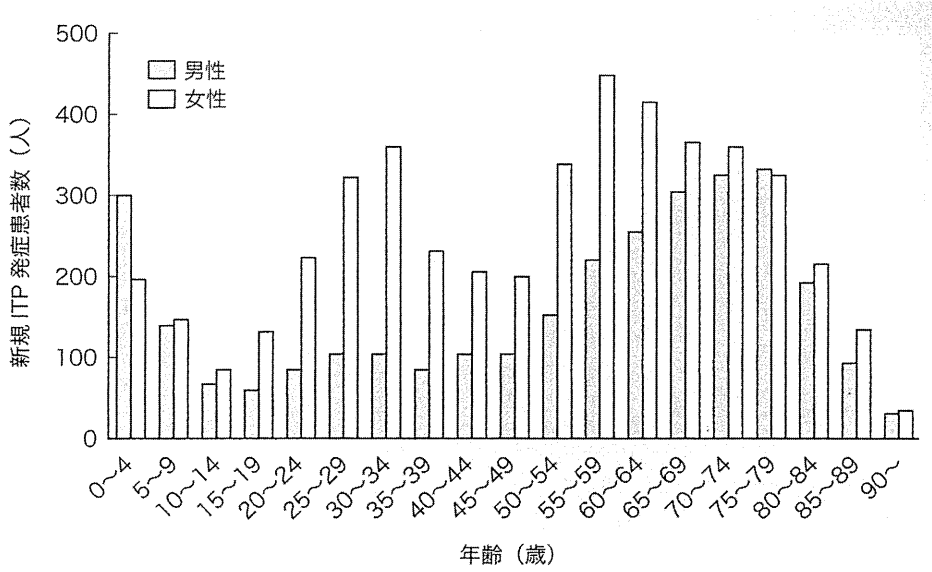


❶ 新規登録 ITP 症例の年齢分布

血液凝固異常症に関する調査研究班による2004～2007年度臨床調査個人票の解析結果の平均を示している．従来の20～40歳代の若年女性での発症に加え，60～80歳代での発症ピークが認められる．10万人あたり年間2.16人が新規に発症している（男性10万人あたり1.72人，女性10万人あたり2.58人）．
（Kurata Y, et al. Int J Hematol 2011[3] より）

小児 ITP では急性 ITP が約75～80％を占め，ウイルス感染や予防接種を先行事象として有する場合が多い．

ITP における血小板減少の主たる病態は，血小板の破壊亢進である．

慢性 ITP では，血小板は抗血小板自己抗体（主に IgG）により感作されており，自己抗体に感作された血小板は早期に脾臓を中心とした網内系においてマクロファージの Fc 受容体を介して捕捉・破壊され血小板減少をきたす．抗血小板自己抗体の主要な標的抗原は，血小板膜糖蛋白である GP Ⅱb-Ⅲa および GP Ib-Ⅸである．

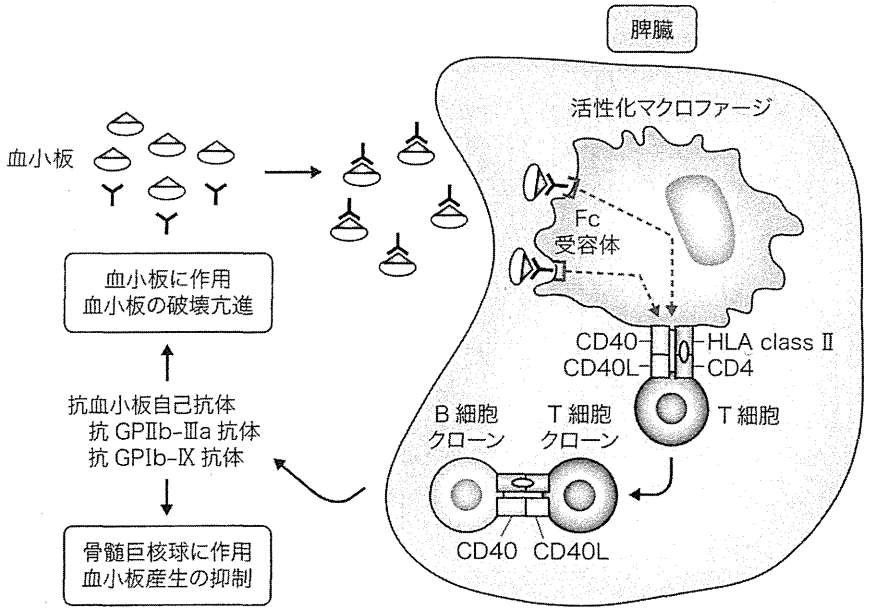
ITP においては，脾臓が主な血小板破壊部位であるとともに，血小板抗体産生部位でもある（❷）[4]．

血小板破壊亢進に加え，ITP においては巨核球の成熟障害や細胞障害を生じており，血小板産生も抑制されている．血小板自己抗体が骨髄巨核球にも結合し，血小板の産生障害を引き起こしている（❷）[4]．

ITP の主な臨床症状は，皮下出血，歯肉出血，鼻出血，性器出血など皮膚粘膜出血である．

一般的な血小板数と出血症状の関係を以下に示す（個人差あり）．

- 5万/μL 以上あれば出血傾向は明らかではなく，打撲時に四肢を中心に紫斑が出現する程度である．
- 3～5万/μL であれば易出血性を自覚することが多い．
- 3万/μL 未満であれば出血傾向が明らかとなる．
- 血小板数が1万/μL 未満になると血尿，消化管出血，吐血，網膜出血を認



❷ ITP の病態生理

主に脾臓で産生された抗血小板自己抗体（主に IgG）は，血小板膜 GPⅡb-Ⅲa あるいは GP Ib-Ⅸに結合し，感作血小板は主として脾臓内でマクロファージ上の Fc 受容体を介して捕捉され，破壊される．血小板を取り込んだマクロファージは，GPⅡb-Ⅲa あるいは GP Ib-Ⅸの抗原ペプチドを HLA 抗原上に表出し，HLA classⅡ-CD4 に加え副刺激経路（ここでは CD40-CD40L を提示）などを介して自己反応性ヘルパー T 細胞を活性化し，さらには B 細胞を活性化し抗体産生を誘導する．一方では，これらの抗体は巨核球の成熟障害などを誘導し，血小板産生を抑制する．
（冨山佳昭．臨床血液 2011[4] より）

めることがある．

血友病など凝固因子欠損症では関節内出血や筋肉内出血を生じるが，ITP では通常これら深部出血は認めない．

検査・診断

ITP の診断は除外診断が主体である．

血小板減少の基準は，10万/μL 未満である．出血の持続により貧血を示すことがある．凝固検査は正常値である．骨髄検査は必須ではないが，高齢者（60歳以上）や骨髄異形成症候群などが疑われる場合は，積極的に行うべきである．

PAIgG（platelet-associated IgG；血小板関連 IgG）は，ITP の補助診断として2006年に保険収載された．ITP の90％以上で PAIgG が上昇しており，その疾患感度は高いが特異度は低く，PAIgG の診断的意義は少ない．

ITP の病態に即した新たな診断法として網状血小板比率測定，血漿トロンボポエチン（TPO）濃度測定，GPⅡb-Ⅲa もしくは GP Ib-Ⅸに対する自己抗体検出があるが，これらの検査は2015年現在保険未収載である．

MEMO

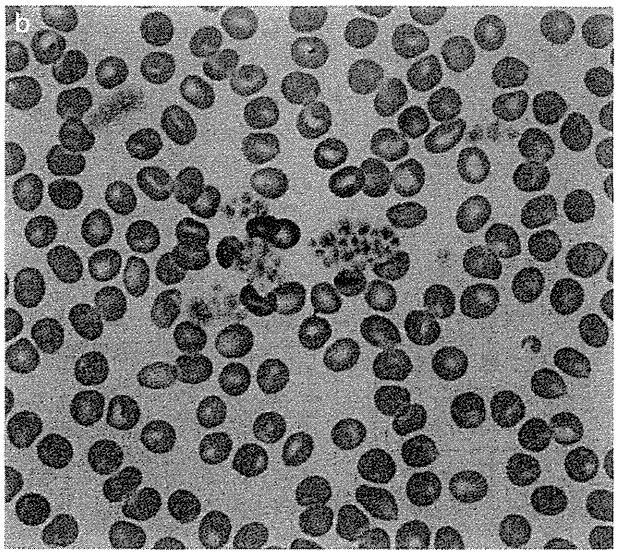
血小板数が5万/μL 未満の症例で出血傾向がまったくみられない場合や，末梢血の検査コメントに血小板凝集（＋）とある場合は，EDTA（エチレンジアミン四酢酸）依存性偽性血小板減少症を積極的に疑うべきである（❸）．

