was added to the test tube and 1 ml Isoton II® (Beckman-Coulter) solution was added to the control tube. After incubation for 1 h in the dark at room temperature, they were immediately read in the cytometer (FACSCalibur, Becton Dickinson SA). Platelets were identified on their logarithmic side scatter (SSC) and CD61 positivity expression. Analysis was performed with computer software (CellQuest Pro, Becton Dickinson SA). A dot plot cytogram (CD61-PerCP® versus TO fluorescence) was generated, and the RP rate was expressed as a percentage of both a TO and CD61-PerCP®-positive population among 10,000 identified platelets. The threshold of TO fluorescence was chosen so that more than 99% of the CD61-PerCP®-positive population was negative for TO. In each session, a sample with a normal number of platelets was used as a control.

2.3. Statistical analysis

RP percentage and total RP number were determined in all samples. Results are expressed as means with 95% confidence interval (CI). Differences between groups were compared by means of the Student’s t-test. A P value below 0.05 was considered significant. SPSS 11.5 for Windows computer software was used for the statistical analysis.

3. Results

RP were determined in 53 controls. Forty-four had a normal platelet count and 9 had thrombocytosis. There were 53 thrombocytopenic patients. In group A, 12 had central thrombocytopenia (4 myelodysplastic disorders, 3 bone marrow...
infiltration, 2 multiple myeloma IgG-κ, 1 aplastic anemia, 1 post-chemotherapy, and 1 acute promyelocytic leukemia) and 2 were secondary to hypersplenism. In group B, 13 had ITP and 4 thrombocytopenia due to enhanced platelet destruction by a non-immunological mechanism (3 DIC and 1 hemolytic-uremic syndrome). In group C, 8 had chronic autoimmune thrombocytopenias and 14 chronic thrombocytopenias with no clear etiology. Sex and age for each group in which RP were determined is shown in Table 1. Mean peripheral platelets in each group, RP percentage, and RP absolute number with 95% CI are presented in Table 2. A comparative analysis of thrombocytopenia with increased thrombopoietic activity (group B) and thrombocytopenia with low or normal thrombopoietic activity (group A) showed significant differences in RP percentage (\(P < 0.0001\)) and RP absolute number (\(P < 0.0001\)). The highest RP percentage was present in ITP. RP in group C were slightly higher than in the central group, but clearly lower than in group B (Fig. 1).

4. Discussion

We measured RP using a very simple technique. We determined RP by flow cytometry directly from whole blood, avoiding any manipulation, and a dual-labelling method for platelet identification. Our preliminary data in this small thrombocytopenic group allowed us to discriminate between thrombocytopenia due to increased thrombopoietic activity and thrombocytopenia with normal or decreased thrombopoietic activity. Reference range values with this simple technique are in agreement with those reported in the literature [2,6,7].

RP measurement is a non-invasive test that provides indirect information about thrombopoietic activity in bone marrow. Even though RP values are related to the sort of technique utilized, it is generally agreed that they are a good marker of platelet turnover [2,6,8,9]. RP measured from

Table 1
Demographic data of thrombocytopenic and control groups

<table>
<thead>
<tr>
<th>Groups</th>
<th>No.</th>
<th>Age (years) Mean (range)</th>
<th>M/F</th>
<th>(% males)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal controls</td>
<td>44</td>
<td>55 (3–81)</td>
<td>25/19 (56.8%)</td>
<td></td>
</tr>
<tr>
<td>Central thrombocytopenia</td>
<td>12</td>
<td>70 (50–83)</td>
<td>8/4 (66.7%)</td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>13</td>
<td>40 (3–84)</td>
<td>5/8 (38.5%)</td>
<td></td>
</tr>
<tr>
<td>Chronic immunological thrombocytopenia</td>
<td>8</td>
<td>52 (11–78)</td>
<td>5/3 (62%)</td>
<td></td>
</tr>
<tr>
<td>Peripheral platelet destruction by a non-immunological mechanism</td>
<td>4</td>
<td>55 (8–91)</td>
<td>3/1 (75%)</td>
<td></td>
</tr>
<tr>
<td>Hypersplenism</td>
<td>2</td>
<td>62 (58–65)</td>
<td>2/0 (100%)</td>
<td></td>
</tr>
<tr>
<td>Chronic thrombocytopenia without clear etiology</td>
<td>14</td>
<td>56 (28–82)</td>
<td>3/11 (21.4%)</td>
<td></td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>9</td>
<td>65 (17–89)</td>
<td>6/3 (66.7%)</td>
<td></td>
</tr>
</tbody>
</table>

