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ORIGINAL ARTICLE

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 ± 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 ± 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 ± 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/μl)	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 ± 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 ± 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 ± 34 × 10^3/μl for ITP, 43 ± 28 × 10^3/μl 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

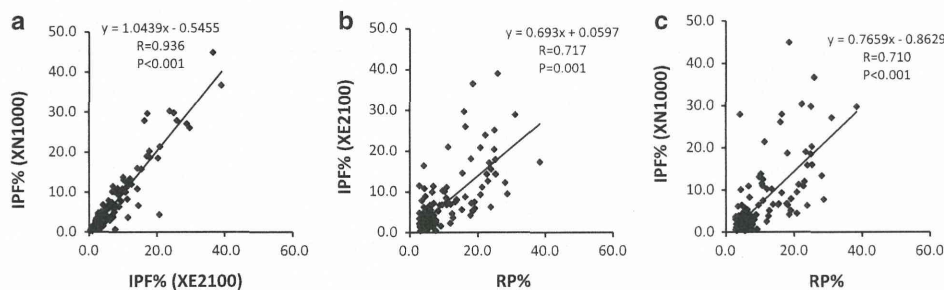

Fig. 1 Correlations between IPF% (XE-2100), IPF% (XN-1000), and RP% by flow cytometry. **a** IPF% (XE-2100) versus IPF% (XN-1000), **b** RP% versus IPF% (XE-2100), and **c** RP% versus IPF% (XN-1000)

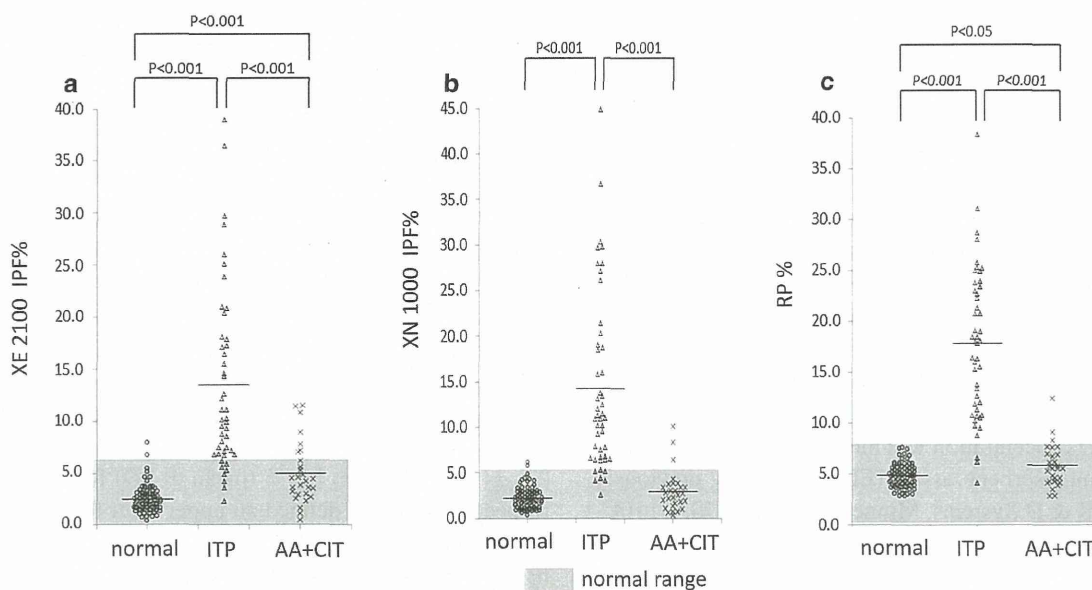

Fig. 2 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in healthy controls, patients with primary immune thrombocytopenia (ITP), and patients with aplastic anemia (AA) or chemotherapy-induced thrombocytopenia (CIT). *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy 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 ± 21.8 pg/ml (mean ± 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 ± 1.0 fl, $n = 28$) were significantly larger than 80 controls (10.5 ± 1.0 fl, $p < 0.001$) and 23 AA/CIT (10.7 ± 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].