MEMO

網状血小板比率（％）は，新たに産生された幼若血小板の指標として用いられる．ITP など血小板破壊亢進時では網状血小板比率が増加するが，再生不良性貧血など血小板造血障害においては増加しない．一方，血漿 TPO 濃度は，ITP では正常ないしは軽度増加しているのみであるが，再生不良性貧血など造血障害による血小板減少では著増する（❹）[1]．

a

	血小板数
抗凝固薬（－）	22.6万/μL
EDTA-2K	
採血後　1分	20.4万/μL
15分	8.5万/μL
30分	5.2万/μL
1時間	3.5万/μL
2時間	3.3万/μL
3時間	3.2万/μL

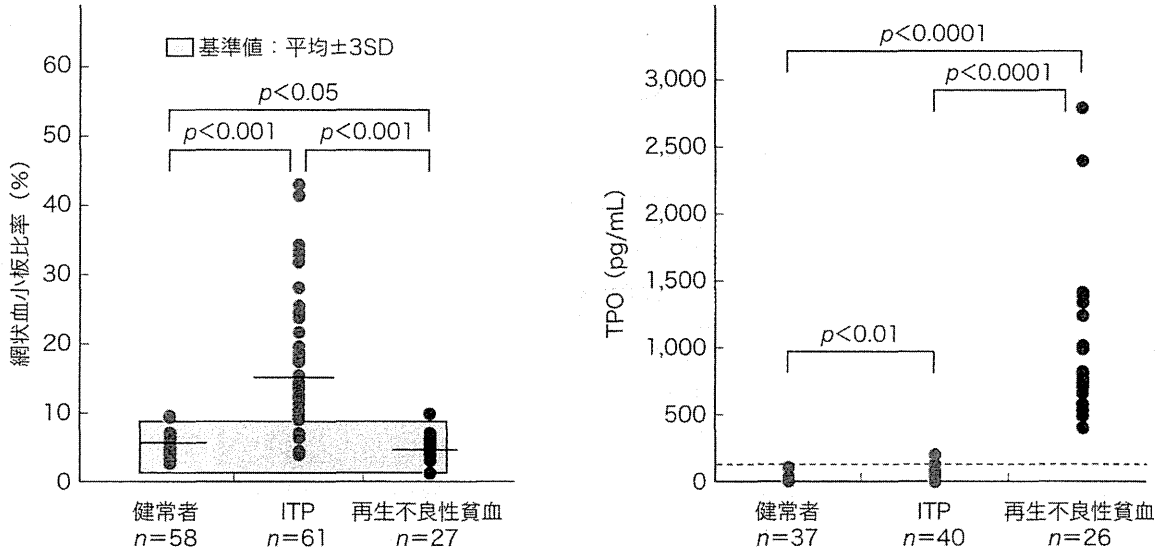


❸ EDTA 依存性偽性血小板減少症

EDTA 採血時，EDTA 依存性の抗体により血小板が採血後時間とともに凝集するため，みかけ上血小板数が低値となる．治療は不要である．

a：自験例における血小板減少の経時的変化．

b：採血1時間後の末梢血塗抹標本．



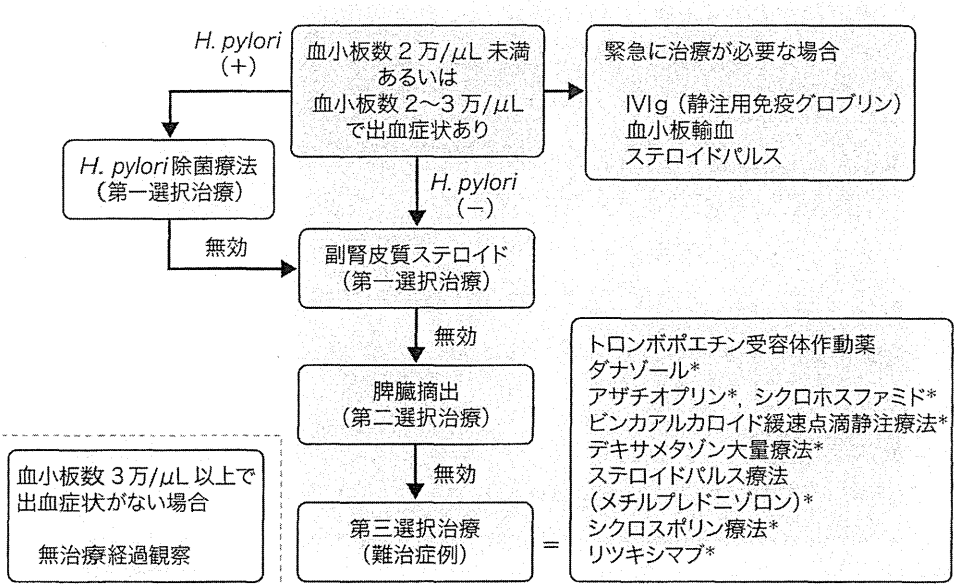
a．網状血小板比率　　b．血漿 TPO 濃度

❹ ITP および再生不良性貧血における網状血小板比率および血漿トロンボポエチン（TPO）濃度の比較検討

a：網状血小板（reticulated platelets；RP）は RNA が豊富に存在する大型血小板で，巨核球から新たに産生された幼若血小板である．患者血小板数あたりの網状血小板比率（RP%）を検討すると，ITP では RP%は著明に増加しているが，再生不良性貧血ではそのような増加はみられない．

b：TPO はその大部分が肝臓で産生されており，血小板数の変動に関係なくその産生量は一定に保たれている．TPO 受容体である c-Mpl は血小板/巨核球系に発現しており，c-Mpl による TPO 吸着が血漿 TPO レベルを制御している．再生不良性貧血では巨核球が減少し血小板産生が低下しているため血漿 TPO 濃度は著増する．一方，ITP においては，血小板減少にもかかわらず血漿 TPO 濃度は正常ないしは軽度増加しているのみであることが特徴である．-----は基準値上限を示す．

（冨山佳昭ほか．臨床免疫・アレルギー科 2013[1] より）



❺ 血液凝固異常症に関する調査研究班が作成した『成人特発性血小板減少性紫斑病治療の参照ガイド2012年版』の概要

*現時点で保険適用のない薬剤

治療[5)]

● ガイドラインの現況

- 厚生労働科学研究費補助金（難治性疾患克服研究事業）血液凝固異常症に関する調査研究班により『成人特発性血小板減少性紫斑病治療の参照ガイド（2012年版）』が作成され公開されている（https://www.jstage.jst.go.jp/article/rinketsu/53/4/53_433/_pdf）.
- 海外のガイドラインでは，アメリカ血液学会2011年版としてエビデンスに基づいたITP診療ガイド，国際作業部会が作成したITP診療に関するコンセンサスレポートが作成されている.
- 治療目標は，血小板数を正常化させることではなく，危険な出血を予防することである.
- 当面の目標は血小板数3万/μL以上であり，可能なら5万/μL以上を目指す．一方，初診時に血小板が3万/μL以上あり出血傾向を認めない場合は，無治療での経過観察とする．血小板数を正常に維持するために高用量の副腎皮質ステロイドを長期に使用すべきではない．❺に成人特発性血小板減少性紫斑病治療の参照ガイド（2012年版）の概要を示す[5)].

