incorporate reliable laboratory tests in the existing guidelines for the diagnosis of ITP. In this regard, the presence of antiplatelet antibodies is a hallmark of this autoimmune disease, and the detection of antibody responses to platelet surface glycoproteins, especially to GPIIb/IIIa, is useful to identify ITP [5–7]. In addition, the percentage of reticulated platelets and the circulating thrombopoietin (TPO) level are useful in discriminating accelerated platelet destruction from decreased platelet production [8,9].

Recently, two of us (M.K. and Y.I.) conducted a prospective study to identify initial laboratory findings that were useful for predicting a diagnosis of ITP and identified six: normal erythrocyte count, normal leukocyte count, increased anti-GPIIb/IIIa antibody producing B cell frequency, increased platelet-associated anti-GPIIb/IIIa antibody level, increased proportion of reticulated platelets, and normal or slightly increased plasma TPO [10]. These results led us to propose diagnostic criteria for ITP that depend solely on non-invasive laboratory tests using peripheral blood samples (Table 1). To diagnose ITP using these criteria, thrombocytopenia without other morphologic abnormalities in the peripheral blood film and the presence of three or more of the six ITP-associated laboratory findings listed above, including at least one of three positive findings, for example, increased anti-GPIIb/IIIa antibody producing B cell frequency, increased platelet-associated anti-GPIIb/IIIa antibody level, and increased proportion of reticulated platelets, are required. As ITP also occurs secondary to an underlying disorder, such as SLE [11], antiphospholipid syndrome [12], lymphoproliferative disorders, infection with HIV [13], and liver cirrhosis [14], a detailed clinical evaluation is still necessary to discriminate idiopathic from secondary forms. Invasive procedures, such as bone marrow examination are not necessary, although these tests are recommended for patients over 60 years or who show findings that are atypical for ITP, as previously mentioned [3].

Here, we report the results of a multi-center prospective study performed to verify the usefulness and accuracy of our preliminary laboratory based criteria in routine hematology clinics.

Table 1 Preliminary laboratory based criteria for the diagnosis of immune thrombocytopenic purpura (ITP) at presentation

1. Thrombocytopenia ($< 100 \times 10^9$ L^{-1}) without morphologic evidence for dysplasia in the peripheral blood film
2. The presence of any three or more, including at least one of (iii), (vi), and (iv), of the following laboratory findings:
   (i) absence of anemia,
   (ii) normal leukocyte count,
   (iii) increased anti-GPIIb/IIIa antibody producing B cell frequency,
   (vi) increased platelet-associated anti-GPIIb/IIIa antibody level,
   (v) elevated percentage of reticulated platelets, and
   (iv) normal or slightly increased plasma TPO level (< 300 pg mL^{-1})

For the diagnosis of ITP, both criteria must be met. Idiopathic or primary ITP can be diagnosed in the absence of conditions that potentially cause secondary ITP, for example SLE, antiphospholipid syndrome, lymphoproliferative disorders, infection with HIV, and liver cirrhosis. Bone marrow examination is recommended in patients over 60 years or with findings that are atypical for ITP. TPO, thrombopoietin; SLE, systemic lupus erythematosus; HIV, human immunodeficiency virus.

Materials and methods

Study design

This multi-center prospective study was conducted at nine medical centers in Japan from November 2002 to April 2004, and involved 127 consecutive patients who had been referred to tertiary care medical centers because of low platelet count or a question of ITP. At the first visit, detailed assessments of complete medical histories, physical examination, and routine laboratory tests, including complete blood count, a peripheral blood film, chemistry profiles, serologic evaluations such as tests for antinuclear antibody, antidouble stranded antibody, anti-β_2-glycoprotein I antibody, were performed for all patients. Serologic tests for HIV, and hepatitis B and C virus were performed only in patients with risk of these pathogens. Additional evaluations, including imaging studies and/or biopsies, were carried out in selected patients who were suspected of having underlying diseases that can cause secondary ITP, such as SLE, antiphospholipid syndrome, lymphoproliferative disorders, infection with HIV, or liver cirrhosis. The inclusion criteria were: (i) *de novo* patients; (ii) thrombocytopenia (platelet count $< 100 \times 10^9$ L^{-1}); (iii) the absence of any morphologic evidence for dysplasia in the peripheral blood film; and (iv) no previous treatment with corticosteroids or immunosuppressants. Patients who were diagnosed as having definitive diseases that can cause secondary ITP were excluded at entry. Diagnoses of SLE, antiphospholipid syndrome, and liver cirrhosis were made according to the published criteria [15–17] and lymphoproliferative disorders were diagnosed based on cytomorphologic analysis and immunophenotyping, while infection with HIV was diagnosed solely based on serologic analysis. The patients who had some features of diseases that can cause secondary ITP, but did not have definitive diagnosis were included in this study. These patients were carefully followed, and were excluded if they meet the above criteria during the observation period.

At the study entry, two tubes of peripheral blood [one anticoagulated with heparin and the other with ethylenediaminetetraacetic acid (EDTA)] were obtained for the evaluation of the anti-GPIIb/IIIa antibody response and platelet turnover. Heparinized blood was sent to Keio University School of Medicine for the measurement of anti-GPIIb/IIIa antibody-producing B cell frequency, platelet-associated anti-GPIIb/IIIa antibody, and plasma TPO, and EDTA-anticoagulated blood was sent to Osaka University Hospital for the measurement of the percentage of reticulated platelets. Investigators at the individual participating centers recorded the gender, age at first visit, erythrocyte count, leukocyte count, and platelet count for each patient at study entry. An erythrocyte count of < 4.3 (male) or < 3.7 (female) $\times 10^{12}$ L^{-1} was regarded as anemia. Investigators at the participating centers were blinded to the

results of the four specialized tests for evaluating the anti-GPIIb/IIIa antibody response and platelet turnover, so the clinical diagnoses were not influenced by these laboratory findings. In addition, all other clinical data were kept at the participating centers, and the investigators at the laboratories were blinded to them. All blood samples were obtained after the patients gave their written informed consent, as approved by the corresponding Institutional Review Boards.