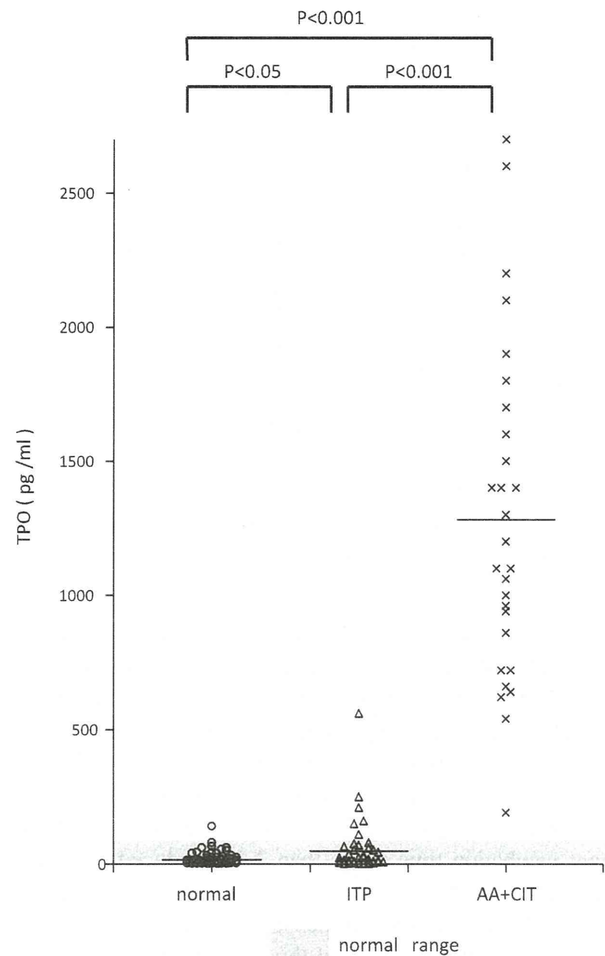

Fig. 3 Plasma thrombopoietin (TPO) levels in healthy controls, patients with ITP, and patients with AA/CIT. Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels

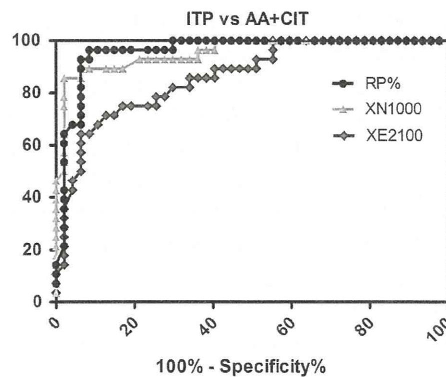

Fig. 4 Receiver operating characteristic (ROC) analysis. ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients. ROC curve for IPF% (XE-2100), IPF% (XN-1000), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively

