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Clinical significance of IPF% or RP% measurement in distinguishing primary immune thrombocytopenia from aplastic thrombocytopenic disorders

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Abstract The diagnosis of primary immune thrombocytopenia (ITP) is based on differential diagnosis. Although the measurement of percentages of reticulated platelets (RP%) by flow cytometry is useful as a supportive diagnostic test, this method is nonetheless a time-consuming, laboratory-based assay. To identify alternative assays that are useful in daily practice, we compared three methods in parallel, IPF% measured by XE-2100 [IPF% (XE), Sysmex Corp.], IPF% measured by new XN-1000 [IPF% (XN)], and RP%. We examined 47 patients with primary ITP, 28 patients with aplastic thrombocytopenia (18 aplastic anemia and 10 chemotherapy-induced thrombocytopenia) and 80 healthy controls. In a selected experiment, we examined 16 patients with paroxysmal nocturnal hemoglobinuria (PNH) to examine the effect of hemolysis. As compared with IPF% (XE), IPF% (XN) showed better within-run reproducibility. The sensitivity and specificity for the diagnosis of ITP were 83.0 and 75.0 % for IPF% (XE), 85.1 and 89.3 % for IPF% (XN), and 93.6 and 89.3 % for RP%, respectively. Examination of PNH patients revealed that hemolysis and/or red blood cell fragments interfered with IPF% (XE) values, but not with IFP % (XN) values. Our results suggest that IPF% measured by XN-1000 may be of comparable value with RP% as a supportive diagnostic test for ITP.

Keywords Immune thrombocytopenia · Reticulated platelets · Differential diagnosis · Paroxysmal nocturnal hemoglobinuria · Thrombopoietin

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Introduction

Primary immune thrombocytopenia (ITP) is an autoimmune disease characterized by early platelet destruction due to anti-platelet autoantibodies and slightly impaired platelet production [1–3]. Despite recent advances in understanding of its pathophysiology, the diagnosis of ITP is still mainly based on differential diagnosis [4]. However, it is sometimes very difficult to distinguish ITP from isolated thrombocytopenia due to aplastic thrombocytopenic disorders such as aplastic anemia (AA) and amegakaryocytic thrombocytopenia. To resolve this issue, several laboratory-based assays have been developed: detection of anti-platelet autoantibodies, measurement of percentage of reticulated platelets (RP%) and plasma thrombopoietin (TPO) concentrations [3]. Regarding detection of platelet-associated autoantibodies, it has been shown that its specificity for the diagnosis of ITP is very high (80–90 %) in prospective studies. However, the drawback in this assay is its relatively low sensitivity as well as being time-consuming, laboratory-based assay: platelet-associated anti-GPIIb/IIIa and/or anti-GPIb/IX antibodies are detected in only 51–55 % of ITP [5–7]. Alternatively, measurement of RP% and plasma TPO concentrations is useful to distinguish between ITP and aplastic thrombocytopenic disorders [8–10]. RPs are reported to be younger platelets (i.e., immature platelets) that have been released recently into

the circulation and are probably analogous to reticulocytes reflecting erythropoiesis. RPs can be distinguished from mature platelets by their RNA contents using flow cytometry with an RNA-binding fluorochrome, such as thiazole orange, and RP% and absolute number of RPs are reflecting platelet production and hence platelet turnover [11, 12]. In ITP patients RP% was markedly increased compared with healthy controls, whereas RP% in patients with AA or chemotherapy-induced thrombocytopenia (CIT) was within normal range [10–12]. In contrast, plasma TPO levels in ITP patients are within normal range or only slightly increased, whereas those in patients with aplastic thrombocytopenic disorders are markedly increased [8–10]. Accordingly, Japanese ITP working group including us proposed preliminary diagnostic criteria for ITP by incorporating anti-platelet autoantibody detection, RP%, and plasma TPO level. In a multi-center prospective study, the criteria showed high sensitivity and specificity for the diagnosis of ITP [13]. However, the method for the measurement of RP% is nonetheless a time-consuming, laboratory-based assay and has not been standardized yet.