Clinical diagnosis

At study entry, the patients were first evaluated for tentative clinical diagnosis (ITP or non-ITP) by at least one of the authors (skilled hematologists) on the basis of clinical history, physical examination, complete blood test, and bone marrow findings if available, according to the guidelines proposed by the ASH [3], as usually performed in regular hematology clinics. Patients who might have ITP, but had atypical features, such as unexplained leukopenia and reduced megakaryocytes in the bone marrow without morphologic evidence for dysplasia, were included in the non-ITP group. Final clinical diagnosis was re-evaluated > 6 months after the first visit, taking account of clinical course over at least 6 months, especially therapeutic responses to corticosteroids, splenectomy, and *Helicobacter pylori* eradication. It is important that tentative and final clinical diagnoses were made independent of the specialized studies performed as part of this study. Patients whose follow-up period was < 6 months were excluded from the study.

Sample preparation

Platelet-rich plasma (PRP) was prepared from heparinized venous blood by centrifugation, followed by separation into platelets and platelet-poor plasma. The remaining cell components were subjected to centrifugation through a Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient to isolate the peripheral blood mononuclear cells. PRP was also prepared by centrifugation of blood that was anticoagulated with EDTA.

Evaluation of anti-GPIIb/IIIa antibody responses

B cells producing IgG anti-GPIIb/IIIa antibodies were detected using the enzyme-linked immunospot assay as described [7]. Each experiment was conducted in five independent wells, and the results represent the mean of the five values. The frequency of circulating anti-GPIIb/IIIa antibody-producing B cells was calculated as the number per 10^5 peripheral blood mononuclear cells, and the cut-off value was defined as 2.0 [7]. IgG anti-GPIIb/IIIa antibodies in platelet eluates (from 5×10^7 platelets) were measured by enzyme-linked immunosorbent assay using purified human GPIIb/IIIa as the antigen [18,19]. Antibody units were calculated from the OD_{450} results, based on a standard curve obtained from serial concentrations of pooled plasma with a high titer of IgG anti-GPIIb/IIIa antibodies. All samples were examined in duplicate, and the results were calculated as the mean of the two values. The cut-off value for platelet-associated anti-GPIIb/IIIa antibodies was 3.3 U [19].

Evaluation of platelet turnover

Reticulated platelets were detected by staining paraformaldehyde-fixed platelets with thiazole orange (Retic-COUNT; Becton Dickinson, San Jose, CA, USA) followed by flow-cytometric analysis, as described previously [9]. The cut-off for the percentage of reticulated platelets was 9.3%. The plasma TPO level was measured using a commercially available enzyme-linked immunosorbent assay kit (Quantikine; R&D Systems®, Minneapolis, MN, USA) according to the manufacturer's protocol.

Statistical analysis

All continuous variables were expressed as the mean ± SD, and compared using the Mann–Whitney *U*-test. Differences in the frequency between two groups were compared using the chi-squared test or Fisher's exact test when applicable. A stepwise multiple regression analysis was conducted to identify independent variables associated with the diagnosis of ITP, and the odds ratio (OR) and its 95% confidence intervals (95% CI) were calculated for all statistically significant differences. All statistical procedures were performed using the STATVIEW software (SAS Institute, Cary, NC, USA).

Results

Clinical diagnoses

A total of 127 consecutive patients were enrolled in this study. After the final clinical diagnosis, which was made > 6 months after the first visit, the clinical information recorded at study entry by the investigators at the individual participating centers and the results for the four specialized laboratory tests for the anti-GPIIb/IIIa antibody response and platelet turnover were combined and analyzed. Fifteen patients were excluded from the analysis because of a platelet count > 100×10^9 L^{-1} at entry ($n = 2$), the subsequent diagnosis of a lymphoproliferative disorder or liver cirrhosis ($n = 3$), spontaneous resolution of thrombocytopenia ($n = 1$), previous treatment with corticosteroids ($n = 6$), and a follow-up period of < 6 months ($n = 3$). In the end, we analyzed data for 112 patients between 1 and 80 years of age (45.7 ± 22.6), including 18 children. The final clinical diagnosis after > 6 months of follow-up included ITP in 88 patients (79%), aplastic anemia in 11 (10%), myelodysplastic syndrome (MDS) in 10 (9%), Fanconi anemia in one (1%), May-Hegglin anomaly in one (1%), and myelofibrosis in one (1%).

Application of the preliminary diagnostic criteria

As all 112 patients were selected because they had thrombocytopenia without any other morphologic evidence for dyspla-

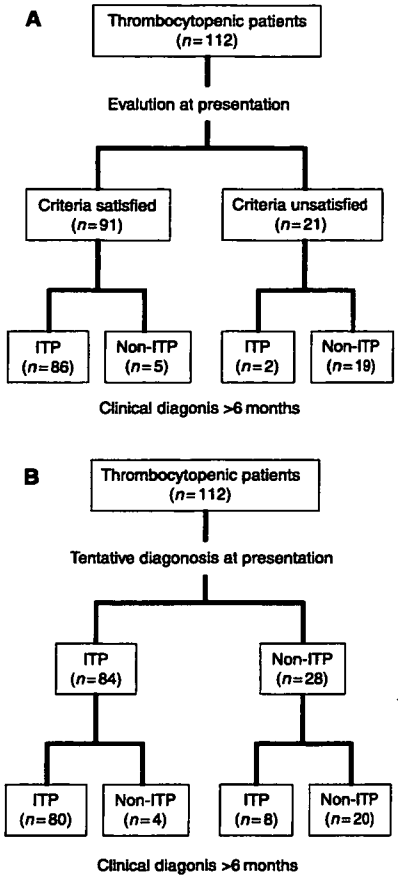

Fig. 1. Classification of the 112 patients with thrombocytopenia and a normal peripheral blood film at study entry, according to the preliminary laboratory based criteria for the diagnosis of immune thrombocytopenic purpura (A) or tentative clinical diagnosis made by skilled hematologists (B).

sia in the peripheral blood film (item no. 1 of the criteria), we focused on the six ITP-associated laboratory findings obtained at study entry. We found that 91 patients (81%) had three or more of the ITP-associated laboratory findings at first visit, and thus satisfied the preliminary criteria for the diagnosis of ITP (Fig. 1A). Of these, 86 patients were given a final clinical diagnosis of ITP and five a diagnosis of a non-ITP disorder: three had aplastic anemia and two had MDS. On the other hand, 19 out of the 21 patients who did not satisfy the criteria had a non-ITP disorder, but the remaining two were given a clinical diagnosis of ITP. In summary, our preliminary criteria for the diagnosis of ITP had a sensitivity of 98%, specificity of 79%, positive predictive value of 95%, and negative predictive value of 90%. When 18 children were separately analyzed, our criteria had a sensitivity of 77%, specificity of 100%, positive predictive value of 100%, and negative predictive value of 63%.