Helicobacter pylori（*H. pylori*）除菌療法

H. pylori 除菌療法はわが国におけるユニークな治療法である．*H. pylori* 感染陽性の場合，緊急時を除き，血小板数に関係なく，*H. pylori* 除菌療法を行う．除菌療法奏効例のうち約60～70％において血小板増加が認められる．2010年6月から保険適用されている.

- わが国では *H. pylori* 除菌療法の有効性は高いが，アメリカやスペインでは除菌療法のITPへの有効性は低く，除菌療法の効果は一定ではない．

副腎皮質ステロイド療法（第一選択治療）

- 血小板数2万/μL未満の症例，2～3万/μLで出血症状を伴う症例が対象である．特に口腔内や鼻腔内の出血を認める場合は積極的に治療を行う．50～75％において血小板が増加するが，多くは副腎皮質ステロイド減量に伴い血小板が減少する．4～6週間投与後，血小板数の増加がなくても徐々に減量する．血小板数および出血症状をみながら5 mgの割合でゆっくり減量し10 mg/日で維持し，経過が良ければさらに減量する．

脾臓摘出術（脾摘）（第二選択治療）

- 発症後6か月以上経過し，ステロイドの維持量にて血小板数3万/μL以上を維持できない症例，ステロイドの副作用が顕著な症例は積極的に脾摘を行う．寛解率は約60％である[6]．

難治ITP症例への治療法（第三選択治療）

- 対象は，副腎皮質ステロイドおよび脾摘療法が無効の症例，脾摘の了解が得られない症例もしくは合併症により脾摘が困難な症例，副腎皮質ステロイド不耐容症例でかつ血小板数は3万/μL未満であり出血症状を伴う症例，である．第三選択治療薬で，保険収載されているのは現時点ではTPO受容体作動薬のみである．

TPO受容体作動薬には，経口薬のエルトロンボパグ（毎日内服〈空腹時服用〉）と皮下注製剤のロミプロスチム（週1回投与）の2種類がある．

【処方例】

- レボレード®（経口薬），12.5～50 mg/日，1日1回，毎日内服（空腹時服用）
- ロミプレート®（皮下注製剤），1～10 μg/kg，毎週1回，皮下注射

- TPO受容体作動薬はc-Mplに結合し，巨核球の成熟を促進し血小板産生を亢進させる薬剤である（❻）[7]．いずれの薬剤も用量依存的に血小板増加反応を示す．一定用量投与により5～7日目から血小板数が増加し始め，12～16日目に最大の血小板数となる．難治症例の80％以上に有効で，出血が回避される．
- 現時点では，TPO受容体作動薬は数年間の投与においても比較的安全に用いられている．しかし留意すべき副作用として，

①血栓塞栓誘発（特に脳梗塞，心筋梗塞，肺塞栓などの血栓症の既往のある症例や抗リン脂質抗体陽性症例，がん患者などの血栓合併症のハイリスク患者に対して），

②骨髄でのレチクリン（細網）線維の増生（投与を中止すれば回復するとの報告がある），

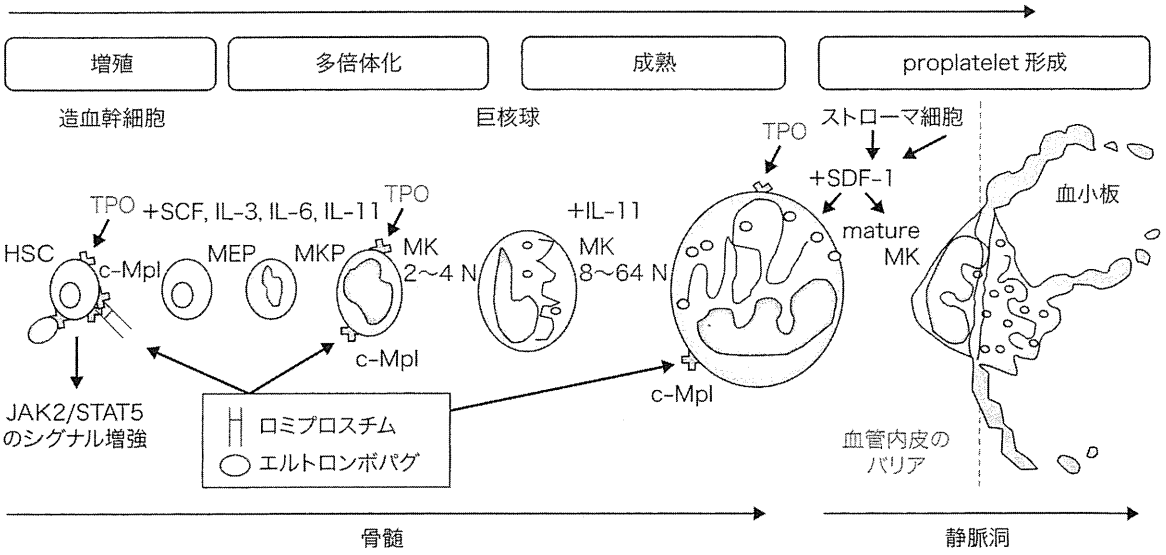
③使用中止後のリバウンドによる血小板減少，

などがあげられる．小児および妊婦には使用を避けるべきである．

MEMO

抗CD20抗体（リツキシマブ）（保険適用外）：リツキシマブは自己抗体産生B細胞に対しても細胞傷害作用を有することから，現在までに種々の自己免疫疾患に対してその有効性が示されている．欧米における後方視的解析では，60％に部分寛解以上（5万/μL以上）の効果を誘導しうるとされている．しかしながら，肝炎ウイルス再活性化などに留意する必要がある．

2013年，わが国で血小板数3万/μL未満の難治ITPを対象に，リツキシマブ375 mg/m^{2}を1週間ごとに4回投与し，6か月後の有効性（血小板数＞5万/μL）を検討した（医師主導型治験）．有効率は30.8％．2015年現在いまだ保険収載には至っていない．



❻ トロンボポエチン（TPO）受容体作動薬の巨核球系細胞への作用

TPO 受容体作動薬は，巨核球のみならず造血幹細胞にも作用し巨核球分化を促進する．血小板数のピークは薬剤開始後，約10～14日で得られる．

c-Mpl：TPO 受容体，HSC：hematopoietic stem cell（造血幹細胞），MEP：megakaryocyte-erythroid progenitor（赤芽球/巨核球系前駆細胞），MKP：megakaryocyte-committed progenitor（巨核球系前駆細胞），MK：megakaryocyte（巨核球），SDF-1：stromal cell-derived factor-1（ストローマ細胞由来因子1）

（Nurden AT, et al. Lancet 2009[7] より）

緊急時の治療

診断時に消化管出血や頭蓋内出血などの重篤な出血を認める症例や，脾摘など外科的処置が必要な症例には，γグロブリン大量療法やメチルプレドニゾロンパルス療法にて血小板数を速やかに増加させ出血をコントロールする必要がある．

血小板輸血は一般には行わないが，急性 ITP の重症例では治療抵抗性であることもあり，このような場合には血小板輸血も考慮する．

（冨山佳昭）

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ORIGINAL ARTICLE

Efficacy and safety of rituximab in Japanese patients with relapsed chronic immune thrombocytopenia refractory to conventional therapy

Yoshitaka Miyakawa[1] · Shinya Katsutani[2] · Takahiro Yano[3] · Shosaku Nomura[4] · Kaichi Nishiwaki[5] · Yoshiaki Tomiyama[6] · Masaaki Higashihara[7] · Yukari Shirasugi[8] · Masakatsu Nishikawa[9] · Katsutoshi Ozaki[10] · Takayuki Abe[11] · Kayoko Kikuchi[11] · Yuzuru Kanakura[12] · Kingo Fujimura[13] · Yasuo Ikeda[14] · Shinichiro Okamoto[15]

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Abstract Primary immune thrombocytopenia (ITP) is an autoimmune disease mediated by the production of autoantibody against platelets. Rituximab, an anti-CD20 antibody, is reported to be useful for treatment of ITP. In Japan, however, robust evidence on this treatment has not been accumulated. Hence, we conducted this open-label phase III clinical trial to confirm the efficacy and safety of rituximab, administered at 375 mg/m^2 once per week at weekly intervals for 4 consecutive weeks in Japanese patients with chronic ITP, who had relapsed and were refractory to conventional therapy. The primary endpoint was defined as the percentage of patients with a platelet count above 50 × 10^9/L at week 24 after the first dose of rituximab, which was 30.8 % of 26 patients (95 % confidence interval 14.3–51.8 %). Although the lower confidence limit of primary endpoint failed to meet the pre-specified threshold of 20 %, the clinical efficacy of rituximab is substantial in consideration of the 2 % response rate in the placebo arm in other clinical studies in patients with chronic ITP. We conclude that rituximab is clinically useful and safe in the treatment of Japanese patients with chronic ITP, achieving the goal of maintaining platelet count and reducing risk of bleeding while minimizing treatment-related toxicity.