We have been seeking alternative assays to measure RP% that are useful in daily practice, although measurement of RP% by flow cytometry is the gold standard method. One candidate is measurement of percentage of immature platelet fraction (IPF%) using Sysmex XE-2100 (or XE-5000) automated hematology analyzer (Sysmex Corp., Kobe, Japan). This IPF% method becomes very popular because of its convenience [14]. However, we previously demonstrated that IPF% measured by XE-2100 showed less sensitivity and specificity as compared to RP% method to distinguish between ITP and AA patients [3, 15]. Thus, IPF% measured by XE-2100 was neither so accurate nor satisfactory in daily practice. To improve the accuracy of IPF% method, new generation analyzer, XN-1000 has been developed and become commercially available. In this study, we compared these three methods in parallel, IPF% measured by XE-2100 [IPF% (XE)], IPF% measured by XN-1000 [IPF% (XN)], and RP%, for their utility in differential diagnosis between ITP and aplastic thrombocytopenia (AA and CIT). In addition, effects of hemolysis in patients with paroxysmal nocturnal hemoglobinuria (PNH) on IPF% (XE), IPF% (XN), and RP% were examined.

Materials and methods

Subjects

For a period of 6 months (October 2013 through March 2014) we examined 47 patients with primary ITP [9 males and 38 females, age 59 ± 17 years, platelet count $57 \pm 34 \times 10^3/\mu l$ (mean $\pm$ SD)], 28 patients with aplastic (or hypoplastic) thrombocytopenia [18 AA and 10 chemotherapy-induced thrombocytopenia (CIT)] [11 males and 17 females, age 50 ± 15 years, platelet count $43 \pm 28 \times 10^3/\mu l$ (mean $\pm$ SD)], and 80 healthy controls [35 males and 45 females, age 34 ± 12 years, platelet count $269 \pm 58 \times 10^3/\mu l$ (mean $\pm$ SD)]. Diagnosis of primary ITP and aplastic anemia was based on reports from an international working group and International Agranulocytosis and Aplastic Anemia Study group, respectively [4, 16]. With regard to management of 47 patients with ITP, 19 patients managed with observation alone, 14 patients mainly with prednisolone, 9 patients with TPO receptor agonist (TPORA) and prednisolone, and 5 patients with TPORA only. Thus, 14 ITP patients treated with TPO receptor agonist such as eltrombopag and romiplostim were included, and 12 of these patients still showed thrombocytopenia less than $100 \times 10^3/\mu l$. Patients with CIT include 6 patients with AML, 2 patients with ALL, and 2 patients with MDS, and samples were obtained on day 1 or day 2 for myeloablative allogeneic hematopoietic stem cell transplantation. We obtained informed consent from all subjects, in accordance with the declaration of Helsinki. This study was approved by Osaka University Institutional Review Board.

In a selected experiment, we examined 16 patients with PNH to investigate effects of hemolysis and/or fragmentation of red blood cells (RBC) on the measurement of IPF% and RP%.

Measurement of RP%

RP% was measured as previously described with a slight modification [15]. In brief, 15-μl aliquots of whole blood anti-coagulated with ethylenediamineteraacetic acid (EDTA) were incubated with 5 μl of phycoerythrin-conjugated anti-CD42b monoclonal antibody (BD Pharmingen, Tokyo, Japan) and 20 μl of 2 % paraformaldehyde for 15 min at room temperature. After adding 1 ml of thiazole orange (Retic-COUNT; Becton–Dickinson, San Jose, CA, USA) diluted to 8 times by phosphate-buffered saline, the whole blood samples were centrifuged at 350 g for 30 s to remove red blood cells, and then the platelet-rich suspensions were incubated at room temperature for 90 min. RP% was analyzed on a flow cytometer (FACScan, Becton–Dickinson) by measuring 10,000 events in the CD42b-positive fraction. To exclude cell autofluorescence and instrument background, platelet-rich suspension without thiazole orange was prepared as a negative control for each sample.