We further compared the tentative diagnosis made at study entry and the final diagnosis made > 6 months after first visit (Fig. 1B). Eighty-four patients (75%) were tentatively diagnosed as having ITP at presentation, and 80 of these were given a final diagnosis of ITP. On the other hand, 20 out of the 28 patients who were tentatively diagnosed as a non-ITP disorder were given a final diagnosis of a non-ITP disorder. Therefore, tentative diagnosis made by skilled hematologists at presentation had a sensitivity of 91%, specificity of 83%, positive predictive value of 95%, and negative predictive value of 71%. This clearly indicates that 6 months of observation improved the ability to establish a clinical diagnosis.

Clinical and laboratory findings at study entry in clinically diagnosed ITP vs. non-ITP

The clinical and laboratory findings at entry of the 88 patients who later received a diagnosis of ITP and the 24 whose diagnosis was non-ITP were compared statistically (Table 2). There was no difference in the distribution of sex, age, and platelet count between these two patient groups, but individual laboratory findings included in the criteria (absence of anemia, normal leukocyte count, increased anti-GPIIb/IIIa antibody-producing B cell frequency, increased platelet-associated anti-GPIIb/IIIa antibody levels, elevated percentage of reticulated platelets, and normal or slightly increased plasma TPO) were significantly more frequent in patients who later received a diagnosis of ITP compared with those who received a diagnosis of a non-ITP disorder. Patients who later received a diagnosis of ITP predominantly had microcytic anemia, but those who received a diagnosis of a non-ITP disorder had various types of anemia, including microcytic, normocytic and macrocytic anemia. In fact, non-microcytic anemia was significantly less frequent in patients with ITP compared with other diagnoses (2% vs. 46%, $P < 0.0001$).

Table 2 Clinical and laboratory findings at first visit of 88 patients who later received a diagnosis of ITP and 24 who had a non-ITP disorder diagnosed

Clinical and laboratory findings	ITP (n = 88)	Non-ITP (n = 24)	P-value*
Female (%)	59	50	0.5
Age at first visit (years; mean ± SD)	45.4 ± 21.6	46.7 ± 26.6	0.8
Platelet count ($\times 10^9$ L^{-1}; mean ± SD)	43 ± 25	39 ± 22	0.5
Absence of anemia (%)	80	13	< 0.0001
Normal leukocyte count (%)	85	50	0.0006
Increased anti-GPIIb/IIIa antibody-producing B cell frequency (%)	84	42	0.0001
Increased platelet-associated anti-GPIIb/IIIa antibodies (%)	49	21	0.03
Elevated percentage of reticulated platelets (%)	56	17	0.001
Normal or slightly increased plasma TPO (< 300 pg mL^{-1}) (%)	95	54	< 0.0001

*Differences between two groups were compared using the Mann–Whitney U-test, chi-squared test, or Fisher's exact test when applicable.
ITP, immune thrombocytopenic purpura; TPO, thrombopoietin; GP, glycoprotein.

The six laboratory tests associated with the later diagnosis of ITP were further analyzed by a stepwise multiple regression model. The initial laboratory findings that were independently associated with a future diagnosis of ITP included the presence or absence of anemia ($P = 0.005$, OR = 0.05, 95% CI 0.006–0.41), anti-GPIIb/IIIa antibody-producing B cell frequency ($P = 0.02$, OR = 1.2, 95% CI 1.02–1.41), a percentage of reticulated platelets ($P = 0.04$, OR = 1.3, 95% CI 1.01–1.66), and plasma TPO ($P = 0.005$, OR = 0.02, 95% CI 0.002–0.32).

Clinical features of patients whose clinical diagnosis differed from the prediction of the preliminary diagnostic criteria

Detailed clinical and laboratory findings at study entry in patients who showed a false-negative or false-positive result on the preliminary laboratory based diagnostic criteria are listed in Table 3. The two patients who later received a diagnosis of ITP had only one or two of the ITP-associated laboratory findings at entry. Both patients had increased anti-GPIIb/IIIa antibody producing B-cell frequency. They were slightly anemic and leukopenic at entry, but their erythrocyte and leukocyte counts were normal at several follow-up evaluations, without any treatment. On the other hand, the five patients who satisfied three or more ITP-associated laboratory findings at the first visit, but later received a clinical diagnosis of a non-ITP disorder, included three with aplastic anemia and two with MDS. Notably, four of these patients had a marked increase in anti-GPIIb/IIIa antibody producing B cells, and three also had elevated platelet-associated anti-GPIIb/IIIa antibodies.

Elimination of one costly laboratory test from the preliminary criteria

We further examined whether one of the quite costly tests, that is, the anti-GPIIb/IIIa antibody-producing B cells, platelet-associated anti-GPIIb/IIIa antibody, and plasma TPO, could be omitted from the original criteria without reducing the predictive reliability (Table 4). Only one test, the platelet-associated anti-GPIIb/IIIa antibody, could be excluded without a marked decrease in the specificity or negative predictive value (< 70%). When the presence of three or more of the five ITP-associated laboratory findings, excluding the platelet-associated anti-GPIIb/IIIa antibody test, was regarded as diagnostic for ITP, the modified criteria showed a sensitivity of 93%, specificity of 83%, positive predictive value of 95%, and negative predictive value of 77%.

