A Clinical Evaluation of High Fluorescent Platelet Fraction Percentage in Thrombocytopenia

Thomas S. Kickler, MD, Sinichiro Oguni, and Michael J. Borowitz, MD, PhD

Key Words: Platelets; Thrombocytopenia; Cell analyzers

Abstract

We evaluated an automated method (XE-2100 automated blood cell counter equipped with a laser and upgraded software, Sysmex, Kobe, Japan) to quantify the high fluorescent platelet fraction percentage (HFPF%) as an indicator of platelet production. The mean HFPF% value was 3.1 in 80 healthy subjects (95% confidence interval, 2.8%-3.5%). The coefficient of variation was less than 10% for elevated values and 10% to 20% for normal values. Sample stability was up to 48 hours. We tested the HFPF% in 171 patients with thrombocytopenia. The highest values were found in patients with autoimmune thrombocytopenia and disseminated intravascular coagulation, although HFPF% also was increased in patients with regenerating marrows. In other patient groups with decreased platelet production, the HFPF% was within the normal range.

The HFPF% was predictive in the evaluation of thrombocytopenia. We found elevated HFPF% values in disorders associated with increased platelet production, particularly associated with platelet destruction, and normal values in disorders associated with decreased platelet production. The results are precise: testing can be performed as readily as a CBC count using an EDTA-anticoagulated blood sample.

In the evaluation of thrombocytopenic patients, characterization of the pathophysiologic process leading to thrombocytopenia is important diagnostically. The primary pathologic processes to be distinguished are platelet consumption and decreased platelet production. Increased platelet destruction can be a consequence of autoimmune thrombocytopenia, disseminated intravascular coagulation (DIC), sepsis, and microangiopathic disorders. In these consumptive thrombocytopenic disorders, thrombopoietin production is increased, leading to increased platelet production by megakaryocytes. As a result, newly formed platelets are released into the circulation. As first shown by Ingram and Coopersmith, these platelets contain residual amounts of megakaryocyte-derived RNA. These early studies used the term reticulated platelets, analogous to reticulocytes, a marker of increased erythropoiesis. Subsequent reports showed that flow cytometry could be used to measure platelets with increased RNA by using RNA binding dyes, most often thiazole orange.

Clinical studies of thrombocytopenic patients using thiazole orange staining and flow cytometry showed that the percentage of reticulated platelets may increase in consumptive disorders and decrease or remain normal when marrow suppression is present. However, this method is not easily integrated into clinical practice because it not only requires a flow cytometer but also demands precise manual preparatory steps for reproducible staining. Even with optimal care in preparing the sample, large variability in reference ranges and imprecision in test results remain. It would be ideal to have a simple method for measuring these RNA-containing platelets that used a standard cell analyzer in use in the laboratory, rather than requiring specialized equipment.
We evaluated a method that measures the high fluorescent
platelet fraction percentage (HFPF%), which was derived as
an extension of a standard method used to measure reticulo-
cytes and reticulocyte fractions on the XE-2100 automated
hematology analyzer (Sysmex, Kobe, Japan). The method per-
mits the staining of platelet RNA and its analysis in whole
blood without preparatory steps.7,8 We report our results in
healthy subjects and in patients with a variety of thrombocy-
topenic disorders.

Materials and Methods

HFPF Percentage

We used the XE-2100 automated hematology analyzer to
measure the HFPF%. The proprietary and US Food and Drug
Administration–licensed name for this parameter is the IPF%,
an abbreviation for immature platelet fraction percentage.
This analyzer uses optical properties and a proprietary fluores-
cent polymethine dye and fluorescent flow cytometry to sepa-
rate platelets and reticulocytes from other cells and from each
other. The analyzer is equipped with software that arbitrarily
but reproducibly defines highly fluorescent platelets as those
with the highest 3% intensity of fluorescence.

Patients

We tested blood samples collected into K2 EDTA tubes
(Becton Dickinson, Franklin Lakes, NJ). The Johns Hopkins
University Investigational Review Board (Baltimore, MD)
approved the study. To measure the reference range for the
HFPF%, we obtained 80 blood samples from healthy adult
subjects with normal CBC counts.

