Assessment of an immature platelet fraction (IPF) in peripheral thrombocytopenia

**Summary**

A new automated method to reliably quantify reticulated platelets, expressed as the immature platelet fraction (IPF), has been developed utilizing the XE-2100 blood cell counter with upgraded software (Sysmex, Kobe, Japan). The IPF is identified by flow cytometry techniques and the use of a nucleic acid specific dye in the reticulocyte/optical platelet channel. The clinical utility of this parameter was established in the laboratory diagnosis of thrombocytopenia due to increased peripheral platelet destruction, particularly autoimmune thrombocytopenic purpura (AITP) and thrombotic thrombocytopenic purpura (TTP). Reproducibility and stability results over 48 h were good. An IPF reference range in healthy individuals was established as 1.1–6.1%, with a mean of 3.4%. Patients in whom platelet destruction might be abnormal, were studied and two of these patients followed serially during the course of treatment. The IPF was raised in several disease states. The most significant increases in IPF values were found in patients with AITP (mean 22.3%, range 9.2–33.1%) and acute TTP (mean 17.2%, range 11.2–30.9%). Following patients during treatment demonstrated that as the platelet count recovered the IPF% fell. These results show that a rapid, inexpensive automated method for measuring the IPF% is feasible and should become a standard parameter in evaluating the thrombocytopenic patient.

**Keywords:** reticulated platelet, immature platelet fraction, Sysmex XE-2100, autoimmune thrombocytopenic purpura, thrombotic thrombocytopenic purpura.
cytometry. This modification, still using thiazole orange, but in combination with an antibody to anti-glycoprotein 1b (CD42) directly conjugated to phycoerythrin, offered a simple, rapid method for whole blood analysis of reticulated platelets. Although thiazole orange has been the most widely used fluorochrome, other dyes have also been employed. There is much variation in the published reference ranges in healthy subjects for this parameter using different flow cytometric methods and even between laboratories using the same methodology (Harrison, 1997; Robinson et al., 1998).

A number of clinical papers on reticulated platelet analysis have appeared over the last decade (Ault et al., 1992; Rinder et al., 1993a; Richards & Baglin, 1995; O’Malley et al., 1996), which clearly showed that, under conditions of thrombocytopenia, platelet RNA content correlated directly with megakaryocyte activity. Patients with low megakaryocyte activity have no RNA elevation in their platelets while those with increased megakaryocyte activity have significantly elevated RNA-stained platelets. This offers the ability to determine whether thrombocytopenia is due to marrow failure or to increased peripheral destruction/loss, thus avoiding the need for bone marrow examination. Other parameters of platelet production/turnover have also been studied, i.e. platelet lifespan (de Vries et al., 1993) and the measurement of glycocalcin (Steinberg et al., 1987) and thrombopoietin (Porcelijn et al., 1998). However platelet lifespan is not widely used due to problems associated with the manipulation of platelets and the use of radioactivity (van Reenan et al., 1980; Danpure et al., 1982). The glycocalcin index (glycocalcin level normalized for individual platelet count) is an indicator of platelet turnover but falsely elevated results have been reported in aplastic anaemia and hypoplastic anaemia (Hayashi et al., 2000) and very large ranges are observed in autoimmune thrombocytopenic purpura (AITP) (Steffan et al., 1998). Thrombopoietin is the main regulator of megakaryocyte development and platelet production (Kaushansky et al., 1996), so is a useful assay for assessing platelet kinetics. All of these assays have generally been limited to research units and are not readily available to monitor routine clinical material.

A further way of expressing reticulated platelets would be to separate them as an immature platelet fraction (IPF), which may be a more representative term due to the technical difficulties in precisely determining platelet RNA. This is now possible using a commercial blood cell counter and is the subject of this report.