Discussion

To develop consistent and reproducible criteria for diagnosis of ITP, we modified the existing guidelines by incorporating laboratory tests found useful for discriminating between future diagnoses of ITP and non-ITP disorders, and evaluated their usefulness by multi-center prospective study. Our

Table 3 Clinical and laboratory findings at study entry in patients who showed a false-negative or false-positive result for the preliminary laboratory based diagnostic criteria for ITP

Patient no.	Gender	Age (years)	Anti-GPIIb/IIIa antibody-producing B cells* (> $2.0/10^5$ PBMCs)	Platelet-associated anti-GPIIb/IIIa antibody* (> 3.3 U)	Reticulated platelets* (> 9.3%)	TPO* (< 300 pg mL^{-1})	Leukocyte count* (3.5–9.0 × 10^9 L^{-1})	Erythrocyte count* (male ≥ 4.3; female ≥ 3.7 × 10^{12} L^{-1})	Number of findings satisfied	Clinical diagnosis
Patients who did not satisfy the criteria, but ITP was later diagnosed										
No.59	Male	14	7.6	0.4	4.5	370	2.7	4.1	1	ITP
No.115	Male	53	23.0	0.5	3.0	94	3.1	3.5	2	ITP
Patients who satisfied the criteria, but a non-ITP disorder was later diagnosed										
No.10	Female	70	10.2	17.3	8.4	113	2.7	2.7	3	Aplastic anemia
No.63	Female	64	14.8	64.0	14.6	134	7.1	3.8	5	MDS
No.80	Male	55	22.8	0.3	4.3	649	4.9	4.6	3	Aplastic anemia
No.88	Male	50	8.0	23.8	8.0	1183	4.0	3.2	3	Aplastic anemia
No.123	Female	38	2.2	2.5	3.6	222	4.5	2.6	3	MDS

*The range in parenthesis denotes the laboratory test result that meets the preliminary criteria. Individual laboratory results that meet the preliminary diagnostic criteria are underlined. MDS, myelodysplastic syndrome. ITP, immune thrombocytopenic purpura; TPO, thrombopoietin; GP, glycoprotein.

Table 4 Sensitivity, specificity, positive predictive value, and negative predictive value of the preliminary laboratory-based criteria for the diagnosis of immune thrombocytopenic purpura (ITP) and the effect on these values of eliminating one of the costly tests

Combinations of ITP-associated laboratory tests	Number of findings required	Patients who met the criteria (number)		*P*-value*	Sensitivity %	Specificity %	Positive predictive value %	Negative predictive value %
		ITP ($n = 88$)	Non-ITP ($n = 24$)					
All six laboratory tests	3 or more	84	5	< 0.0001	98	79	95	90
Minus anti-GPIIb/IIIa antibody-producing B cells	2 or more	84	11	< 0.0001	95	54	88	76
Minus anti-GPIIb/IIIa antibody-producing B cells	3 or more	76	2	< 0.0001	86	92	97	65
Minus platelet-associated anti-GPIIb/IIIa antibody	2 or more	87	14	< 0.0001	99	42	86	91
Minus platelet-associated anti-GPIIb/IIIa antibody	3 or more	82	4	< 0.0001	93	83	95	77
Minus TPO	2 or more	85	9	< 0.0001	97	63	90	83
Minus TPO	3 or more	73	3	< 0.0001	83	88	96	58

*Differences between two groups were compared using the chi-squared test or Fisher's exact test when applicable. ITP, immune thrombocytopenic purpura; TPO, thrombopoietin; GP, glycoprotein.

laboratory based criteria showed high concordance with the clinical diagnosis made by very skilled hematologists according to the ASH guidelines after > 6 months of follow-up (specificity 98%), but the sensitivity was rather low (79%). Both the positive and negative predictive values were fairly high (≥ 90%), indicating that our preliminary laboratory based criteria are reliable for the diagnosis of ITP at presentation. The present study confirmed our previous study, which was conducted in a single medical center using only adult patients. In our previous study, we also found higher frequencies of the six ITP-associated laboratory findings in the patients later diagnosed as ITP compared with non-ITP than we did here [10].

Our study indicates that none of clinical skills or laboratory tests performs better than 6 months of observation at making or excluding the diagnosis of ITP. However, our laboratory based criteria are able to predict future diagnosis of ITP in the majority of thrombocytopenic patients at presentation, and their sensitivity and specificity were comparable to, or even better than, the clinical diagnosis made by skilled hematologists at presentation according to the ASH guidelines. Therefore, our consistent criteria, which do not rely on the skill of the clinicians, are useful for hematologists, especially for those who are not skilled at diagnosing ITP, to make the diagnosis in patients with thrombocytopenia and to decide whether they should be or should not be treated with ITP-based therapies.

The major weakness of our criteria is the relatively low specificity (79%): five non-ITP patients with aplastic anemia or MDS gave a false-positive result. Interestingly, four of these patients had a remarkably increased frequency of anti-GPIIb/IIIa antibody-producing B cells, and three of them also had increased platelet-associated anti-GPIIb/IIIa antibodies, suggesting that these patients might also manifest ITP. In this case, actual specificity of our criteria would be better than observed specificity. Moreover, our criteria may be useful in diagnosing ITP even in patients with MDS or aplastic anemia. In this regard, it has been reported that MDS patients with detectable antiplatelet autoantibodies have ITP and potentially respond to treatment used for ITP [20]. Moreover, autoimmune-mediated mechanisms contribute to the pathogenic processes of aplastic anemia [21]. Therefore, a bone marrow examination should be performed, even in patients who satisfy three or more ITP-associated laboratory findings at presentation, if they have atypical findings during the course of the disease, such as the appearance of persistent anemia or leukopenia, as stated in the ASH guidelines [3].

We believe that this study is a necessary first step toward establishing diagnostic criteria for ITP, but it had several limitations. Firstly, our criteria need validation with a larger number of patients who have confirmed diagnoses of non-ITP disorders. Especially, there is little information on whether the proposed criteria would be useful in distinguishing ITP from rare mimicking disorders, such as hereditary thrombocytopenia, chronic disseminated intravascular coagulation, or pregnancy associated thrombocytopenia. In addition, it would be interesting to know if these diagnostic criteria successfully discriminated patients with ITP from patients who were subsequently discovered to have drug induced thrombocytopenia, which is often a major and difficult differential diagnostic point [22]. Applying our criteria to these disorders would provide interesting and important information about the specificity of these tests. If these criteria are specific to ITP, that would be helpful for the diagnosis of ITP. If they are not specific, we would learn more about the pathogenesis of the other disorders.

Second, we excluded thrombocytopenic patients with conditions that potentially cause secondary ITP and focused on the diagnosis of idiopathic or primary ITP in this study. It is expected that our proposed criteria are also useful in identifying patients with various forms of secondary ITP. In fact, our preliminary analysis revealed that 27 (84%) out of 32 patients with SLE and thrombocytopenia satisfied our preliminary criteria (M. Kuwana, unpubl. obs.). Further studies to evaluate usefulness of our preliminary criteria in various forms of secondary ITP are underway.