Keywords Immune thrombocytopenia · Platelets · Rituximab

✉ Yoshitaka Miyakawa
miyakawa@saitama-med.ac.jp

1 Department of General Internal Medicine, Saitama Medical University, 38 Morohongo, Moroyamamachi, Iruma-gun, Saitama 350-0495, Japan

2 Department of Hematology and Oncology, Hiroshima University, Hiroshima, Japan

3 Hematology, Internal Medicine, National Hospital Organization Tokyo Medical Center, Tokyo, Japan

4 First Department of Internal Medicine, Kansai Medical University Hirakata Hospital, Osaka, Japan

5 Department of Oncology and Hematology, The Jikei University School of Medicine, Kashiwa Hospital, Chiba, Japan

6 Department of Blood Transfusion, Osaka University Hospital, Osaka, Japan

7 Department of Hematology, Kitasato University Hospital, Kanagawa, Japan

8 Department of Hematology and Oncology, Tokai University Hospital, Kanagawa, Japan

9 Clinical Research Support Center, Mie University Hospital, Mie, Japan

10 Division of Hematology, Jichi Medical University, Tochigi, Japan

11 Center for Clinical Research, Keio University School of Medicine, Tokyo, Japan

12 Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Osaka, Japan

13 Yasuda Women's University, Hiroshima, Japan

14 Faculty of Science and Engineering, Waseda University, Tokyo, Japan

15 Division of Hematology, Department of Internal Medicine, Keio University School of Medicine, Tokyo, Japan

Introduction

Primary immune thrombocytopenia (ITP) is a thrombocytopenia-causing autoimmune disease. Approximately 20,000 patients are suffered from this disease in Japan, and about 3000 new cases occur per year [1]. The causes of decrease in the platelet count are known to be destruction of platelets, to which auto-antibody is attached, in the spleen, and defects of proliferation and maturation of megakaryocyte due to the relative shortage of thrombopoietin (TPO). The major clinical symptoms and signs of ITP are petechiae and mucosal hemorrhage. ITP is classified by duration into newly diagnosed (within 3 months of onset), persistent (3–12 months' duration) and chronic (12 months or more in duration). Of ITP with onset in adult age, about 90 % of the patients are chronic and its male-to-female ratio is 1:2. Massive hemorrhage is relatively uncommonly seen in chronic ITP, whereas seen in the other types of ITP.

Patients who show no effects on standard therapy and have platelet count $\leq 30 \times 10^9$/L are diagnosed with refractory ITP, and those are about 10 % of total patients with ITP. Such patients have a 4.2-fold higher risk of death than healthy population [2]. In Japan, nearly half of patients with ITP are infected with *Helicobacter pylori*, and *H. pylori* eradication therapy is effective for restoring the platelet count in 60 % of patients [3].

The first-line treatment of standard therapy is corticosteroid administration. Corticosteroid administration can only be discontinued in about 10–20 % of the patients. For most of the patients, the steroid therapy is continued especially in Japan. Splenectomy is chosen as a second-line treatment for patients who fail to respond or have poor tolerability to corticosteroids. A radical cure can be expected from splenectomy in about 70 % of patients, while for the remaining 30 % of patients this surgical intervention makes to be ineffective. Splenectomy has several concerns such as complications in the perioperative phase (mortality rate at 0.1 % and complication rate at 10 %), postoperative depression of immune functions, and relapses in about 20 % of patients [4]. Since it is difficult to predict the efficacy of surgical removal of the spleen in patients with ITP, both patients and physicians tend to avoid splenectomy in Japan, as well as in Europe and the United States. Non-responders to splenectomy die at about 10 % from serious hemorrhage such as cerebral hemorrhage. Therefore, the goal of ITP treatment is to increase the platelet count to $\geq 30 \times 10^9$/L to avoid such fatal bleeding [5, 6].

Immunosuppressants such as azathioprine and cyclosporine, and anticancer agents such as cyclophosphamide and vincristine, in off-label use, are empirically prescribed for patients who are non-responding or ineligible for splenectomy. With these drugs, response rates are as low as about 30 %, and adverse events are rather frequently observed. Recently, thrombopoietin receptor agonists (romiplostim and eltrombopag) have been approved for refractory ITP and showed efficacy in nearly 60 % of patients. However, some concerns arise from these agonists, including offset of drug effect back to baseline level in about 2 weeks after discontinuation of the medication, a high cost of drug expense as high as 2–3 million yen per year, a high incidence of thrombotic complications in some of patients with a certain background, and disease progression to myelofibrosis or acute leukemia after long-term treatment.

Rituximab is a chimeric monoclonal antibody against the CD20 antigen and prepared by recombinant DNA technology. Rituximab was approved in Japan for the treatment of B cell non-Hodgkin's lymphoma, microscopic polyangiitis (MPA), and granulomatous polyangiitis (GPA; Wegener granulomatosis). In addition to these indications, this drug was approved for the treatment of chronic lymphoid leukemia and rheumatoid arthritis in the United States and Europe. Rituximab specifically eliminates CD20-positive B lymphocytes; therefore, its efficacy for various disorders relevant to B cell abnormalities is anticipated [7, 8]. Recent studies have demonstrated that B cells are involved in the onset and maintenance of autoimmune diseases, and the efficacy of treatment with rituximab has been reported in autoimmune disorders such as systemic lupus erythematosus (SLE), multiple sclerosis, nephrotic syndrome, and thrombotic thrombocytopenic purpura (TTP). Outside of Japan, rituximab is extensively prescribed and accepted as the second-line treatment for refractory ITP [5, 9–11]. A systematic review on the efficacy and safety of rituximab in approximately 300 patients with ITP by Arnold et al. [11] showed that the response rate was 62.5 %, and the time to therapeutic response was 5.5 weeks. A phase II clinical trial of rituximab in 60 patients with refractory ITP in France showed that the response rate was 40 % after 1 year of the treatment [12]. As seen in a number of reports outside Japan, the efficacy and safety of rituximab in patients with refractory ITP have been extensively evaluated and the effectiveness of the drug has been demonstrated.

In Japan, there are several case reports to indicate the efficacy of rituximab in the treatment of refractory ITP, but robust evidence on this treatment has not yet been accumulated such as from clinical studies. Rituximab is then prescribed off-label for rescuing patients with refractory ITP, but the medication cost of rituximab is not reimbursed under the Japanese National Health Insurance program.

Hence, we conducted this open-label phase III clinical trial to confirm the efficacy and safety of rituximab, administered at 375 mg/m^2 (body surface area) once a week, at weekly intervals for 4 consecutive weeks in Japanese patients with chronic refractory ITP. This study was implemented in accordance with the International

Consensus Guidelines for Diagnosis and Treatment of ITP [10], and the Japanese Guidelines for Treatment of ITP. This study was registered with the Japan Medical Association Center for Clinical Trials (JMACTR; CTR Number: JMA-IIA00070, https://dbcentre3.jmacct.med.or.jp/jmactr/default.aspx?JMACCTID=JMA-IIA00070).

Materials and methods

Patient population

Patients included in this study were: Japanese, aged ≥20 years, and diagnosed with chronic refractory ITP at least 12 months before the enrollment of this study. The definition of the term *refractory* in this study was as follows: platelet counts ≤30 × 10^9/L (measured at weeks 4 and 2 before enrollment), ineffective or intolerable for steroids, ineffective or judged as inappropriate by investigators for splenectomy, and ineffective, intolerable or judged as inappropriate by investigators for thrombopoietin receptor agonists.