Methods and patients

Automated technology

The Sysmex XE-2100 (Sysmex, Kobe, Japan) is a widely used modern routine fully-automated haematology analyser (Briggs et al., 2000). Using a carefully designed gating system in the XE-2100 optical (fluorescence) reticulocyte/platelet channel it is now possible to reliably quantify the IPF, employing upgraded software (XE-Pro Series; Sysmex). The flow cytometric IPF determination uses a proprietary fluorescent dye containing polymethine and oxazine. These two dyes penetrate the cell membrane, staining the RNA in the red cell and platelet reticulocytes. The stained cells are passed through a semiconductor diode laser beam and the resulting forward scatter light (cell volume) and fluorescence intensity (RNA content) measured. Figure 1 illustrates optical (fluorescence) platelet scattergrams with forward scattered light on the y-axis and fluorescence on the x-axis. A computer algorithm discriminates the mature and IPF by the intensity of forward scattered light and fluorescence. The XE-2100 instrument used in the study was equipped with upgraded software for the data analysis of the IPF, which applies a preset gate to separate the two platelet populations. Mature platelets appear as blue dots and the immature platelets are displayed as green dots, the latter constituting the IPF parameter. Figure 1 illustrates the optical platelet scattergram from a healthy individual and one from an AITP pregnant patient with a high IPF.

Imature platelet fraction is usually expressed as a proportional value of the total optical platelet count to indicate the rate of platelet production, although an absolute count can also be obtained. The IPF measurement can now be performed.

Fig 1. Optical platelet scattergrams from a healthy individual with a normal IPF and a patient with a high IPF. Mature platelets appear as blue dots, green dots represent the IPF with increased cell volume and higher fluorescence intensity compared to mature platelets.
as part of the routine blood count analysis and the results are available at the same time.

At present no widely acceptable reticulated platelet reference method has been established and therefore the IPF data have not been compared with a flow cytometric method in the present study.

Patients

Peripheral blood samples collected into K₂EDTA (Beckton Dickinson, Franklin Lakes, NJ, USA) were analysed at University College London. Residual patient samples were taken from the routine haematology laboratory after testing was complete. All samples were analysed within 2–4 h after collection.

Fifty samples from apparently healthy adults (all routine full blood count parameters including platelets within the healthy reference range) were used to establish a normal reference range for the IPF%.

The initial patient diagnostic groups studied consisted of 22 with AITP diagnosed according to the British Committee for Standards in Haematology (BCSH) guidelines for investigation and management of idiopathic thrombocytopenia purpura in adults, children and pregnancy (BCSH, 2003), 11 with thrombotic thrombocytopenia purpura (TTP) diagnosed according to BCSH guidelines on the diagnosis and management of thrombotic microangiopathic anaemias (Allford et al., 2003), 12 patients in the third trimester of pregnancy (BCSH, 2003), 11 with thrombotic thrombocytopenia purpura (TTP) diagnosed according to BCSH guidelines on the diagnosis and management of thrombotic microangiopathic anaemias (Allford et al., 2003), 12 patients in the third trimester of pregnancy [six normal pregnancies and six patients with pregnancy-induced hypertension (PIH)] and 13 patients undergoing chemotherapy with falling platelet counts. Two patients were followed serially over their course of treatment and recovery (one patient with acute TTP and one presenting with a low platelet count 9 months post bone marrow transplantation). Intercurrent episodes of infection were recorded, as were plasma exchanges or drug therapy, to indicate their effect on the IPF.

For this first evaluation all samples with platelet counts <50×10⁹/l were analysed in duplicate and the mean IPF% result calculated and used for analysis.

Results

Reproducibility/precision

The reproducibility of the method of 10 repeat analyses on seven different samples was good, as shown in Table 1. The average coefficients of variation (CV) for IPF in the three samples from individuals with platelet counts within the healthy reference range was 9.05%; for the high platelet count samples the IPF CV was 2.04%; and for the three low platelet count samples (mean 44×10⁹/l) with a high IPF, the CV was 10.78%. The numerical value of the IPF in all samples was very small and consequently higher CVs were to be expected, but lower CVs occurred when samples had a high IPF%.

Table 1. Reproducibility results for 10 repeat analyses on seven different individuals with varying platelet count and IPF% values.