Third, four of the ITP-associated laboratory tests included in the criteria are not currently available in many clinical laboratories. To make these criteria routinely available, it is

necessary to develop convenient, reliable, commercial kits or systems for these tests. In this regard, a rapid, inexpensive, and automated method to reliably quantify reticulated platelets has already been developed utilizing a widely used fully automated hematology analyzer with upgraded software [23]. A sensitive sandwich enzyme-linked immunosorbent assay for measuring TPO has also been established [24], and several commercial kits are currently available. In contrast, the evaluation of the anti-GPIIb/IIIa antibody response is not currently feasible in most laboratories, although an enzyme-linked immunospot assay kit for detecting anti-GPIIb/IIIa antibody-secreting B cells has been manufactured and is expected to be available soon. However, assays for evaluating platelet-associated anti-GPIIb/IIIa antibodies reportedly have significant inter-laboratory variability [25], and standardization of the test is required before its widespread use can be recommended.

Finally, it remains unclear whether all six ITP-associated laboratory tests are necessary for the reliability of these criteria. Of these tests, the measurement of erythrocyte and leukocyte counts is very easy to do, and the measurement of reticulated platelets appears to be cheap and readily available. The remaining three tests are costly, difficult to perform, and much less available. Our analysis showed that the measurement of platelet-associated anti-GPIIb/IIIa antibodies could be omitted with a minimal reduction in the reliability of the criteria, although our platelet-associated anti-GPIIb/IIIa antibody assay was not sensitive or specific enough for a rigorous assessment of its contribution.

In summary, our results indicate that the development of the diagnostic criteria for ITP is feasible by combining laboratory findings that are useful for predicting a future diagnosis of ITP. However, our laboratory based criteria are still being developed, and additional modifications and studies will be necessary to establish accurate and practical criteria that can be used widely in clinical settings.

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Disclosure of Conflict of Interests

The authors state that they have no conflict of interests.

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ORIGINAL ARTICLE

Prolonged thrombocytopenia after allogeneic hematopoietic stem cell transplantation: associations with impaired platelet production and increased platelet turnover

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To evaluate the mechanisms underlying prolonged thrombocytopenia after allogeneic hematopoietic stem cell transplantation (SCT), an index for plasma glycocalicin normalized for the individual platelet count (GCI), plasma thrombopoietin (TPO), and circulating B cells producing anti-GPIIb-IIIa antibodies were measured in 50 SCT recipients with or without prolonged thrombocytopenia, 42 patients with idiopathic thrombocytopenic purpura, nine patients with aplastic anemia, and 22 healthy individuals. All three indices were significantly higher in the SCT recipients with thrombocytopenia than in those without ($P<0.01$ for all comparisons), and were significantly correlated with the platelet count in SCT recipients. Stepwise multiple regression analysis of the samples from the SCT recipients revealed that GCI and TPO independently pointed to specific mechanisms of thrombocytopenia. The GCI and TPO status in SCT recipients with thrombocytopenia had a pattern similar to that seen in aplastic anemia, suggesting a major role for impaired thrombopoiesis. An antiplatelet antibody response was frequently detected in SCT recipients, but the development of thrombocytopenia is likely to depend on additional factors, such as reticuloendothelial function. In summary, post transplant prolonged thrombocytopenia is associated with complex mechanisms, including impaired thrombopoiesis and increased platelet turnover.
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Introduction

Prolonged thrombocytopenia is a late complication of allogeneic hematopoietic stem cell transplantation (SCT), and it occurs in 5–37% of SCT recipients.[1–3] Isolated thrombocytopenia after SCT is mainly attributed to engraftment failure, recurrence of the underlying malignancy, microangiopathy, drugs, or viral infection,[4] but its underlying mechanism is uncertain in some cases, especially in patients who have survived for 6 months or more after SCT. Several studies have suggested that isolated thrombocytopenia that occurs late in the post transplant period is associated with chronic graft-versus-host disease (GVHD)[1,5] or with the production of antiplatelet alloantibodies of recipient origin.[6,7]

Recently, measurement of the glycocalicin index (GCI) and circulating thrombopoietin (TPO) has been useful for discriminating increased platelet turnover from impaired platelet production.[8–12] Glycocalicin is a fragment cleaved from the extracellular domain of the platelet-specific glycoprotein (GP) Ibα, and its plasma level normalized to the platelet count (referred to as the GCI) has been proposed as a useful parameter for evaluating platelet turnover.[13] The GCI is increased in hyperdestructive states, such as in immune thrombocytopenia. In contrast, TPO has been identified as a key cytokine for megakaryogenesis and thrombopoiesis.[14] A large increase in circulating TPO is detected in conditions in which bone marrow megakaryocytes are absent or present at low levels, such as aplastic anemia and amegakaryocytic thrombocytopenia. In addition, the detection of autoantibodies reactive with platelet surface GPs, such as GPIIb-IIIa, is a hallmark of immune thrombocytopenia, especially idiopathic thrombocytopenic purpura (ITP).[15] We recently established a convenient and sensitive assay for detecting circulating B cells that produce anti-GPIIb-IIIa antibodies,[16] which is useful for identifying patients with immune thrombocytopenia. In this study, we used these parameters, GCI, TPO, and anti-GPIIb-IIIa antibody-producing B cells, to assess the processes underlying prolonged thrombocytopenia in SCT recipients.