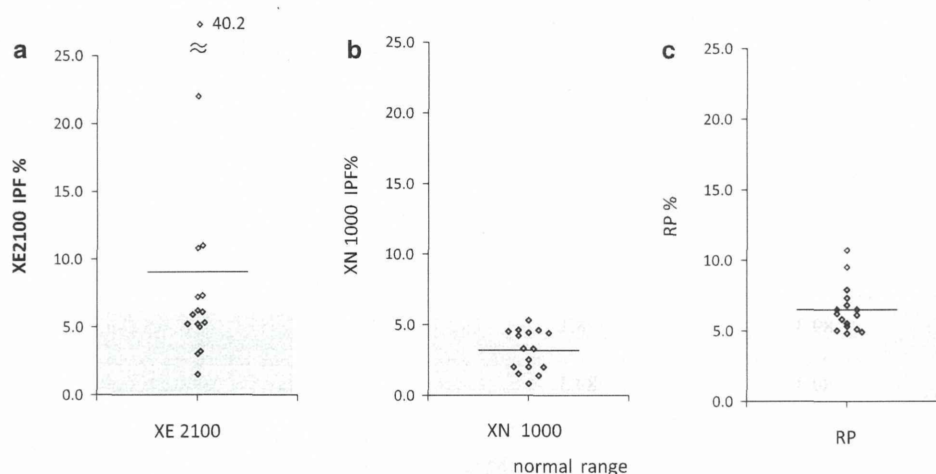

Fig. 5 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in patients with PNH. *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy controls)

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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Conflict of interest Automated hematology analyzers, XE-2100 and XN-1000, were supplied by Sysmex Corp. During this study the authors have no other COI to declare.

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PNH患者におけるC5遺伝子多型

西村純一，金倉　譲

Eculizumab投与を受けた日本人PNH患者345例中11例が不応症であったが，この分子的機構は不明であった。全11例で共通のC5のミスセンスヘテロ接合性変異c.2654 G>Aを認め，p.Arg885Hisが予測された。患者の変異保有率（3.2%）は，健常人の保有率（3.5%）と同程度で，漢民族においても確認された。この変異型C5は野生型C5とともに *in vitro* 溶血を引き起こしたが，野生型C5のみがeculizumabと結合し，eculizumabによる阻害を受けた。この *in vitro* 溶血は，eculizumabとは異なるC5部位に結合する抗体N19-8を用いると，野生型C5，変異型C5ともに完全に阻害された。Arg885 His変異を伴うC5遺伝子多型の機能的特性は，eculizumabによる阻害を受けず，この変異を有する患者のeculizumab不応症を説明し得ることが示された。
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はじめに

発作性夜間ヘモグロビン尿症（paroxysmal nocturnal hemoglobinuria, PNH）の主要症状である補体介在性溶血に対する治療薬として，補体蛋白C5を標的とするヒト化モノクローナル抗体であるeculizumabが開発され，長期使用における有効性と安全性のデータが海外[1]と日本[2]から相次いで報告された。さらに，小児患者においても，成人と同様の有効性と安全性を示すデータが公表された[3]。その一方で，溶血の指標である血清LDH値が全く低下しない不応症が，日本人PNH患者の一部の集団に見いだされ問題となっていたが，その分子機構が解明された[4]。本稿ではこの分子機構を中心に概説するとともに，C1[5]やC3[6]といったC5より上流の分子を標的にした治療薬開発の動きも加速しつつあり，それら候補薬剤の可能性についても言及したい。

PNHの病態

1. 補体介在性血管内溶血

PNH患者の血液細胞では，補体制御因子であるCD55（decay-accelerating factor, DAF）とCD59（membrane inhibitor of reactive lysis, MIRL）の発現が欠損または低下している。CD55はC3/C5転換酵素の崩壊を促進することによって補体活性化経路の前半の段階を調節するのに対し[7]，CD59はC9に作用して膜侵襲複合体（membrane attack complex, MAC）の形成を阻害する[8,9]。PNH患者では，平常でもわずかな補体活性化による持続的な溶血がみられるが，感染症，睡眠，手術，妊娠，鉄剤投与など様々な誘因により強い補体活性化が起こると，短時間で大量溶血（溶血発作）をきたす。これら誘因の中でも，臨床的にしばしば問題となるのは感染症である。PNH患者は，補体介在性の血管内溶血，骨髄不全および血栓症を3大症状とするが，他にも，腹痛，嚥下障害，男性機能不全などの多彩な症状を示す。溶血により血漿中に放出された遊離ヘモグロビンが，Nitric Oxide（NO）を強力に捕縛し，NOの作用を阻害する結果，腹痛，嚥下障害，男性機能不全，血栓症などの症状を誘発，増幅する機序が明らかになった[10]。