Study design

This study was an open-label multicenter phase III clinical trial conducted between October 2011 and July 2013 in ten clinical institutions in Japan. The study consisted of screening (4 weeks), treatment (4 weeks), and follow-up periods (20 weeks).

Rituximab was administered at 375 mg/m^2 in once a week consecutively for 4 weeks (weeks 0, 1, 2, and 3). To prevent infusion reactions associated with rituximab infusion, patients received pre-medications of oral antipyretic–analgesics, oral antihistamines, and intravenous hydrocortisone at 30 min before each administration of rituximab.

During the study period, the following concomitants or therapies were prohibited: immunoglobulin preparations, drugs which stimulate platelet production, splenectomy, *H. pylori* eradication therapy, hematopoietic factors, antineoplastics and platelet transfusion.

Efficacy and safety analysis

The primary efficacy endpoint was a response rate: the percentage of patient with the platelet count ≥50 × 10^9/L at 24 weeks after the first administration of the study drug.

The major secondary efficacy endpoints included the percentage of patients with the platelet count ≥100 × 10^9/L and who did not have bleeding at week 24, the percentage of patients with the platelet count ≥30 × 10^9/L and ≥two-fold higher than the baseline value and who did not have bleeding at week 24, and the improvement rate of bleeding symptoms [World Health Organization (WHO) bleeding scale]. In addition, the changes of peripheral blood B cells (CD19 and CD20) and T cells (CD3), and changes of serum IgG, IgM, and IgA levels were evaluated as exploratory endpoints. Safety parameters (adverse events and clinical laboratory data) were also assessed.

Statistical considerations

Sample size and its rationale were pre-specified in the study protocol. Response rate in the primary endpoint was assumed to be 50 % based on results of clinical studies [11, 12]. Twenty-four patients were needed to have ≥80 % power to show that the lower limit of 95 % confidence interval (CI) for the response rate is greater than a threshold (20 %). The threshold was determined conservatively taking account of a response rate, 2 % (1/42 patients, 95 % CI 0–12.6 %) in placebo group in the phase III clinical trial of romiplostim in patients with refractory ITP [13]. All enrolled patients were included in the primary efficacy analysis population (full analysis set, FAS). Demographic factors and baseline characteristics were summarized with mean ± standard deviation (SD) or median (interquartile range, IQR) depending on distributions. Exact 95 % CIs for proportions were calculated with the Clopper–Pearson method. Paired proportions were compared with the exact McNemar's test. For continuous valuables, values at each time point were compared with baseline values by signed rank sum test. Significance level was a two-sided 5 % for all tests. All data were analyzed with SAS version 9.2 (SAS Institute Inc., Cary, NC, USA).

Ethical considerations

This clinical trial was conducted in compliance with the ethical principles of the Declaration of Helsinki, the Japanese Guidelines for Good Clinical Practice, and other relevant regulatory requirements. The investigator or co-investigator gave a full explanation of the clinical trial to patients prior to participation in the study and, upon confirming that the patients gained a good understanding of the nature of the study, obtained written informed consent for voluntary participation in the study. Prior to conduct of this clinical trial, the institutional review board (IRB) of each participating medical facility reviewed the ethical, scientific, and medical propriety of this clinical trial and approved this study.

Table 1 Patient demography and disease characteristics

	Analysis set: $n = 26$
Sex (females)[a]	23 (88.5 %)
Age (years)[b]	39.7 ± 13.0 (23, 69)
Body weight (kg)[b]	56.5 ± 9.7 (37.9, 73.1)
Duration of ITP (years)[c]	5.9 (1.9, 11.2)
Hemorrhagic symptoms (WHO bleeding scale)	
Grade 0[a]	11 (42.3 %)
Grade 1[a]	14 (53.8 %)
Grade 2[a]	1 (3.8 %)
Baseline platelet count (10^9/L)[c]	22 (17, 24)
Baseline CD3 cells (/μL)[c]	1035.5 (798, 1588)
Baseline CD19 cells (/μL)[c]	97 (63, 147)
Baseline CD20 cells (/μL)[c]	91.5 (59, 145)
Had splenectomy (yes)[a]	4 (15.4 %)
Had *H. pylori* eradication (yes)[a]	9 (34.6 %)
Had complications (yes)[a]	21 (80.8 %)
Previous therapy for ITP	
Had corticosteroids (yes)[a]	18 (69.2 %)
Had high-dose immunoglobulin therapy (yes)[a]	10 (38.5 %)
Had thrombopoietin receptor agonists (yes)[a]	7 (26.9 %)
Number of previous therapies for ITP[c]	2 (1, 3)

[a] Number of patients (%)

[b] Mean ± standard deviation (range)

[c] Median (25 % point, 75 % point)

Results

Patient characteristics

Written informed consent was obtained from 49 patients in this clinical trial. Of them, 26 patients who met the inclusion criteria were enrolled. All the 26 patients completed a total of four doses of rituximab infusion and were included in the FAS. None of the patients discontinued the study treatment. The following measured values of the platelet count were partially excluded from the FAS: the platelet counts of one patient at week 4 and week 0 when platelet aggregation was seen in the sample of the patient, and the platelet count of other patient at the time when the platelet count was increased due to emergency treatment (high-dose immunoglobulin therapy plus platelet transfusion) at week 2.

Although protocol deviations were seen in 12 patients, none were relevant to patient eligibility or discontinuation criteria. None of these patients were excluded from the efficacy and/or safety analysis, as the deviations in this study were examined at the case-conference meeting and judged not to significantly influence on the evaluation of the study.

Most of the patients with refractory ITP enrolled in this study were female (88.5 %). The mean age was 39.7 ± 13.0 years (Table 1). The median duration of ITP was 5.9 years (IQR 1.9–11.2), and Grade 0, 1, and 2 hemorrhagic symptoms in severity at baseline were 42.3, 53.8, and 3.8 % of patients, respectively. The median platelet count at baseline was 22 × 10^9/L (IQR 17–24). Of the enrolled patients, 15.4 % had previously received splenectomy and 34.6 % underwent *H. pylori* eradication. The percentages of patients who had previously received corticosteroids, high-dose immunoglobulin therapy, and thrombopoietin receptor agonists, as prior therapy for ITP, were 69.2, 38.5, and 26.9 %, respectively.

Primary efficacy endpoint

The percentage of patients who had achieved the platelet count ≥50 × 10^9/L at week 24 was 30.8 % (8/26 patients). The 95 % CI of the response rate was 14.3–51.8 %, and the lower limit of CI did not exceed the threshold of 20 %. However, in comparison with the response rate of 0–2 % in placebo group reported in other clinical studies with a similar target population to this study, it was suggested that the efficacy of rituximab observed in this study is substantial. The number of patients who achieved the platelet count ≥50 × 10^9/L at each time point for the assessment is shown in Table 2. Box plots of the trajectory of the platelet count over 24 weeks in a subgroup which consists of eight responders is shown in Fig. 1. In these eight responders, mean platelet count reached ≥50 × 10^9/L at week 4 and continued to increase throughout the follow-up period (Fig. 1).

Secondary efficacy endpoints

The percentage of patients with the platelet count ≥100 × 10^9/L and who did not have bleeding at week 24 was 15.4 % (4/26 patients; 95 % CI 4.4, 34.9 %). The percentage of patients with the platelet count ≥30 × 10^9/L and ≥twofold higher than the baseline value and who did not have bleeding at week 24 was 26.9 % (7/26 patients; 95 % CI 11.6, 47.8 %).

As for the percent changes of the platelet count, the median platelet count was significantly increased compared with the baseline at every time point from week 1 to week 24 ($P < 0.01$, signed rank sum test; Fig. 2). The median platelet count exceeded ≥30 × 10^9/L at week 8 and remained stable throughout the follow-up period.