Patients and methods

Patients and controls

We studied 23 SCT recipients who had prolonged thrombocytopenia (platelet count $<100 \times 10^9$/l for more than 3 months) without sustained anemia or leukopenia, but no apparent cause for thrombocytopenia, such as engraftment failure, recurrence of the underlying malignancy, microangiopathy, or drugs. To minimize the potential influence of procedure-related complications, we selected patients who had survived for >100 days after SCT. As a control, we selected 27 SCT recipients who had not been thrombocytopenic after day 100 and did not have anemia or leucopenia at entry. Factors potentially affected the platelet count were matched between the study and control groups as possible. All these patients underwent SCT for various hematological malignancies between February 1996 and November 2005. The transplanted grafts were granulocyte colony-stimulating factor-mobilized peripheral blood stem cells from sibling donors ($n=7$), bone marrow cells from unrelated donors or siblings ($n=43$). All the patients received cyclosporin or tacrolimus in combination with a short-term methotrexate for prophylaxis of GVHD. The clinical information for individual patients was retrospectively collected by the chart review. Chronic GVHD was defined according to the published criteria.[17]

Blood samples from 42 adult patients with ITP and nine with aplastic anemia were served as disease controls for thrombocytopenia. At the time of the blood examination, all patients with ITP or aplastic anemia had a platelet count $<100 \times 10^9$/l. ITP was defined as thrombocytopenia persisting for longer than 6 months, normal or increased bone marrow megakaryocytes without morphologic evidence of dysplasia, and no secondary immune or non-immune diseases that could account for the thrombocytopenia.[18] The diagnosis of aplastic anemia was based on the following criteria: pancytopenia, absence of splenomegaly or lymph node adenopathy, and reduced cellularity of the marrow without dysplasia.[19] Twenty-two healthy individuals were also used as a control subjects. The study protocol conformed to the ethical principles of the World Medical Association Declaration of Helsinki as reflected in *a priori* approval from the Keio University Institutional Review Board. Peripheral blood samples were obtained after the patients and control subjects gave their written informed consent.

Bone marrow evaluation

Forty-five SCT recipients received bone marrow evaluation at the time of blood collection. The number of megakaryocytes on bone marrow films was rated as described elsewhere.[20] Thirty randomly selected high-power fields were examined by three independent observers, and the results were calculated as the mean of these values. One megakaryocyte per >5 low power fields was regarded as megakaryocytic hypoplasia.

Cell separation

Heparinised venous blood was obtained from all subjects at study entry. After separation of the platelet-rich plasma, the residual cell components were applied to a Lymphoprep (Nycomed Pharma AS, Oslo, Norway) density gradient for centrifugation to isolate peripheral blood mononuclear cells (PBMCs). Freshly isolated PBMCs were resuspended in RPMI1640 containing 10% heat-inactivated fetal bovine serum. Platelets were isolated from the platelet-rich plasma by centrifugation, and the supernatant was used as plasma.

GCI

The glycocalicin level was measured in duplicate using an enzyme-linked immunosorbant assay (ELISA) kit (Takara Biomedical, Ohtsu, Japan) according to the manufacturer's instructions. The lower detection limit of the kit is 10 ng/ml. The GCI was calculated using this formula: glycocalicin (ng/ml) $\times 250 \times 10^6$ divided by the individual platelet count per l.[13] The cutoff value for normal GCI was defined as the mean plus three times the standard deviation (s.d.) of 30 healthy individuals (2.30).

Plasma TPO

The plasma TPO level was measured in duplicate using a commercially available ELISA kit (R&D System, Minneapolis, MN, USA). The lower detection limit of the kit was 31.2 pg/ml. The cutoff value for normal plasma TPO was defined 142 pg/ml.[21]

Circulating B cells producing IgG anti-GPIIb-IIIa antibodies

The antiplatelet antibody response was evaluated by the detection of circulating B cells producing anti-GPIIb-IIIa antibodies, using an enzyme-linked immunospot assay, as described previously.[16] In brief, polyvinylidene difluoride-bottomed 96-well plates were coated with 30 μg/ml purified pooled human GPIIb-IIIa (Enzyme Research Laboratories, South Bend, IN, USA). PBMCs (10^5 cells/well) were cultured on GPIIb-IIIa-coated wells at 37°C in a humidified atmosphere of 5% CO_2 for 4 h and subsequently incubated with alkaline phosphatase-conjugated goat anti-human IgG (ICN/Cappel, Aurora, OH, USA). Antibodies bound to the membranes were visualized as distinct spots by incubation with nitro blue tetrazolium and 5-bromo-4-chloro-indolyl phosphate. Each experiment was conducted in five independent wells, and the results represent the mean of the five values. The frequency of circulating anti-GPIIb-IIIa antibody-producing B cells was presented as the number of 10^5 PBMCs, and the cutoff value was defined as 2.0.[16]

Platelet-associated and plasma IgG anti-GPIIb-IIIa antibodies

IgG anti-GPIIb-IIIa antibodies in platelet eluates and plasma were measured in some samples using ELISA according to the method described elsewhere.[22] Platelet eluates were prepared by incubating the platelets with 0.1 mol/l HCl followed by immediate neutralization with 0.2 mol/l NaOH. Antibody units were calculated from the OD_{450} results, based on a standard curve obtained from serial concentrations of pooled plasma with a high level of IgG anti-GPIIb-IIIa antibodies. All samples were examined in duplicate. The cutoff values for normal levels of platelet-

associated and plasma IgG anti-GPIIb-IIIa antibodies were 3.3 and 5.0 units, respectively.[23]

Statistical analysis

All continuous variables were expressed as the mean ± s.d., and compared using the Mann–Whitney *U*-test. Differences in frequency between two groups were compared using the χ^2-test or Fisher's exact test when applicable. The correlation coefficient was determined using the single regression model. A stepwise multiple regression analysis was conducted to identify independent variables associated with the presence of thrombocytopenia. All statistical procedures were performed using the StatView software (SAS Institute, Cary, NC, USA).

Results

Patient characteristics

The demographic and clinical characteristics of SCT recipients with and without thrombocytopenia are summarized in Table 1. All characteristics except the period between SCT and blood examination were nearly equally represented in the study and control groups. The length of time after SCT tended to be shorter in patients with thrombocytopenia than in those without, but this difference did not reach statistical significance.

Platelet turnover

Platelet turnover was evaluated by measuring the GCI (Figure 1a). The GCI was significantly higher in SCT recipients with thrombocytopenia than in those without or in healthy controls ($P<0.0001$ for both comparisons), but the GCI in SCT recipients with thrombocytopenia was significantly lower than that in patients with ITP, a disease characterized by increased platelet turnover ($P=0.001$). Moreover, the increased GCI was detected less frequently in SCT recipients with thrombocytopenia than in ITP patients (52 versus 90%, $P=0.001$). There was a negative correlation between the platelet count and GCI in all SCT recipients combined ($R=0.57$, $P<0.001$).