2. PNHクローンの拡大機序

PNHは，一つ（または数個）の造血幹細胞の *phosphatidylinositol glycan class A*（*PIGA*）遺伝子に後天的変異が起こり，その変異細胞がクローン性に拡大する造血幹細胞疾患である[11]。*PIGA* 遺伝子変異によりglycosylphosphatidylinositolアンカー型蛋白（GPI-AP）の合成障害をきたし，CD59やCD55などのGPIアンカー型補

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体制御因子を欠損するPNH赤血球が，補体の活性化に伴い血管内溶血を起こす。最近，GPI-AP合成に関わる他の遺伝子*PIGT*の変異によっても，PNHを発症することが報告された[12]。症例は典型的なPNH症状を呈していたが，*PIGA*に変異がみつからず，GPI-AP合成に関わる遺伝子群の全エクソンシークエンスを行ったところ，GPIトランスアミダーゼの構成成分である*PIGT*遺伝子において，ヘテロの先天性変異にPNHタイプ血球特異的な体細胞突然変異が付加されていることが証明された。

PNHクローンの拡大には，動物実験の成績から*PIGA*遺伝子変異だけでは不十分であることが判明している[13~17]。再生不良性貧血（aplastic anemia, AA）を代表とする造血不全疾患としばしば合併・相互移行すること，AA患者の約50%に微少なPNH血球（>0.003%）が検出されることなど[18]，AAとの深い関連性から，AAにみられるような免疫学的機構がPNHクローンの相対的増加（選択機序）に関与しているとされる。すなわち，造血障害を引き起こす免疫学的傷害のターゲットとしてGPI-APを介していれば，これを発現する正常幹細胞は傷害されるのに対し，PNH幹細胞はこの傷害を免れることになり，PNHクローンの拡大機序を説明する上で大変魅力的な説である。以前からLuzzattoらは，この説を説明する一つの機序として，PNH患者において類似のTCRVβを発現するNKT細胞（CD8+，CD57+）集団の存在を報告していた[19]。彼らは，化学合成した血流型トリパノソーマ由来GPIとヒトGPIを用いた実験から，GPIがCD1dによってPNH患者のNKT細胞に提示され活性化することを示した[20]。GPIアンカー欠損細胞は，一部の健常者において微量ではあるが存在することや[21]，PNH患者では，複数のGPIアンカー欠損クローンが存在することが知られており[22]，1つのクローンが拡大していても，他のクローンは，必ずしも拡大しているとは限らないことから，*PIGA*の変異，免疫学的選択に加え，さらに何らかの付加的異常の関与が疑われていた。

井上らは，脂肪種や子宮筋腫等の良性腫瘍の原因遺伝子である*High-mobility group AT-hook 2*（*HMGA2*）が，2例でPNHクローンに異所性に発現し，腫瘍性性格を与えている事を報告していた[23]。その後，βサラセミア患者の骨髄細胞に正常なβグロビン遺伝子をレンチウイルスベクターを用いて遺伝子治療を行ったところ，βグロビン遺伝子を含むベクターは*HMGA2*遺伝子のイントロン3に挿入され，*HMGA2*遺伝子が異所性に発現した結果，クローン性の造血が回復し，輸血も不要となった[24]。さらに，*HMGA2*遺伝子を過剰発現したマウスを作成すると，造血幹細胞/前駆細胞の段階でクローン性の拡大を伴う造血能を獲得していることが示された[25]。これらのことは，*HMGA2*遺伝子の高発現がクローン性増殖をもたらすことを直接証明するもので，今後のさらなる解析が注目される。