<table>
<thead>
<tr>
<th>Sample number</th>
<th>Mean IPF</th>
<th>SD</th>
<th>CV%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal 1</td>
<td>2.4</td>
<td>0.16</td>
<td>6.82</td>
</tr>
<tr>
<td>Normal 2</td>
<td>3.7</td>
<td>0.33</td>
<td>8.94</td>
</tr>
<tr>
<td>Normal 3</td>
<td>2.6</td>
<td>0.30</td>
<td>11.39</td>
</tr>
<tr>
<td>High Plt, high IPF</td>
<td>8.7</td>
<td>0.18</td>
<td>2.04</td>
</tr>
<tr>
<td>Low Plt, high IPF 1</td>
<td>8.4</td>
<td>0.58</td>
<td>6.92</td>
</tr>
<tr>
<td>Low Plt, high IPF 2</td>
<td>35.1</td>
<td>3.92</td>
<td>11.15</td>
</tr>
<tr>
<td>Low Plt, high IPF 3</td>
<td>43.9</td>
<td>6.26</td>
<td>14.27</td>
</tr>
</tbody>
</table>

The IPF is expressed as percentage of total platelet (plt) count. Normal 1, 2 and three are samples from normal healthy donors.

Stability

The IPF remained stable over 2 d when blood samples were stored at room temperature; there was no consistent increase or decrease in the IPF values from 0 to 48 h after sampling (Fig 2). It should be noted that the standard deviation (SD) and CV% for these samples were only slightly higher than those for the reproducibility results.

IPF values in healthy controls and patients

IPF was measured in 50 healthy adults. The mean platelet count was 260×10⁹/l, range 154–391×10⁹/l; the mean IPF was 3.4% (absolute count, 8.6×10⁹/l), range 1.1–6.1% (absolute count, 3.1–16.4×10⁹/l).

IPF values in thrombocytopenia – diagnosis

Autoimmune thrombocytopenic purpura patients demonstrated the highest IPF% results of all the patient groups studied, indicating active platelet production. The highest IPF, 54%, was from a pregnant patient with AITP. Patients with active TTP also demonstrated very high IPF% results but, like the ITP patients, due to the low platelet counts the absolute IPF values were not raised. Both AITP and TTP patients in remission had generally normal IPF results but both the mean IPF% and absolute results from these groups could be slightly higher than in healthy individuals.
The IPF% results for the patients undergoing cytotoxic chemotherapy were at the lower end of the healthy reference range, however the association with thrombocytopenia indicated a failure of platelet production by the marrow.

Overall, the third trimester pregnancy patients demonstrated a raised IPF% and absolute count. Six patients had PIH and demonstrated higher IPF% values than those from normal pregnancies (mean IPF 10.8%, range, 7.2–14.6%). For the six normal pregnancies these values were mean IPF 6% (3.2–9.7%) (four were within the healthy reference range and two were slightly raised). The mean total platelet count for the patients with PIH was 192 × 10⁹/l, range 110–216 × 10⁹/l, and for the normal pregnancies 230 × 10⁹/l, range 176–310 × 10⁹/l.

Figure 3 shows the IPF% in various patient groups; AITP with platelet counts below 50 × 10⁹/l, AITP with platelet counts above 50 × 10⁹/l, acute TTP prior to therapy, TTP in stable remission, a group of third trimester antenatal patients and patients undergoing cytotoxic chemotherapy.

Table 2 lists the mean values, ranges and the percentage of patients with abnormal results for each patient group.

There was an inverse correlation between platelet count and IPF% in patients with AITP (P < 0.0001), see Fig 4.

**IPF values – role in patient monitoring**

The potential value of regular IPF% analysis was followed in two patients undergoing a prolonged course of treatment.

Figure 5 sequentially follows a 60-year old female who presented with acute TTP with transitory neurological weakness.

The IPF% is illustrated against the platelet count over the course of 82 d. Treatment consisted of daily plasma exchange for 30 d, then at less frequent intervals until initial remission and finally sustained remission was obtained. A high or rising IPF% represented increased platelet consumption on each of the three occasions before normalization of the platelet count followed.

Figure 6 follows a 50-year-old male who had received a mismatched unrelated donor reduced-intensity transplant for myelofibrosis 9 months prior to this event of acute thrombocytopenia.