Measurement of IPF% by Sysmex automated hematology analyzer XE-2100 and XN-1000

EDTA-anti-coagulated whole blood samples were also used to measure IPF% employing automated hematology

Table 1 Within-run reproducibility for measuring IPF% by XE-2100 and XN-1000

Sample	Platelet count ($10^3/\mu l$)	Number	IPF (%)	CV (%)
XE-2100				
Control-1	312.2 ± 7.2	10	2.29 ± 0.41	17.7
Control-2	339.8 ± 7.0	10	0.76 ± 0.13	16.9
Control-3	336.9 ± 6.8	10	1.76 ± 0.21	11.7
Control-4	256.8 ± 5.0	10	1.98 ± 0.21	10.6
ITP-1	37.8 ± 1.8	10	8.97 ± 1.26	14.0
ITP-2	24.7 ± 1.3	10	12.85 ± 1.83	14.3
XN-1000				
Control-1	293.5 ± 2.3	10	2.78 ± 0.07	2.7
Control-2	362.0 ± 2.8	10	0.56 ± 0.05	8.8
Control-3	314.9 ± 2.5	10	1.76 ± 0.09	5.2
Control-4	251.5 ± 2.4	10	2.68 ± 0.16	6.0
ITP-1	37.4 ± 0.5	10	11.84 ± 0.60	5.0
ITP-2	20.5 ± 0.8	10	13.40 ± 0.77	5.7

CV coefficient of variation

analyzer XE-2100 (XE, Sysmex) and the newer generation analyzer, XN-1000 (XN, Sysmex). XE used polymethine and oxazine to stain nucleic acid and RET-channel for the measurement of IPF, whereas XN used only oxazine and PLT-F channel to more accurately detect platelets and IPF [17]. All samples were measured within 7 h of venesection.

Measurement of plasma TPO concentrations

Plasma TPO concentration was measured using an enzyme-linked immunosorbent assay (ELISA) kit as previously described (R & D Systems, Minneapolis, MN, USA) [10].

Statistical analysis

The differences between mean values were evaluated using Student t test and a p value less than 0.05 was considered statistically significant. We analyzed sensitivity and specificity of IPF% (XE), IPF% (XN), and RP% for the diagnosis of ITP as previously described [10]. Constructed receiver operating characteristics (ROC) curves were analyzed using GraphPad Prism (GraphPad Software Inc., La Jolla, CA, USA).

Results

Within-run reproducibility

We first examined within-run reproducibility for IPF% measurement in 4 control subjects and 2 ITP patients employing XE and XN in parallel. Each sample was measured 10 times, and mean intra-assay coefficients of variation [CV(%)] for these samples were 14.2 ± 2.8

and 5.6 ± 2.0 % for XE and XN, respectively ($p < 0.001$) (Table 1).

IPF%, RP%, and plasma TPO levels in thrombocytopenic disorders due to either accelerated platelet destruction or deficient platelet production.

We examined 47 patients with ITP as a thrombocytopenic disorder due to early platelet destruction and 18 patients with aplastic anemia and 10 patient with CIT as aplastic (or hypoplastic) thrombocytopenic disorders. IPF% values obtained from 80 control subjects were 2.5 ± 1.3 and 2.2 ± 1.2 % (mean $\pm$ SD) for XE and XN, respectively. As we defined an upper limit for healthy control subjects as mean $+$ 3SD in this study, the upper limits were 6.4 and 5.8 % for XE and XN, respectively. RP% value obtained from 80 control subjects measured by flow cytometry was 4.8 ± 1.1 % (mean $\pm$ SD) and its upper limit of reference range was defined as 8.1 % (mean $+$ 3SD).