PNHの治療

PNHは補体介在性の血管内溶血，骨髄不全および血栓症を3大症状とするが，それぞれの症状の程度とその全体のバランスは症例ごとに様々である。PNHの根治療法は造血幹細胞移植であるが，疾患の希少性もあり明確な基準はない（致命的な血栓症や重度の造血不全が主な適応と考えられる）。前述の如く，PNH患者は，3大症状のみならず，腹痛，嚥下障害，男性機能不全など多彩な症状を呈するが，これらの症状が溶血に起因していることが近年明らかになり，溶血を抑制することがPNH患者の多くの症状やQoLを著しく改善すると期待されるようになった[10]。このような潮流の中で，PNH溶血に対する治療薬として，新規補体阻害剤（eculizumab）が開発された。

1. 新規補体阻害剤eculizumab（Soliris®，アレクシオンファーマ）の開発

Eculizumabは，ヒトC5上のエピトープと結合し，C5転換酵素の作用を阻害する（Fig. 1）[26]。C5と特異的に結合することにより，炎症性メディエータであるC5a（アナフィラトキシン）の放出を阻害するとともに，C5b（膜侵襲蛋白）に引き続く膜破壊性のC5b-9複合体の生成を阻害するが，病原体のオプソニン化や免疫複合体の除去等には影響を与えない。結合部位である相補性決定領域（complementarity determining region, CDR）はマウスとヒトのキメラになっている。一方，重鎖定常領域（heavy-chain constant region, CH）1にヒトIgG2を，CH2と3にヒトIgG4を用いることにより，Fcレセプターに結合したり補体を活性化したりしないように工夫されているのが特徴である。

本邦での第2相試験AEGISに先立ち3つの臨床試験が海外で先行して行われた。英国で行われた第2相オープンラベルパイロット試験で，溶血に対する劇的な効果が示された[27,28]。引き続き欧米で行われた，有効性を判定するプラセボ対照二重盲検第3相試験TRIUMPH[29]と安全性を判定する非盲検第3相試験SHEPHERD（97例）[30]があり，いずれも共通の継続試験に入り（TRIUMPHのプラセボ群も含む），多くの欧米諸国で承認に至っている。本邦でもAEGIS試験（29例）が2008年に開始され，これまでの成績と同等の有効性と安全性が示され，2010年4月に承認に至った[2,31]。投与方法は25～45分点滴静注で，600 mgを週1回4週続けて投与し，5週目に900 mgを投与，その後2週毎に900 mg