Platelet production

Platelet production was primarily assessed by measuring the plasma TPO. As shown in Figure 1b, plasma TPO was significantly increased in SCT recipients with thrombocytopenia compared with those without, ITP patients, and healthy controls ($P<0.001$ for all comparisons). The plasma TPO level was comparable between SCT recipients with thrombocytopenia and patients with aplastic anemia, a disease characterized by an extremely high level of TPO.[10,11] The frequency of increased TPO was comparable between SCT recipients with thrombocytopenia and patients with aplastic anemia (70 versus 89%). It was noted that eight (30%) SCT recipients lacking thrombocytopenia also had an elevated TPO level. There was a negative correlation between platelet count and TPO in all SCT recipients combined ($R=0.55$, $P<0.001$). Bone marrow films were available for 45 SCT recipients, and megakaryocytic hypoplasia was more frequently detected in the 19 recipients with thrombocytopenia than in the 26 without (63 versus 27%, $P=0.03$).

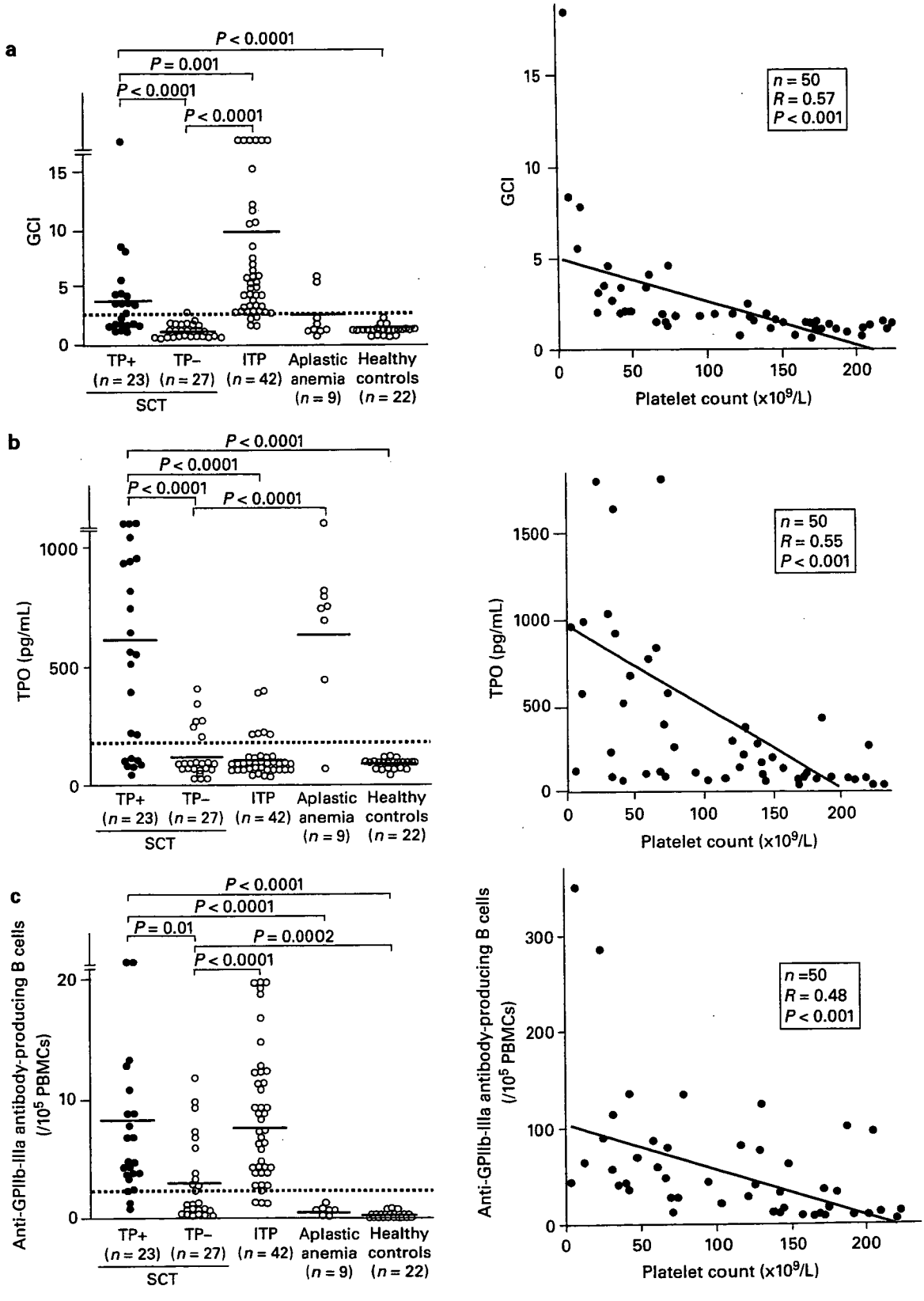
Table 1 Clinical characteristics of SCT recipients with and without prolonged thrombocytopenia[a]

Demographics and clinical features	*Prolonged thrombocytopenia*		P-*value*
	Present (n = *23*)	*Absent* (n = *27*)	
Age at examination (year)	43.4 ± 9.2 (24–60)	40.8 ± 10.2 (22–59)	0.4
Male	12 (52%)	18 (67%)	0.5
Days after SCT	532 ± 661 (103–3365)	721 ± 647 (172–2586)	0.1
Type of donor: related	10 (43%)	9 (33%)	0.7
Stem cell source			0.7
Bone marrow	20 (87%)	23 (85%)	
Peripheral blood	3 (13%)	4 (15%)	
Transplanted stem cell dose (bone marrow grafts only) ($\times 10^8$/kg)	2.4 ± 0.8 (1.0–3.8) ($n=19$)	2.8 ± 0.9 (1.4–4.5) ($n=23$)	0.2
HLA mismatching	3 (14%)	8 (30%)	0.3
Underlying disease			1.0
Acute myelogenous leukemia	8 (35%)	7 (26%)	
Myeloproliferative disease	5 (22%)	9 (34%)	
Acute lymphoblastic leukemia	4 (17%)	6 (22%)	
Myelodysplastic syndrome	6 (26%)	4 (15%)	
Non-Hodgkin lymphoma	0 (0%)	1 (3%)	
Nonmyeloablative regimen	3 (13%)	3 (11%)	1.0
History of acute GVHD[b]	11 (48%)	11 (41%)	0.6
Current extensive chronic GVHD	14 (60%)	13 (48%)	0.4
History of CMV reactivation	12 (52%)	11 (41%)	0.5
Recent CMV reactivation	2 (9%)	1 (4%)	0.6
Recent use of gancyclovir	2 (9%)	1 (4%)	0.6

Abbreviations: CMV = cytomegalovirus; GVHD = graft-versus-host disease; HLA = human lymphocyte antigen; SCT = stem cell transplantation.
[a]All results are shown in number (%) except continuous results, which are shown in the mean ± s.d. (range).
[b]Grade II–IV.