For the different study populations, we chose samples
from patients seen for evaluation of thrombocytopenia in the
hematology clinic or inpatient consultative service. We cate-
gorized the cause of thrombocytopenia with the attending
hematologist’s diagnosis and used this diagnosis to assess the
test performance. The diagnostic criteria for autoimmune
thrombocytopenia were those recommended in the American
Society of Hematology practice guidelines.9 In addition, all
blood samples from patients with autoimmune thrombocy-
topenia also had positive antiplatelet glycoprotein antibody
results, as determined by an enzyme-linked immunosorben-
t assay for antibodies to platelet glycoproteins using a kit (GTI,
Milwaukee, WI).

Patients with DIC had acquired coagulopathy in a clinical
setting of sepsis and demonstrated elevated D dimers (≥5
µg/mL). Patients in the chemotherapy group were those receiv-
ing myeloablative chemotherapy for hematologic malignant
neoplasms and generally underwent sampling near the nadir
of the platelet count. Patients in the cancer group had malignant
neoplasms that were treated with nonmyeloablative therapy. The
patients with aplastic anemia/paroxysmal nocturnal hemoglo-
binuria were not receiving nonmyeloablative therapy.

Results

Because accurate measurement of the HFPF% is depend-
ent on the optical platelet count, we first compared optical
platelet counts with impedance platelet counts in 171 blood
samples from patients with thrombocytopenia. We found
excellent correlation between the measurements in thrombo-
cytopenic patients. Figure 1.

We tested the precision of the HFPF% by testing 8 patients
and healthy subjects 10 times and calculated the coefficient of
variation. The reproducibility of the assay was relatively good,
with coefficients of variation (CVs) ranging from 4.9% to 22.1%
(Table 1). As might be expected, patients with lower HFPF% val-
ues had higher CVs, whereas patients with elevated HFPF% val-
ues all had CVs less than 8%. We also tested the 48-hour stabil-
ity of the test results for 8 samples. In room temperature–stored
blood samples, there was no change during this period.

The reference range was measured in 80 healthy subjects,
18 men and 62 women, whose ages ranged from 24 to 57 years.
The subjects all had normal blood counts and no history of blood
dyscrasias. The mean value of the HFPF% was 3.1%, and a reference range (± 2 SD) was approximately 0% to 6%. The HFPF% values for thrombocytopenic patients are shown in Table 2. Figure 2 shows the distribution of the HFPF% values for different diagnostic groups and healthy subjects.

The patient groups in Table 2 were organized further to compare those with increased destruction (autoimmune thrombocytopenia and DIC) with those with marrow reconstitution (following myeloablative chemotherapy or bone marrow transplantation) and with those with marrow suppression. For the latter group, we included patients with aplastic anemia/paroxysmal nocturnal hemoglobinuria along with patients with solid tumors and thrombocytopenia; none of the latter patients was receiving myeloablative therapy.

Except for patients with solid tumors, all thrombocytopenic patient groups had elevated levels of HFPF% compared with levels in healthy subjects. However, the highest levels of HFPF% were seen in patients with autoimmune thrombocytopenia and DIC, with the very highest values seen in autoimmune thrombocytopenia. The 37 patients with autoimmune thrombocytopenia were adults who were receiving steroid therapy. The patients with DIC were being treated for sepsis or postoperative infection. None of these patients had a history of underlying blood dyscrasias.

As a group, patients with reconstituting marrows, after chemotherapy or bone marrow transplantation, also had elevated HFPF% values compared with the values in healthy subjects. However, both groups of patients, alone or combined, had significantly lower values than patients with autoimmune thrombocytopenia or DIC (P < .0001; Mann-Whitney U test). Patients with solid tumors did not have HFPF% values significantly different from those of healthy subjects, and overall,