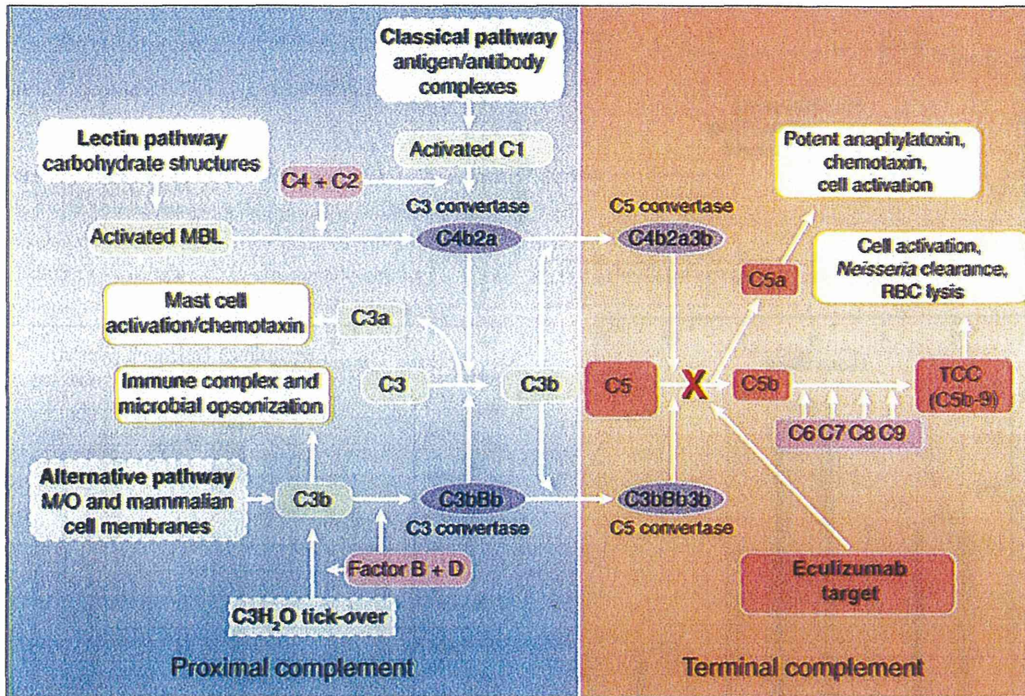

Fig. 1 The complement pathway and these regulatory factors. Eculizumab targets blockade of complement protein C5[29].

を継続投与する。投与に先立ち髄膜炎菌に対するワクチン接種が推奨される。

2. 本邦 AEGIS 試験における臨床成績

本邦では 12 週間の AEGIS 試験と[31]，引き続き 2 年間の継続試験が行われた[2]。AEGIS 試験には 29 人が参加し，溶血の指標である LDH の平均値は，投与前値の 1,845 U/*l* から投与 12 週後には 399 U/*l* まで低下した（P<0.001）（Fig. 2A）。LDH の低下は eculizumab 投与後 1 週目から見られ，投与期間中その効果は持続した。ヘモグロビン値も投与前中央値 7.6 g/d*l* に対し，投与後 9 g/d*l* と有意な改善（P<0.001）を認め，輸血量も投与前後 12 週で比較すると，5.2 U（平均）から 1.5 U（平均）へと有意に減少した（P=0.006）。投与前 12 週に輸血歴のあった 21 例のうち，14 例（66%）は投与後 12 週に輸血は不要となった（P=0.001）。このように輸血量は有意に減少するものの，輸血依存から脱却できない症例も存在することがわかった。疲労度について FACIT 疲労スコアを用いて調べると，投与後速やかに効果を認め，臨床的に有意な改善とされる 3 ポイント以上の改善を，1 週目に 38%，2 週目に 62%，12 週目に 66% の症例に認めた（Fig. 2B）。PNH 患者の慢性溶血による慢性腎臓病（CKD）は重要な予後因子であるが，CKD の病期分類を行うと，66%（19/29）の症例に CKD を認めた（Fig. 2C）。eculizumab 投与後 12 週で，19 例の CKD 合併症例のうち 12 例（63%）において病期の改善を認めた（Fig. 2D）。この改善効果は，軽度の CKD（1～2 期）においてより顕著であった。5 例において血栓・塞栓の既往を認めたが，12 週の投与期間中に血栓・塞栓を発症した症例はなかった。29 例中 11 例（38%）において，D ダイマーの投与前値が高値であったが，5 例（45%）は投与後 12 週で正常値に復した（P<0.001）。血栓症の予防効果については，欧米例でより顕著であった。欧米で行われた 3 つの臨床試験を合わせた継続試験における，治療前後の血栓症発症頻度に関する後方視的研究によって，eculizumab による血栓症予防効果を調べた[32]。継続試験の 195 例について，治療前後で血栓症発症頻度（事象/100 症例-年）を比較すると，治療前 7.4 に対し，治療後 1.1 と有意な予防効果を認めた（P<0.001）。

有害事象のほとんど（98.3%）は，軽度～中等度であった。試験期間中の死亡例，有害事象による脱落例，髄膜炎感染症，重篤な血管病変，溶血発作などの発症はなかった。重篤な有害事象としては，明らかな関連性が指摘できない発熱が 1 例報告されたのみであった。主な副作用としては，頭痛（52%），鼻咽頭炎（41%）及び悪心（21%）などで，頭痛を訴えた 15 例のうち 14 例は投与開始後 24 時間以内に発現した。すべての頭痛は軽度～中等度で，通常の頭痛薬で対処可能であった。2 年間