Figure 1 shows correlations between IPF% (XE), IPF% (XN), and RP%. Good linear correlation between IPF% (XE) and IPF% (XN) was obtained ($r = 0.94$), whereas only moderate correlation between RP% and IPF% (XE) ($r = 0.72$) or IPF% (XN) ($r = 0.71$) was obtained. IPF and RPs were measured with oxazine and thiazole orange, respectively, and they were thought to be equivalent. However, our data suggested that IPF and RPs were similar, but not quantitatively identical. Next, we examined the sensitivity and specificity of IPF% and RP% to distinguish ITP from AA/CIT. There was no significant difference in platelets count between two groups ($57 \pm 34 \times 10^3/\mu l$ for ITP, $43 \pm 28 \times 10^3/\mu l$ for AA/CIT). Figure 2 shows IPF% (XE), IPF% (XN) and RP% in ITP patients and AA/CIT patients. As expected, IPF% (XE), IPF% (XN) and RP% showed clear difference between ITP and AA/CIT. However, elevated values were detected in 83.0, 85.1, and 93.6 % of ITP measured by IPF% (XE), IPF% (XN) and
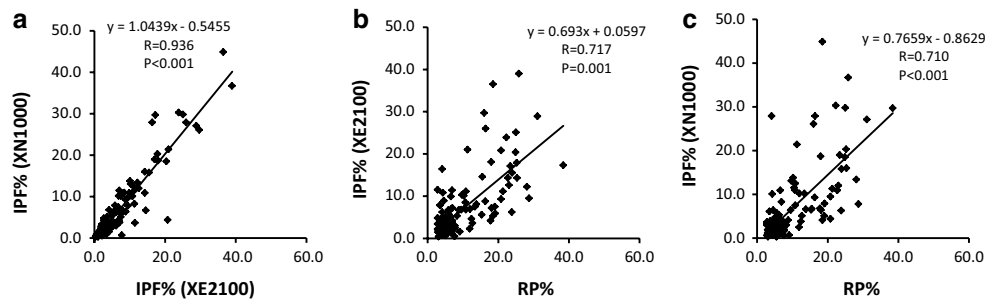
Fig. 1 Correlations between IPF% (XE-2100), IPF% (XN-1000), and RP% by flow cytometry. **a** IPF% (XE-2100) versus IPF% (XN-1000), **b** RP% versus IPF% (XE-2100), and **c** RP% versus IPF% (XN-1000)
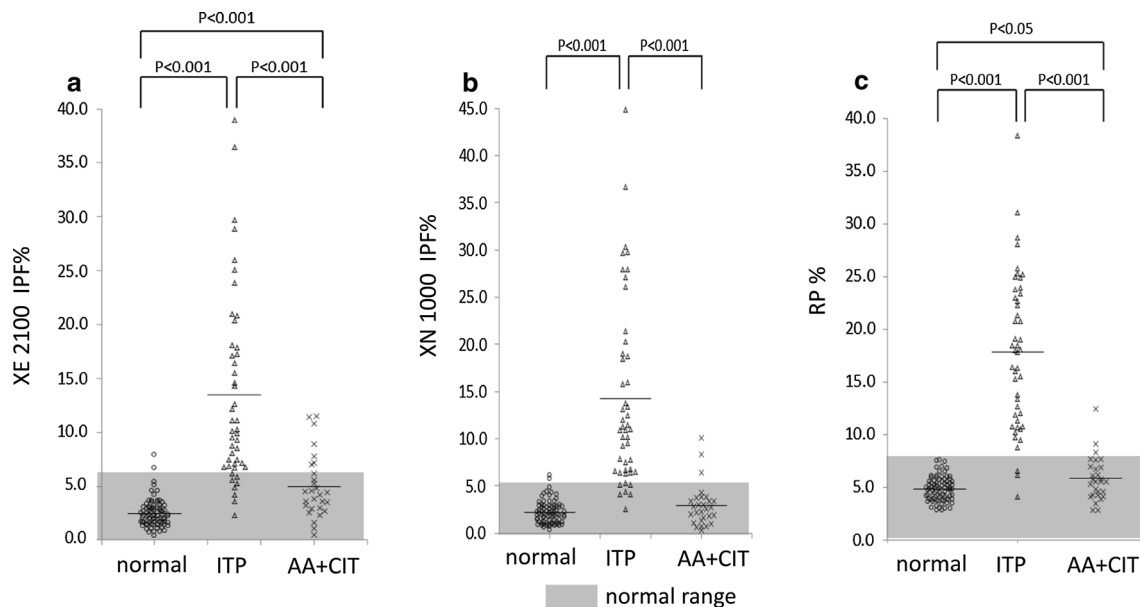

Fig. 2 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in healthy controls, patients with primary immune thrombocytopenia (ITP), and patients with aplastic anemia (AA) or chemotherapy-induced thrombocytopenia (CIT). *Shaded area* indicates normal range (mean $\pm$ 3SD obtained from 80 healthy controls)

RP%, respectively. In sharp contrast, the elevated values were detected in 25.0, 10.7, and 10.7 % of AA/CIT by IPF% (XE), IPF% (XN) and RP%, respectively (Fig. 2). As compared with IPF% (XN) and RP%, IPF% measured by XE-2100 appeared to be less frequently elevated in ITP and much frequently elevated in AA/CIT. Thus, the sensitivity and specificity of the elevation of IPF% or RP% for the diagnosis of ITP were 83.0 and 75.0 % for IPF% (XE), 85.1 and 89.3 % for IPF% (XN), and 93.6 and 89.3 % for RP%, respectively (Table 2).