### Table 1

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Coefficient of Variation (%)</th>
<th>× 10^3/µL (× 10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal CBC count</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>7.46</td>
<td>0.36</td>
<td>7.0</td>
<td>8.0</td>
<td>4.9</td>
<td>292 (292)</td>
</tr>
<tr>
<td>2</td>
<td>3.58</td>
<td>0.45</td>
<td>2.9</td>
<td>4.2</td>
<td>12.5</td>
<td>223 (223)</td>
</tr>
<tr>
<td>3</td>
<td>1.63</td>
<td>0.25</td>
<td>1.3</td>
<td>2.2</td>
<td>15.6</td>
<td>265 (265)</td>
</tr>
<tr>
<td>4</td>
<td>1.12</td>
<td>0.18</td>
<td>0.8</td>
<td>1.4</td>
<td>16.2</td>
<td>396 (396)</td>
</tr>
</tbody>
</table>

Abnormal CBC count

<table>
<thead>
<tr>
<th>Case No.</th>
<th>Mean</th>
<th>SD</th>
<th>Minimum</th>
<th>Maximum</th>
<th>Coefficient of Variation (%)</th>
<th>× 10^3/µL (× 10^9/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>16.20</td>
<td>1.15</td>
<td>14.1</td>
<td>18.5</td>
<td>7.1</td>
<td>86 (86)</td>
</tr>
<tr>
<td>2</td>
<td>11.73</td>
<td>0.63</td>
<td>10.5</td>
<td>12.4</td>
<td>5.4</td>
<td>100 (100)</td>
</tr>
<tr>
<td>3</td>
<td>3.66</td>
<td>0.81</td>
<td>2.1</td>
<td>4.6</td>
<td>22.1</td>
<td>29 (29)</td>
</tr>
<tr>
<td>4</td>
<td>1.34</td>
<td>0.18</td>
<td>1.0</td>
<td>1.6</td>
<td>13.3</td>
<td>713 (713)</td>
</tr>
</tbody>
</table>

* Cases 1 and 2 were patients with autoimmune thrombocytopenia, case 3 was a patient undergoing chemotherapy and bone marrow suppression, and case 4 was a patient with essential thrombocytopoiesis.

### Table 2

<table>
<thead>
<tr>
<th>Subject</th>
<th>No. of Cases</th>
<th>Mean</th>
<th>SD</th>
<th>Range</th>
<th>95% Confidence Interval</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Healthy</td>
<td>80</td>
<td>3.1</td>
<td>1.6</td>
<td>1.0-7.0</td>
<td>2.8-3.5</td>
<td>—</td>
</tr>
<tr>
<td>Destruction</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATP</td>
<td>37</td>
<td>15.0</td>
<td>7.1</td>
<td>3.6-35.4</td>
<td>12.7-17.2</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>DIC</td>
<td>25</td>
<td>9.5</td>
<td>5.2</td>
<td>2.3-18.6</td>
<td>7.5-11.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>All destruction</td>
<td>62</td>
<td>12.8</td>
<td>6.9</td>
<td>2.3-35.4</td>
<td>11.0-14.5</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Recovery</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>79</td>
<td>4.1</td>
<td>2.0</td>
<td>1.2-10.9</td>
<td>3.6-4.5</td>
<td>.001</td>
</tr>
<tr>
<td>BMT</td>
<td>8</td>
<td>8.0</td>
<td>4.2</td>
<td>4.3-15.3</td>
<td>5.1-10.8</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>All recovery</td>
<td>87</td>
<td>4.4</td>
<td>2.5</td>
<td>1.2-15.3</td>
<td>3.9-4.9</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Suppression</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA/PNH</td>
<td>3</td>
<td>6.1</td>
<td>2.5</td>
<td>3.9-8.8</td>
<td>3.2-8.9</td>
<td>.019</td>
</tr>
<tr>
<td>Cancer</td>
<td>16</td>
<td>3.8</td>
<td>2.0</td>
<td>0.8-7.1</td>
<td>2.8-4.7</td>
<td>NS</td>
</tr>
<tr>
<td>All suppression</td>
<td>19</td>
<td>4.1</td>
<td>2.2</td>
<td>0.8-8.8</td>
<td>3.1-5.1</td>
<td>.05</td>
</tr>
</tbody>
</table>

ATP, autoimmune thrombocytopenia; AA/PNH, aplastic anemia/paroxysmal nocturnal hemoglobinuria; BMT, bone marrow transplantation; DIC, disseminated intravascular coagulation; NS, not significant.