Platelet engraftment post-transplant was initially prompt and robust, maintaining a normal platelet count without need for platelet support. He then presented with acute purpura and was found to have an isolated thrombocytopenia. His platelet count had been normal 2 weeks previously. The initial management dilemma of whether this was immune destruction or graft failure necessitated a bone marrow aspirate, trephine and chimaerism analysis. The initial marrow aspirate supported peripheral destruction, the trephine result received 5 d later confirmed this and full donor chimerism was confirmed 6 d later. He was treated with oral prednisolone (1 mg/kg) and intravenous gamma globulin with initial improvement, but then required Rituximab after relapsing whilst the steroid was being tapered. The persistently raised IPF% for 35 d reflected the prolonged period of severe

<table>
<thead>
<tr>
<th>Group</th>
<th>IPF%</th>
<th>Percentage of patients with a high IPF</th>
<th>IPF (%×10⁹/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Minimum</td>
<td>Maximum</td>
</tr>
<tr>
<td>Normal</td>
<td>3–4</td>
<td>1–1</td>
<td>6–1</td>
</tr>
<tr>
<td>Antenatal</td>
<td>8–4</td>
<td>3–2</td>
<td>14–6</td>
</tr>
<tr>
<td>AITP</td>
<td>16–8</td>
<td>2–3</td>
<td>52–1</td>
</tr>
<tr>
<td>AITP (&lt; 50 × 10⁹/l)</td>
<td>22–3</td>
<td>9–2</td>
<td>48–3</td>
</tr>
<tr>
<td>TTP</td>
<td>17–2</td>
<td>11–2</td>
<td>30–9</td>
</tr>
<tr>
<td>TTP (remission)</td>
<td>5–0</td>
<td>2–3</td>
<td>8–8</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>2–4</td>
<td>1–1</td>
<td>3–8</td>
</tr>
</tbody>
</table>

Antenatal = third trimester pregnancy samples; AITP < 50 = patients with a platelet count less than 50 × 10⁹/l.

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Table 2. Mean and range of IPF (% and × 10⁹/l) in the different groups studied and the percentage of patients in each group that had a high result.
thrombocytopenia followed by normalization of the IPF% as the platelet count recovered. The patient achieved a complete remission after this and his platelet count and IPF% now remain in the normal range. The presenting raised IPF% supported a peripheral immune destruction rather than graft failure.

Discussion

The measurement of reticulated platelets by flow cytometry is currently imprecise, expensive, time consuming, requires considerable expertise and lacks adequate quality control. The healthy reference range for reticulated platelets, as measured by flow cytometry, has still not been clearly defined (Ault et al, 1992; Harrison, 2003) with values quoted of less than 1% (Rinder et al, 1993a) to 16-4% (Robinson et al, 1998).

The type of RNA stain, its concentration, uptake across the platelet membrane, incubation time and gating of the reticulated platelet population are all problematical and need careful standardization before reticulated platelet counting can become a routine laboratory procedure. The International Society of Laboratory Hematology Platelet Task Force is currently undertaking a multi-centre study to define the population of reticulated platelets in healthy samples (Harrison, 2003) but has not yet identified a consensus flow cytometric method giving acceptable intra- and inter-laboratory results.

We have advised on the development and undertaken the initial evaluation of a fully-automated, rapid method for the measurement of reticulated platelets, expressed as the IPF using the Sysmex XE-2100 and an RNA fluorescent dye. Reproducibility and stability were demonstrated to be acceptable.

The reference range for the IPF% in healthy individuals using this method proved to be 1-1-6-1%. Various thrombocytopenic groups, where there may be excessive peripheral consumption/destruction of platelets or failure of platelet production, were studied and have provided some evidence suggesting that the IPF could be useful in the diagnosis and monitoring of such thrombocytopenic patients.