In parallel we measured plasma TPO concentration in each sample, which further confirmed the diagnosis (Fig. 3). TPO levels obtained from 80 control subjects were 16.3 $\pm$ 21.8 pg/ml (mean $\pm$ SD), and the upper limit of reference range for TPO was 81.7 pg/ml (mean + 3SD). Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels. In addition, we measured mean platelet volume (MPV) for ITP and AA/CIT. However, MPV could be measured by XN-1000 in only 28 out of 47 ITP patients, probably because of abnormal size distribution of platelet volume in ITP. Nonetheless, MPVs for ITP (12.5 $\pm$ 1.0 fl, $n = 28$) were significantly larger than 80 controls (10.5 $\pm$ 1.0 fl, $p < 0.001$) and 23 AA/CIT (10.7 $\pm$ 0.9 fl, $p < 0.001$).

Receiver operating characteristic (ROC) analysis

ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients, and IPF% (XE), IPF% (XN), and RP% data were analyzed separately. ROC curve for IPF% (XE), IPF%

Table 2 Sensitivity, specificity, and predictive values of IPF% (XE), IPF% (XN), and RP% for the diagnosis of ITP

	Sensitivity (%)	Specificity (%)	Predictive value (%)	
			Positive	Negative
Upper panel includes ITP patients treated with TPORA (total 47 ITP patients)				
IPF% (XE-2100)	83.0	75.0	84.8	72.4
IPF% (XN-1000)	85.1	89.3	93.0	78.1
RP%	93.6	89.3	93.6	89.3
Lower panel excludes ITP patients treated with TPORA (total 33 ITP patients)				
IPF% (XE-2100)	78.8	75.0	78.8	75.0
IPF% (XN-1000)	81.8	89.3	90.0	80.6
RP%	90.9	89.3	90.9	89.3

(XN), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively (Fig. 4).

IPF% and RP% in patients with PNH

To examine effects of hemolysis on the measurement of IPF% and RP% we examined patients with PNH. Ten patients out of 16 PNH patients were treated with eculizumab, a humanized monoclonal antibody against terminal complement protein C5 that inhibits terminal complement activation. As shown in Fig. 5, 6 PNH patients showed elevated IPF% (XE), whereas none and only two patients showed elevated IPF% (XN) and RP%, respectively. Five out of 6 PNH patients with elevated IPF% (XE) were treated with eculizumab, suggesting active hemolysis may interfere with the measurement of IPF% by XE-2100.