The percentage of patients with Grade ≥1 hemorrhage in the WHO bleeding scale was numerically decreased at every time point compared with the baseline. Statistically significant decrease was observed at week 8 compared with the baseline (26.9 vs 57.7 %, $P = 0.0215$; exact

Table 2 Percentages of patients who had achieved the platelet count $\geq 50 \times 10^9$/L at each time point

Time points	*n*	No. of patients who achieved	%	Two-sided 95 % CI of the percentage
Week 1	26	3	11.5	(2.4, 30.2)
Week 2	25	2	8.0	(1.0, 26.0)
Week 3	26	4	15.4	(4.4, 34.9)
Week 4	26	3	11.5	(2.4, 30.2)
Week 8	26	7	26.9	(11.6, 47.8)
Week 12	26	6	23.1	(9.0, 43.6)
Week 16	26	8	30.8	(14.3, 51.8)
Week 20	26	8	30.8	(14.3, 51.8)
Week 24	26	8	30.8	(14.3, 51.8)

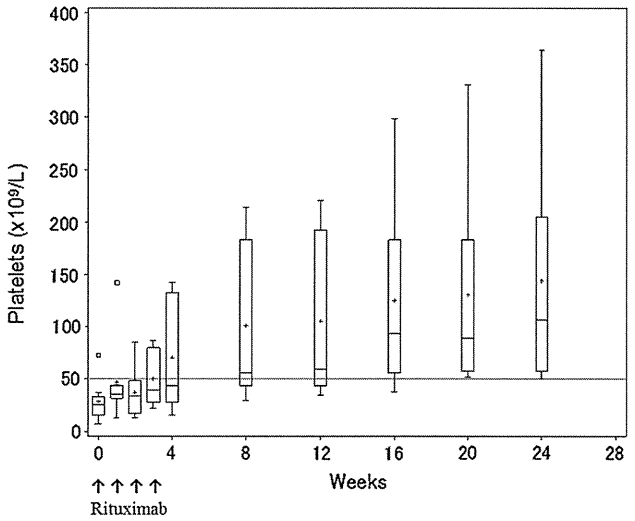

Fig. 1 *Box plots* of platelet counts of the eight patients who met the primary response (platelet count >50 $\times$ 10^9/L at week 24). *Central horizontal bold line* is the median; the *lower* and *upper box* limits are the 1st and 3rd quartiles, respectively; and the *whiskers* extended to the most extreme data points, which do not exceed the 1.5 $\times$ the interquartile of the box. *Plus symbol* represents the mean value

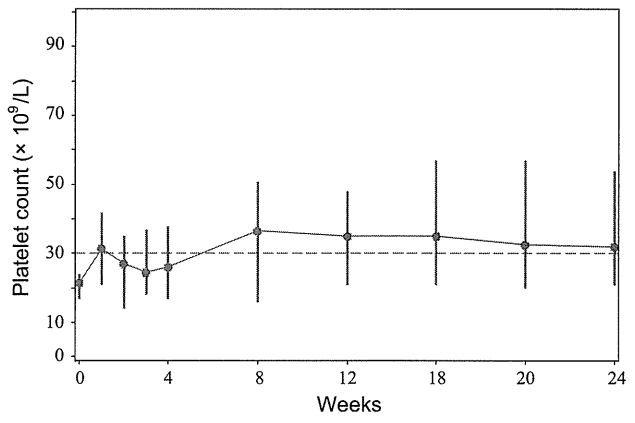

Fig. 2 Time course of median platelet count (FAS). *Black circles* are median values of platelet count. The *lower* and *upper ends* of *vertical lines* are the 1st and 3rd quartiles, respectively

McNemar's test), whereas no significant differences were observed at other time points.

Subgroup analysis

In Table 3, the results of subgroup analyses of the percentage of patients with the platelet count $\geq 50 \times 10^9$/L at week 24 were summarized. Subgroups with higher response rate were patients with duration of ITP shorter than the median duration 5.9 years (46.2 %, 6/13 patients), patients who underwent splenectomy (50.0 %, 2/4 patients), patients who did not have concomitants for ITP at baseline (60.0 %, 3/5 patients), patients who did not previously receive thrombopoietin receptor agonists (36.8 %, 7/19 patients), and patients with previous therapies for ITP less than 3 (41.2 %, 7/17 patients). While factors predictive of response to rituximab have not been consistently identified across studies, shorter duration of ITP was reported to be associated with good response from several studies [14, 15] as we found in this study.

Exploratory efficacy endpoints

Peripheral blood B cells (CD20-positive cells and CD19-positive cells) were significantly decreased at week 2 and subsequent time points compared with the baseline ($P < 0.001$ for both parameters: signed rank sum test). The median absolute B cell count at week 2 was <5 cells/μL and persisted in low during the study. Transient but significant decrease of peripheral blood T cells (CD3-positive cells) was observed at weeks 2, 4, and 12, compared with the baseline ($P < 0.05$, signed rank sum test); however, the median value of absolute cell count of CD3-positive T cells remain >790 cells/μL throughout the study.

Serum IgG levels were significantly increased at week 4 compared with the baseline ($P = 0.023$, signed rank sum test), whereas IgM levels were significantly decreased at weeks 12 and 24, compared with the baseline ($P < 0.001$, signed rank sum test). These changes of serum IgG and IgM, however, were within the normal range. Serum IgA

Table 3 Subgroup analysis of the percentage of patients who had achieved the platelet count $\geq 50 \times 10^9$/L at week 24 after administration of the study drug

Subgroups	*n*	No. of patients who achieved	%	Two-sided 95 % CI of the percentage
Duration of ITP (median: 5.9 years) (years)				
<5.9	13	6	46.2	(19.2, 74.9)
≥5.9	13	2	15.4	(1.9, 45.4)
Had splenectomy				
No	22	6	27.3	(10.7, 50.2)
Yes	4	2	50.0	(6.8, 93.2)
Had concomitant drugs for ITP at baseline				
None	5	3	60.0	(14.7, 94.7)
Yes	21	5	23.8	(8.2, 47.2)
Baseline platelet count (/L)				
$<15 \times 10^9$	6	2	33.3	(4.3, 77.7)
$\geq 15 \times 10^9$	20	6	30.0	(11.9, 54.3)
Previously received thrombopoietin receptor agonists				
No	19	7	36.8	(16.3, 61.6)
Yes	7	1	14.3	(0.4, 57.9)
Number of previous therapies for ITP				
<3	17	7	41.2	(18.4, 67.1)
≥3	9	1	11.1	(0.3, 48.2)

levels were not significantly changed from the baseline over 24-week study period.

Safety

Three serious adverse events required inpatient hospitalization were reported in three patients: one patient with grade 3 viral infection, one with grade 2 viral infection and one with grade 2 hypermenorrhea. All these three events recovered by supportive treatment and the patients discharged from the hospital in a week. The causal relationship of all the serious adverse events with rituximab was not completely ruled out.

The other adverse drug reactions (ADRs) that occurred in two or more patients were upper respiratory tract infection and headache in three patients each, and diarrhea, abdominal pain, malaise, and cough in two patients each. All these ADRs were grade 1 or 2 in severity.

Infusion related reactions were observed in eight patients and those that occured in two or more patients were fever, oropharyngeal pain, headache, pruritus, urticaria, and hypersensitivity, all of which were grade 1 or 2 in severity. Infusion related reactions were most frequently observed at the initial administration of rituximab (at week 0) among the injection-time points in the 4-dose study drug regimen. None of patients had adverse events led to discontinuation of the study drug, and no deaths were reported in this study.

Discussion

The response rate of the primary efficacy endpoint in this study, the percentage of patients with the platelet count $\geq 50 \times 10^9$/L at week 24 after the first administration of rituximab, was 30.8 % (95 % CI 14.3–51.8 %), and failed to meet the pre-determined statistical criteria of the lower confidence limit of 20 %.