ITP, acute idiopathic thrombocytopenic purpura.

Table 2
RP percentage and absolute number in each group analyzed

<table>
<thead>
<tr>
<th>Group</th>
<th>(no)</th>
<th>Platelets (×10^9/L)</th>
<th>RP % (95% CI)</th>
<th>RP×10^9/L (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group D</td>
<td>53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal control</td>
<td>(44)</td>
<td>250.30</td>
<td>1.19 (1.01–1.38)</td>
<td>2.92 (2.48–3.56)</td>
</tr>
<tr>
<td>Thrombocytosis</td>
<td>(9)</td>
<td>922.25</td>
<td>1.8 (1.20–2.41)</td>
<td>17.86 (11.93–23.80)</td>
</tr>
<tr>
<td>Group A</td>
<td>14</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Central thrombocytopenia</td>
<td>(12)</td>
<td>46</td>
<td>4.85* (3.21–6.49)</td>
<td>1.98* (1.33–2.63)</td>
</tr>
<tr>
<td>Hypersplenism</td>
<td>(2)</td>
<td>74.5</td>
<td>2.04 (0.96–3.12)</td>
<td>1.52 (0.85–2.20)</td>
</tr>
<tr>
<td>Group B</td>
<td>17</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ITP</td>
<td>(13)</td>
<td>22.10</td>
<td>29.41* (24.72–34.10)</td>
<td>7.24* (4.97–9.51)</td>
</tr>
<tr>
<td>Peripheral platelet destruction by a non-immunological mechanism</td>
<td>(4)</td>
<td>49</td>
<td>30.67 (24.57–36.76)</td>
<td>5.58 (3.90–7.25)</td>
</tr>
<tr>
<td>Group C</td>
<td>22</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic immunologic thrombocytopenia</td>
<td>(8)</td>
<td>54</td>
<td>6.38 (4.83–7.93)</td>
<td>3.65 (2.71–4.60)</td>
</tr>
<tr>
<td>Chronic thrombo-cytopenia without clear etiology</td>
<td>(14)</td>
<td>63.21</td>
<td>5.41 (3.41–7.28)</td>
<td>3.39 (2.18–4.60)</td>
</tr>
</tbody>
</table>

\(^*P < 0.0001, \#P < 0.0001\).

RP, reticulated platelets; ITP, acute idiopathic thrombocytopenic purpura.
platelet-rich plasma is one of the six laboratory findings that discriminated ITP from other diagnoses in isolated thrombocytopenia [10]. RP determination appears to be a reliable measure to predict imminent platelet recovery in hematologic patients after intensive chemotherapy [11]. RP measurement is not routinely used despite its potential clinical interest for thrombopoietic evaluation. This is due to its technical complexity, variability, and lack of standardization. Simplifying this technique or making it fully automatic [12] would clearly extend its clinical use.

In sum, RP determination is a reliable measure of platelet turnover and may be as good an initial screening test for thrombocytopenic patients as reticulocytes are in studies of anemic patients.

5. Learning points

1. Reticulated platelets may be measured by simple flow cytometry directly from whole blood.
2. Reticulated platelets are an indirect peripheral marker of thrombopoietic bone marrow activity.
3. Determination of reticulated platelets may be as useful an initial screening test in thrombocytopenic patients as reticulocytes are in studies of anemic patients.

Reference

[2] Kienast J, Schmitz G. Flow cytometric analysis of thiazole orange uptake by platelets: a diagnostic aid in the evaluation of thrombocyto-