* P values are vs healthy. Mann-Whitney U test, 2-sided. In addition to the P values given, the high fluorescent platelet fraction percentage was significantly higher (P < .0001) in patients with platelet destruction (ie, ATP or DIC) than in thrombocytopenic patients with recovery marrows or in those with suppressed marrows (P < .0001). Patients with ATP had higher high fluorescent platelet fraction percentage values than did patients with DIC (P = .002).
patients with marrow suppression had significantly lower HFPF% values than seen among patients with platelet destruction. At a cutoff value of 6.5%, the HFPF% was 79% sensitive and 89% specific for distinguishing platelet destruction from suppression. Values more than 9.0% were 100% specific for being found in patients with increased platelet destruction. At this level, all patients with DIC had elevated HFPF% values, so the sensitivity was 100%, whereas in the autoimmune thrombocytopenic patients, 4 of 37 values were not elevated.

**Figure 3** shows serial HFPF% values for several days in 1 patient in relation to the platelet count and mean platelet volume (MPV). As the platelet count increased to normal, the HFPF% decreased to normal. During this decline in HFPF%, the MPV remained unchanged. These results are typical of patients we studied and suggest that increased platelet staining by the dye is not merely a result of an increase in MPV during the thrombocytopenic period.

**Discussion**

In thrombocytopenic patients, a clinically useful method is needed to differentiate impaired platelet production from increased platelet destruction. A variety of approaches have

**Figure 2** The distribution of the high fluorescent platelet fraction percentages is shown for 80 patients with normal CBC counts and 168 thrombocytopenic patients: 37 patients with chronic autoimmune thrombocytopenia (ATP), 3 patients with aplastic anemia/paroxysmal nocturnal hemoglobinuria (AA), 8 bone marrow transplant (BMT) recipients, 79 patients receiving chemotherapy (Chemo), 25 patients with disseminated intravascular coagulation (DIC), and 16 untreated cancer patients with solid tumors (Cancer).

**Figure 3**

**A**, Serial high fluorescent platelet fraction percentages compared with the platelet count. The dotted line shows the mean normal range of the immature platelet fraction percentage. **B**, The mean platelet volume (MPV) and the platelet hematocrit, the product of the platelet count and MPV. This increases with recovery of the platelet count, whereas the MPV stays relatively constant.
been used for this purpose, although they have shortcomings. These include MPV, bone marrow examination for identification of normal or increased megakaryocytes, isotopically labeled platelet survival studies, and plasma glycoalcalcin. An increased MPV may indicate increased platelet production. However, even though this parameter is measured routinely on most current hematology instruments, the MPV cannot be measured accurately in the setting of severe thrombocytopenia and might not be available because the analyzer might not be able to derive the value because of the platelet histogram distribution. The other methods lack the simplicity of a direct marker of increased platelet turnover. For these reasons, there is significant interest in having a platelet parameter analogous to the reticulocyte count that is as simple and precise to perform.

In 1969, Ingram and Coopersmith demonstrated increased RNA in canine platelets after acute blood loss. They used methylene blue to stain platelets in these studies and designated positively stained platelets as reticulated platelets. Subsequently, Kienast and Schmitz showed that human platelets could be stained with thiazole orange and analyzed by flow cytometry in a manner similar to well-established methods for reticulocytes. They showed that thiazole orange–positive platelets correlated with the clinical diagnosis of increased platelet consumption or decreased production. Ault and coworkers subsequently extended these studies in different clinical settings, giving additional support to the earlier studies.

Dale and coworkers provided the first direct evidence that thiazole-orange staining of platelets could be used as a marker for newly synthesized platelets in dogs. This group showed that newly synthesized platelets are thiazole orange–positive and remain positive for less than 24 hours. Despite these findings, thiazole orange platelet analysis has not become a standard hematologic parameter for the estimation of thrombopoiesis. Difficulty in standardizing the assay by providing a cutoff threshold for positivity, absence of a standard against which to assess accuracy, and wide variation in normal ranges and interlaboratory precision have been concerns.