Discussion

Recent in vivo vital imaging as well as biochemical and genetic approaches have revealed the mechanism of platelet production (thrombopoiesis) from mature megakaryocytes. Mature megakaryocytes localized in bone marrow sinusoids extend proplatelets into the lumen of the sinusoids, and then new platelets are shed as fragments from the tips of intravascular proplatelets [18, 19], and newly produced platelets can be distinguished from mature platelets by their content of RNA as RPs by flow cytometry [20]. Despite our progress in understanding of pathophysiology of ITP as well as mechanism of thrombopoiesis, diagnosis of ITP has been still based on differential diagnosis [1–3].
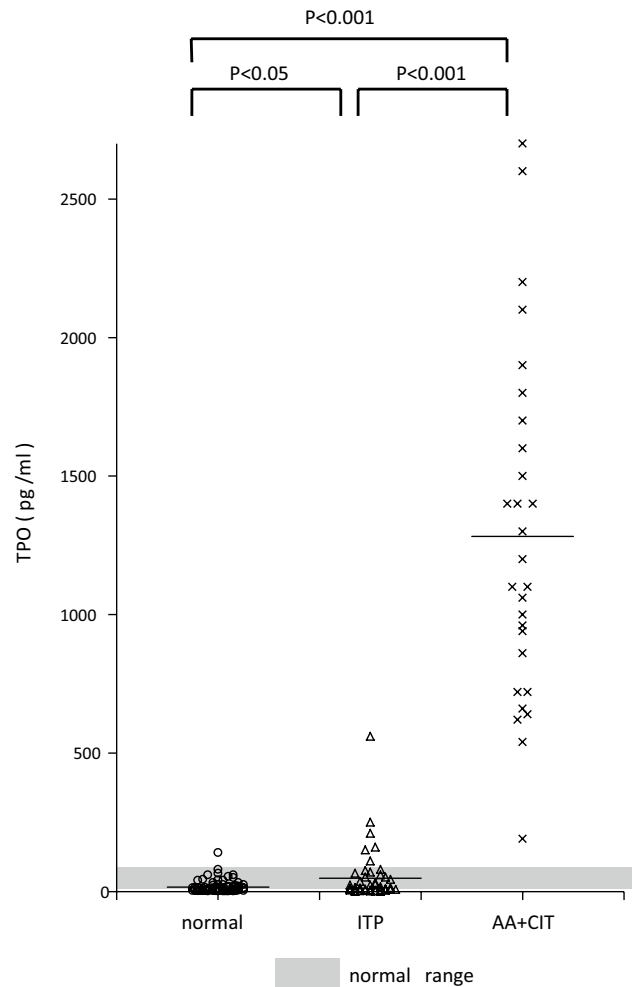

Fig. 3 Plasma thrombopoietin (TPO) levels in healthy controls, patients with ITP, and patients with AA/CIT. Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels
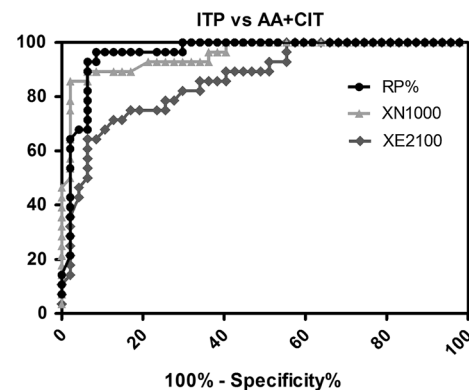

Fig. 4 Receiver operating characteristic (ROC) analysis. ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients. ROC curve for IPF% (XE-2100), IPF% (XN-1000), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively
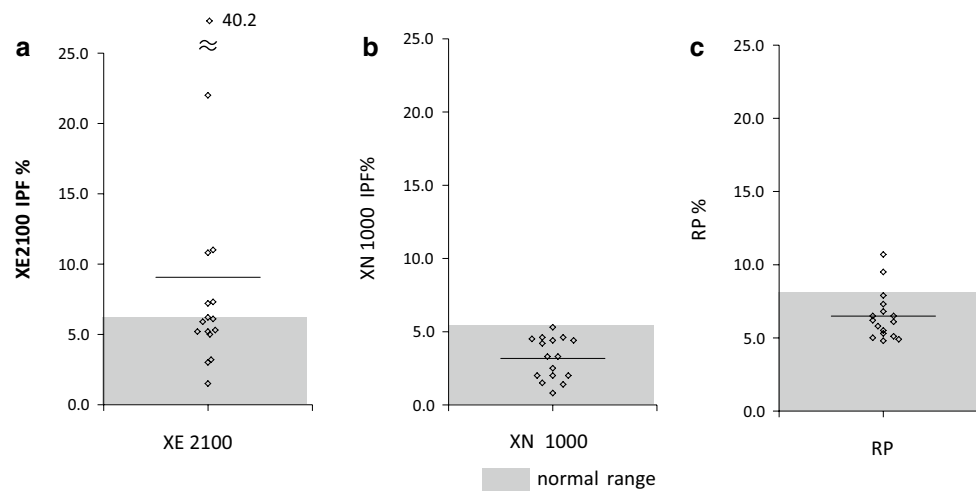
Fig. 5 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in patients with PNH. *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy controls)