However, the efficacy of rituximab in patients with chronic refractory ITP in this study is substantial when compared with the modest response rate of 2 % (1/42 patients; 95 % CI 0, 12.6 %) in placebo group reported in other clinical studies in patients with refractory ITP [13]. Also, as seen in the subgroup analysis, even heavily treated patients with chronic refractory ITP in this study exhibited moderate efficacy, with the platelet count $\geq 50 \times 10^9$/L at week 24, to rituximab as shown below: 50.0 % (2/4 patients) of patients who underwent splenectomy and 14.3 % (1/7 patients) of patients who previously received thrombopoietin receptor agonists. This trend becomes much clearer in this study when considering clinical benefit to patients who are at risk of fatal bleeding (i.e., the platelet count $\leq 30 \times 10^9$/L). As additional analysis, the percentages of patients with the platelet count $\geq 30 \times 10^9$/L at week 24 after administration of the study drug were evaluated, and rituximab then showed considerably high effectiveness in a total of patients (57.7 %, 15/26 patients) as well as patients previously heavily treated, who underwent splenectomy (75.0 %, 3/4 patients) and who received

thrombopoietin receptor agonists (71.4 %, 5/7 patients). These lines of evidence suggest that rituximab can clinically useful for the treatment of Japanese patients with chronic refractory ITP.

The goal of treatment in ITP is to maintain the platelet count which reduces the risk of bleeding while minimizing treatment-related toxicity. To accomplish this goal, thrombopoietin receptor agonists are recently used. However, thrombocytopenia usually recurring shortly after the drug withdrawal is known as one of the drawbacks of these agonists. Thus, these agents are indefinitely used to maintain the platelet count to minimize bleeding [16]. In the sense, this study showed that rituximab's effect lasted longer after completion of treatment; the platelet counts at all the time points exceeded 30×10^9/L until week 24 after the last dose of the study drug at week 3.

Clinical significance of rituximab in patients with ITP is still being investigated extensively outside Japan to position the therapy at an alternative treatment for ITP prior to splenectomy [15–18]. Although the details of study design was varied from study to study in terms of target population (e.g., newly diagnosed or relapsed ITP), concomitant therapy (e.g., with or without steroids), dosage and administration of rituximab (e.g., 4-weekly 375 mg/m^2 or 2-times 1000 mg 2 weeks apart), and endpoints of efficacy analysis, rituximab commonly showed a clinically substantial efficacy and well tolerability in patients with ITP. Especially, several studies showed a clinically meaningful sustained response to have a chance for sparing splenectomy [12, 18–23], supporting our results in this study. Regarding the safety of rituximab, we observed severe adverse events in three patients. Two had viral infection of unknown etiology and one had hypermenorrhea. All three events were resolved by supportive treatment and all patients were discharged from hospital within a week of admission.

We, thus, conclude from the above-mentioned results that rituximab is clinically useful and involves no particular safety concerns in the treatment of Japanese patients with chronic refractory ITP.

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Compliance with ethical standards

Conflict of interest Dr. Miyakawa reports non-financial support from Zenyaku Kogyo, grants from Japan Medical Association Center for Clinical Trials (JMACCT), during the conduct of the study; grants and personal fees from Alexion pharmaceutical, personal fees from Daiichi Sankyo, personal fees from Fuji film, personal fees from GlaxoSmithKline, personal fees from Kyowa-Hakko Kirin, personal fees from Ono pharmaceuticals, outside the submitted work; Dr. Kanakura reports grants from Chugai Pharmaceutical Co. Ltd., personal fees from Zenyaku-Kogyo, during the conduct of the study; grants and personal fees from Kyowa-Hakkyo Kirin Co. Ltd., grants and personal fees from Otsuka Pharmaceutical Co. Ltd., grants from Takeda Pharmaceutical Co. Ltd., grants from Teijin Pharma, grants from Janssen Pharmaceutical Co. Ltd., grants and personal fees from Alexion Pharmaceutical Co. Ltd., personal fees from Shire Co. Ltd., grants from Bristol-Myers Squibb Co. Ltd., outside the submitted work; Dr. Yano has nothing to disclose. Dr. Shirasugi reports personal fees from Kyowa-Hakko Kirin, personal fees from Pfizer, personal fees from Ono pharmaceutical, personal fees from Bristol-Myers Squibb, personal fees from Celgene, personal fees from GlaxoSmithKline, outside the submitted work; Dr. Higashihara reports grants and personal fees from Chugai pharmaceutical, during the conduct of the study; grants and personal fees from Kyowa-Hakko Kirin, grants from MSD, grants from Jannsen pharmaceutical, grants from Takeda, personal fees from Sumitomo Dainippon pharma, grants from Teijin, grants from Astellas pharma, personal fees from GlaxoSmithKline, outside the submitted work; Dr. Nishiwaki reports grants from Zenyaku Kogyo Company, Limited, grants from Chugai Pharmaceutical, grants from Novartis Pharma K.K., outside the submitted work; Dr. Okamoto reports grants from Chugai pharmaceuticals, during the conduct of the study; personal fees from KyowaHakkoKirin, personal fees from Takeda, personal fees from Bristol-Myers Squibb, personal fees from Janssen pharmaceutical, personal fees from Celgene, personal fees from Alexion, personal fees from Eisai, grants from GlaxoSmithKline, outside the submitted work; Dr. Katsutani has nothing to disclose. Dr. Nomura has nothing to disclose. Dr. Kikuchi has nothing to disclose. Dr. Abe has nothing to disclose. Dr. Nishikawa has nothing to disclose. Dr. Ozaki reports grants from Japan Medical Association Center for Clinical Trials, during the conduct of the study; personal fees from Nippon Shinyaku, personal fees from Janssen pharmaceutical companies, personal fees from Chugai pharmaceutical, personal fees from Celgene, personal fees from Kyowa-Hakko Kirin pharmaceutical, outside the submitted work; Dr. Ikeda reports personal fees from Chugai pharmaceuticals, during the conduct of the study; personal fees from Novartis, personal fees from Sanofi, personal fees from Daichi Sankyo, outside the submitted work; Dr. Tomiyama reports grants from JMACCT, during the conduct of the study; personal fees from GSK, personal fees from Novartis, personal fees from Kyowa-Hokko Kirin, personal fees from Eisai, personal fees from Sysmex, outside the submitted work; Dr. Fujimura reports non-financial support from Kyowa-Hakko Kirin, during the conduct of the study; non-financial support from Medical support, outside the submitted work.

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ORIGINAL ARTICLE

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

Mikiko Sakuragi · Satoru Hayashi · Miho Maruyama · Osamu Kabutomori · Tomoko Kiyokawa · Keisuke Nagamine · Hisashi Kato · Hirokazu Kashiwagi · Yuzuru Kanakura · Yoshiaki Tomiyama

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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

M. Sakuragi · S. Hayashi · T. Kiyokawa · K. Nagamine · H. Kato · Y. Tomiyama (✉)
Department of Blood Transfusion, Osaka University Hospital, Suita, Osaka 565-0871, Japan
e-mail: yoshi@hp-blood.med.osaka-u.ac.jp

M. Maruyama · O. Kabutomori
Laboratory for Clinical Investigation, Osaka University Hospital, Suita, Osaka, Japan

H. Kato · H. Kashiwagi · Y. Kanakura · Y. Tomiyama
Department of Hematology and Oncology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan

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 ± 34 × 10^3/μl (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 ± 28 × 10^3/μl (mean ± SD)], and 80 healthy controls [35 males and 45 females, age 34 ± 12 years, platelet count 269 ± 58 × 10^3/μl (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 × 10^3/μl. 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

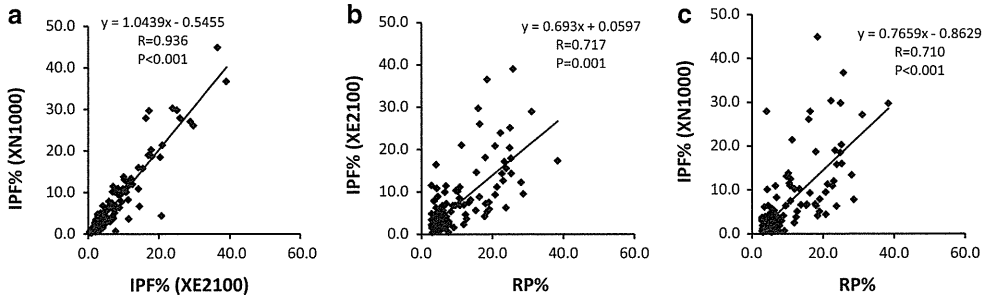

Fig. 1 Correlations between IPF% (XE-2100), IPF% (XN-1000), and RP% by flow cytometry. **a** IPF% (XE-2100) versus IPF% (XN-1000), **b** RP% versus IPF% (XE-2100), and **c** RP% versus IPF% (XN-1000)