Currently, the presence of normal or increased numbers of megakaryocytes in the bone marrow excludes the diagnosis of hypoplastic thrombocytopenia, but bone marrow aspiration is subject to sampling errors, delays and subjective interpretation. There is no simple test to confirm hyperdestructive thrombocytopenia. Bone marrow examination in suspected cases of AITP is contentious. The BCSH guidelines recommend examination if over 60 years old, presence of atypical features or poor response to first line therapy (BCSH, 2003).

On the basis of our data, a suspected AITP without a significantly elevated IPF should have a marrow examination whatever the patient age. We propose that this is an important parameter to prompt early marrow examination in atypical cases and potentially avoid the need for marrow examination in ‘typical’ presentation AITP. It also provides a suitable non-invasive parameter to monitor patient response, particularly when changing immunosuppressive therapy. The measurement of the IPF is simple and non-invasive and provides rapid information about the bone marrow megakaryocyte activity and platelet lifespan. The sensitivity and specificity of reticulated platelet percentage has previously been reported to be 91–96% and 67–100%, respectively (Rinder et al, 1993a; Richards & Baglin, 1995). Seventy-three percent of AITP patients in this study had a raised IPF% and 100% of patients with a total platelet count of $<50 \times 10^9/l$ had a raised IPF%. There was also significant inverse correlation of platelet count with IPF%; the lower the platelet counts, the higher the IPF%. The IPF% value reflected the severity of platelet destruction. Although the IPF% was raised in AITP and acute TTP patients, the total absolute counts were generally decreased or within the normal range.
value of the IPF absolute count as opposed to the percentage count is limited by the low values that are inevitable when the platelet count is low, even if the IPF% is approaching 100%. Our conclusion is that the IPF% provides more useful information on the rate of thrombopoiesis and this may have specific diagnostic and monitoring significance.

However, the absolute reticulated platelet count has been reported to reflect the rate of platelet production (Kienast & Schmitz, 1990). It has previously been reported that, in a minority of patients with AITP, platelet production was decreased compared with healthy subjects as well as having an increased clearance rate (Ballem et al, 1987). The value of the IPF% in diagnosing peripheral immune destruction is clearly illustrated by the patient that presented with a low platelet count post bone marrow transplant. The immediate concern would be that this was due to bone marrow failure but, the first blood count showed that this was clearly not the case, as the IPF was 15.5%; platelet count 11 x 10^9/l; the next count, 2 d later, gave an IPF of 32% with a platelet count of 5 x 10^9/l. As the platelet count recovered, the IPF% fell, consistent with the dilution in the blood of the young platelets with older platelets with increasing lifespan.

The IPF results for the patients with active TTP were similar to those with AITP. The IPF% was high, due to the excessive consumption of platelets and then, after a variable period of plasma exchanges as the disease process began to come under control (Allford et al, 2003), the IPF% fell as the platelet count rose. While the IPF% remains high there will be no recovery in platelet count and treatment will need to be continued. Patients in remission from TTP have relatively normal IPF values.

We have investigated 13 patients undergoing cytotoxic chemotherapy and generally, those with falling platelet counts had the lowest IPF values. We have now started to study patients undergoing peripheral blood stem cell and bone marrow transplants in order to determine whether the IPF% could predict the timing of platelet recovery and guide prophylactic platelet transfusion, providing the potential to reduce the use of platelet concentrates in these patients. The IPF% may be a useful parameter to measure the response to the various growth factors that affect platelet production.

The third trimester antenatal patients, particularly those with PIH, showed quite marked increases in the IPF% and absolute counts. These results agree with other reports that suggested that the reticulated platelet count may be capable of differentiating normal pregnancies from those in which pre-eclampsia or PIH is likely to develop (Rinder et al, 1993b).

In conclusion, this study demonstrates a fully automated, rapid method for the determination of the IPF, using the Sysmex XE-2100 fitted with upgraded software to analyse the highly fluorescent platelets. The results are available at the same time as the full blood count and the IPF is reproducible and stable in an EDTA sample stored at room temperature for at least 48 h. The IPF% provides a valuable diagnostic method to clearly differentiate between the consumptive and aplastic causes of thrombocytopenia.

The IPF should become a standard routine parameter in the diagnosis and serial monitoring of the thrombocytopenic patient.

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References