Several laboratory-based tests, detection of platelet-associated autoantibodies, measurement of RP% and plasma TPO level, could be useful for the diagnosis of ITP [10]. However, the measurement of RP% is laboratory-based assay, and not used in daily practice yet. In addition, methods for RP% measurement have not been standardized. High concentrations of thiazole orange as well as longer incubation time, more than 2.5 h, induced dramatically higher fluorescence intensities, probably due to penetration of the dye into the dense granules [20]. Accordingly, we used 8-times-diluted thiazole orange and shorter incubation time (90 min) to measure RP%. To examine the reliability of automated hematology analyzer-based IPF% method for the differential diagnosis of ITP, we compared IPF% (XE), IPF% (XN), and RP% in parallel. Platelets were precisely monitored by anti-CD42b antibody and 10,000 events were analyzed even under thrombocytopenic conditions in the RP% method, but not in IPF% (XE) or IPF% (XN). However, in the new XN series a novel PLT-F channel was introduced to more specifically gate platelets than in XE series [17, 20]. Actually, we confirmed that data obtained by XN-1000 were much more accurate than XE-2100 regarding within-run reproducibility.

We examined the correlation between IPF% (XE), IPF% (XN), and RP%. Excellent correlation between IPF% (XE) and IPF% (XN) was obtained, while only moderate correlation even between RP% and IPF% (XN) existed. The difference between IPF% and RP% is probably caused by the difference in the fluorescent dyes: oxazine and thiazole orange. Our data first revealed that IFP % (XN) values and RP% values were moderately related, but not quantitatively identical.

In a selected experiment we examined samples obtained from PNH patients, since in XE-2100 RBC fraction and platelet fraction were relatively close each other and both polymethine and oxazine were used as fluorescent dyes. As expected, hemolysis and/or RBC fragments interfered with IPF% (XE) values and 6 out of 16 PNH samples showed elevated IPF% (XE) values. However, none and two samples showed elevated IFP % (XN) and RP%, respectively. In contrast to XE series employing both polymethine and oxazine as fluorescent dyes to measure reticulocytes and immature platelets, XN-1000 employs only oxazine to measure immature platelets more specifically. In addition, the novel PLT-F channel enables us to more accurately differentiate platelets from other cells and interfering particles such as RBC fragments than XE series [21]. The false positive results in IPF% (XE) may be partially caused by non-specific binding of polymethine to RBC fragments. These data suggested that influence of hemolysis and/or RBC fragmentation was only minimal on measurement of IPF% by XN-1000 as well as RP% by flow cytometry.

We then compared the sensitivity and specificity of IPF% (XE), IPF% (XN), and RP% for the differential diagnosis between ITP and AA/CIT. There were clear differences in plasma TPO levels between ITP and AA/CIT. We confirmed our previous data that IPF% (XE) showed less sensitivity and specificity (83.0 and 78.6 %, respectively) as compared with RP% (93.6 and 89.3 %, respectively) [15]. In sharp contrast, IPF% by XN-1000 showed comparable sensitivity and specificity (85.1 and 92.9 %, respectively) with RP%. The sensitivity and specificity of IPF% (XE) was relatively high as compared with our previous study (sensitivity 67 %, specificity 63 %). This difference is probably due to the inclusion of ITP patients treated with TPORA in this study, because TPORA effectively increased the absolute number of RPs (and IPF) and maintained elevated RP% (and IPF%) even after improvement

of thrombocytopenia [14]. In fact, exclusion of 14 ITP patients treated with TPORA led to the decrease in the sensitivity of these assays [IPF% (XE) 78.8 %, IPF% (XN) 81.8 %, and RP% 90.9 %] (Table 2). ROC curve for IPF% (XE), IPF% (XN), and RP% showed area under curve (AUC) of 0.86, 0.96, and 0.96, respectively, indicating that IPF% measured by XN-1000 may be comparable with RP% by flow cytometry.

In summary, the data obtained from our study suggested that IPF% measured by XN-1000 may be of comparable value with RP% as a supportive diagnostic test in distinguishing between thrombocytopenic disorders due to early platelet destruction such as ITP and aplastic thrombocytopenic disorders such as aplastic anemia. In addition, automated hematology analyzer is easy to handle and suitable for daily practice. Limitation of our study is that we examined patients with definitive diagnosis from single institute. Multi-center prospective study would be necessary to further confirm our data.

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