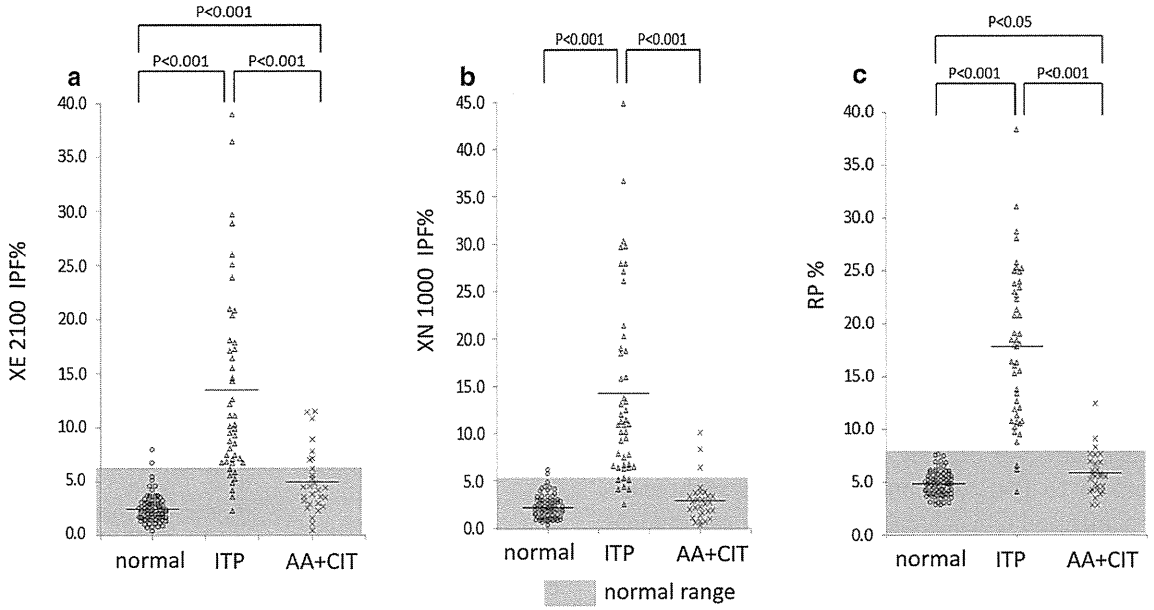

Fig. 2 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in healthy controls, patients with primary immune thrombocytopenia (ITP), and patients with aplastic anemia (AA) or chemotherapy-induced thrombocytopenia (CIT). *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy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

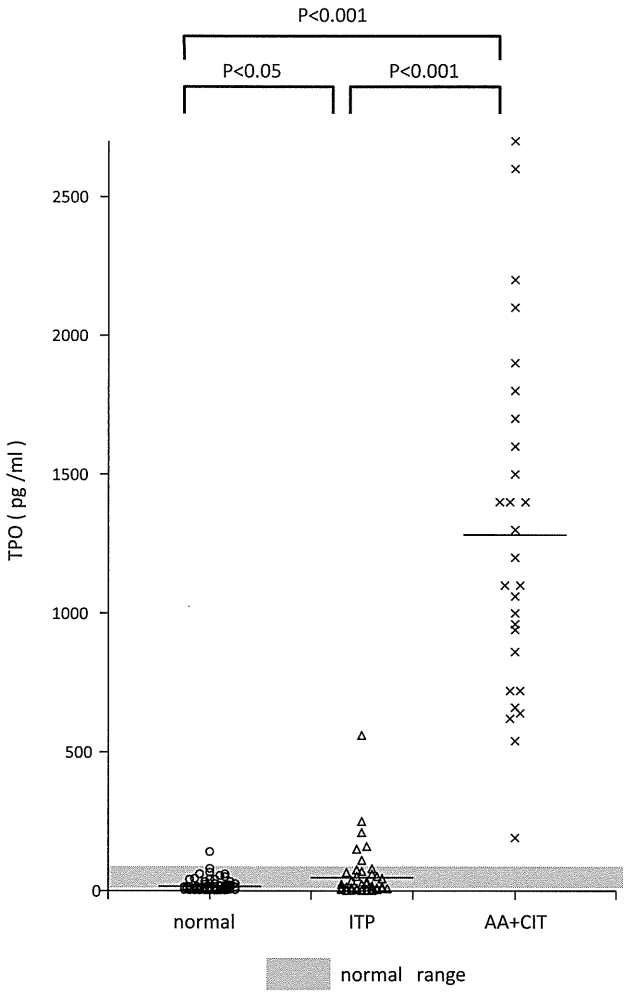

Fig. 3 Plasma thrombopoietin (TPO) levels in healthy controls, patients with ITP, and patients with AA/CIT. Patients with AA/CIT showed markedly increased plasma TPO levels, whereas patients with ITP showed normal or modestly increased TPO levels

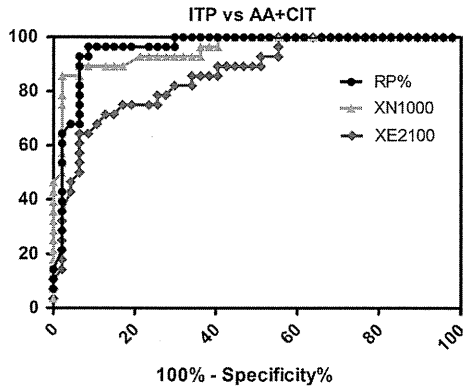

Fig. 4 Receiver operating characteristic (ROC) analysis. ROC curves were constructed for the sensitivity and specificity of the differential diagnosis of ITP from AA/CIT patients. ROC curve for IPF% (XE-2100), IPF% (XN-1000), and RP% showed area under curve (AUC) of 0.863, 0.956, and 0.959, respectively

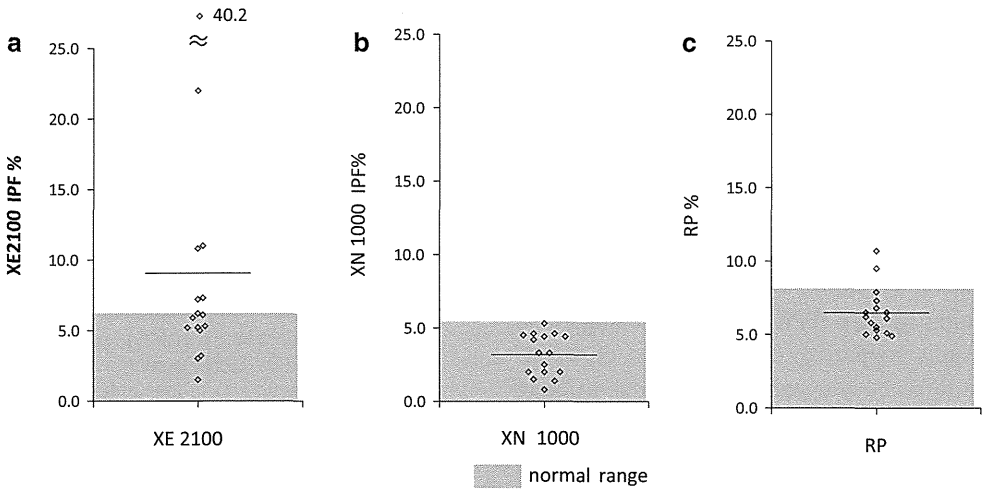

Fig. 5 IPF% (XE-2100) (**a**), IPF% (XN-1000) (**b**), and RP% (**c**) in patients with PNH. *Shaded area* indicates normal range (mean ± 3SD obtained from 80 healthy controls)

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