The method we evaluated is well standardized to measure the HFPF% using an RNA polymethine dye similar to thiazole orange. The XE-2100 analyzer is equipped with software that arbitrarily but reproducibly defines highly fluorescent platelets as those with the highest 3% intensity of fluorescence. It is important to note that the normal range that we observed closely corresponds to that reported by Briggs and coworkers who used the same method that we used. This suggests that the measurement will have good interlaboratory correlation when performed as described. This is in contrast with the highly variable normal range for determining reticulated platelet percentages using thiazole orange and flow cytometry.

With this method, we showed that patients with thrombocytopenia caused by platelet destruction have very high levels of HFPF% and that these are much higher than those seen in healthy subjects or in cancer patients with thrombocytopenia not receiving myeloablative therapy, a population we considered to have decreased marrow production. Elevations in HFPF% values also are seen in patients with regenerating marrows following chemotherapy or marrow transplantation, although these levels were significantly lower than those seen in platelet destruction.

Although our findings support the value of HFPF% values for distinguishing among causes of thrombocytopenia, our study has some potential limitations. First, we do not provide direct evidence of a relationship between the HFPF% and the rate of platelet production or platelet maturity. Experimental studies, similar to those done by Dale and coworkers, would be a more direct way to relate platelet staining with rate of platelet production or maturity.

Because we used the clinical diagnosis to assign patients to categories typically associated with increased or decreased production, it might be argued that our study is flawed because the clinical classifications we used were imprecise or variable. Although we agree that using patients with cancer and thrombocytopenia not receiving myeloablative chemotherapy is something of an assumption, we simply did not have enough patients with aplastic anemia and documented marrow suppression to form a reasonably sized cohort. However, our major conclusions are about the very high levels of HFPF% in destructive thrombocytopenia, and our classification of patients with autoimmune thrombocytopenia and DIC is likely accurate, given that experienced clinicians used standard criteria for diagnosis. Also in support of our conclusions is the fact that our results agree largely with observations by others using the same method in a different study population and using similar diagnostic criteria. It also should be noted that these investigators referred to the parameter as the immature platelet fraction. Because we have no direct proof of this assertion pending further experimental studies, we refer to the parameter as the HFPF%.

Another potential concern about the HFPF% is that increased staining simply might reflect the greater uptake of larger platelets as might occur with increased thrombopoiesis. Although this cannot be excluded completely in some cases, when we observed selected patients over time, we saw a decrease in the HFPF% as the platelet count increased to the normal range, whereas the MPV remained unchanged.

A final possible concern is the issue of the specificity of nucleic acid dyes in platelets. Others have shown variable reduction of staining with polymethine dyes such as thiazole orange after RNase treatment of platelets. Failure by others to completely block staining in these experiments does not prove that polymethine dyes are nonspecific. The variable
decrease in reported RNA staining after RNase treatment may result from poor uptake of RNase into platelets or lack of activity of the enzyme once taken up into platelets. These types of experiments were beyond the scope of our clinical studies and may be useful to perform once standardized experimental methods are developed.

There is evidence in autoimmune thrombocytopenia that impaired thrombopoiesis may be present and that this is associated with refractoriness to therapy.16 It may be interesting in the future to study these patients to determine whether the HFPF% correlates with refractoriness to treatment. It may be possible to categorize patients with disease refractory to therapy because of impaired thrombopoiesis vs because of ongoing platelet destruction at a rate that thrombopoiesis cannot compensate sufficiently. Subsequently, one may be able to use this information to guide therapy for patients with refractory autoimmune thrombocytopenia.

The simplicity of the measurement, along with its availability in an automated mode on a cell counter, makes the measurement of HFPF% potentially useful for detecting evidence of increased platelet production and helpful for the initial evaluation of patients with thrombocytopenia. As we gain further experience with this measurement and as software refinements are made, it may be possible to develop measurements at different levels of fluorescent intensity to improve diagnostic classification.

From the Department of Pathology, Johns Hopkins University School of Medicine, Baltimore, MD.

Address reprint requests to Dr Kickler: Johns Hopkins Hospital, Weinberg Bldg, Room 2333, 600 N Wolfe St, Baltimore, MD 21205.

Sysmex provided an educational grant to Johns Hopkins University for the performance of these studies.

References