Antiplatelet antibody response

The antiplatelet antibody response was evaluated by measuring circulating B cells producing IgG anti-GPIIb-IIIa antibodies. As shown in Figure 1c, SCT recipients with thrombocytopenia had a higher frequency of circulating anti-GPIIb-IIIa antibody-producing B cells than those without, aplastic anemia patients, or healthy controls ($P<0.0001$ for all comparisons). The number of anti-



GPIIb-IIIa antibody-producing B cells was comparable between SCT recipients with thrombocytopenia and patients with ITP, a disease characterized by antiplatelet autoantibodies such as anti-GPIIb-IIIa antibodies.[18] In addition, the frequency of increased anti-GPIIb-IIIa antibody-producing B cells was comparable between SCT recipients with thrombocytopenia and ITP patients (91 versus 90%). It was noted that SCT recipients without thrombocytopenia also had increased anti-GPIIb-IIIa antibody-producing B cells compared with healthy controls ($P<0.0001$). When the results from the SCT recipients with and without thrombocytopenia were combined, the anti-GPIIb-IIIa antibody-producing B cell frequency was negatively correlated with the platelet count ($R=0.48$, $P<0.001$). The frequency of anti-GPIIb-IIIa antibody-producing B cells was increased in all SCT recipients whose GCI was increased.

To evaluate whether the IgG anti-GPIIb-IIIa antibodies produced by B cells were present on the surface of circulating platelets *in vivo* in SCT recipients, both platelet eluates and plasma samples were used to detect anti-GPIIb-IIIa antibodies. The levels of platelet-associated anti-GPIIb-IIIa antibodies were higher in SCT recipients than in healthy controls, independent of the presence or absence of thrombocytopenia ($P<0.0001$ for both comparisons), but were similar to the level in ITP patients (Figure 2a). On the other hand, an increased plasma anti-GPIIb-IIIa antibody level was infrequent in SCT recipients and ITP patients (Figure 2b). These findings indicate that IgG anti-GPIIb-IIIa antibodies are present mainly on the surface of circulating platelets in SCT recipients, as in ITP patients.

Identification of laboratory markers associated with thrombocytopenia in SCT recipients

Variables that significantly differed between SCT patients with and without thrombocytopenia by single regression analysis included GCI, TPO, and frequency of IgG anti-GPIIb-IIIa antibody-producing B cells were analyzed. To identify variables independently associated with thrombocytopenia in SCT recipients, these three parameters were subjected to a stepwise multiple regression analysis. We found that the GCI and TPO were independent laboratory markers that pointed to specific mechanisms for post transplant prolonged thrombocytopenia ($P<0.001$ and 0.01, respectively).

Classification according to the GCI and TPO status

Distribution of the GCI and TPO levels was evaluated in the SCT recipients with and without thrombocytopenia, ITP patients, aplastic anemia patients, and healthy controls (Figure 3). There was no correlation between the GCI and TPO levels in all groups, confirming an independent nature of these two laboratory markers. SCT recipients with thrombocytopenia showed a heterogeneous distribution in terms of their GCI and TPO status: three (13%) with increased GCI and normal TPO; seven (30%) with normal GCI and increased TPO; nine (39%) with increased

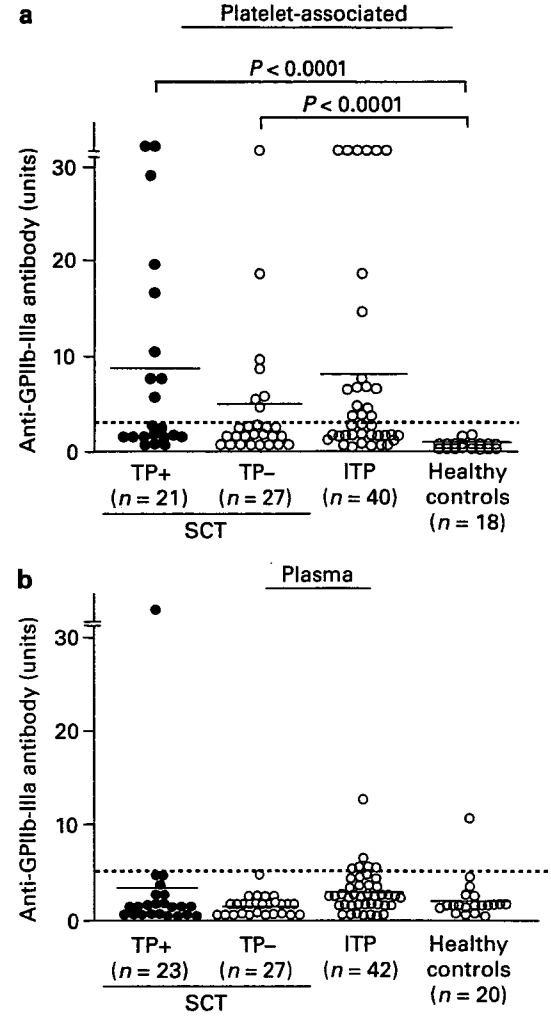


Figure 2 IgG anti-GPIIb-IIIa antibodies in platelet eluates (**a**) and in plasma (**b**) in SCT recipients with thrombocytopenia (TP+) and without thrombocytopenia (TP–), ITP patients, and healthy controls. The bold line in each column indicates the mean. The cutoff values for normal levels of platelet-associated and plasma anti-GPIIb-IIIa antibodies are shown by broken lines (3.3 and 5.0 units, respectively). Differences between two groups were evaluated using the Mann–Whitney *U*-test. Only statistically significant differences between SCT recipients with or without thrombocytopenia and other groups are shown.

Figure 1 Measurement of GCI (**a**), plasma TPO (**b**), and IgG anti-GPIIb-IIIa antibody-producing B cells (**c**). Left, GCI, plasma TPO, and IgG anti-GPIIb-IIIa antibody-producing B cells in SCT recipients with thrombocytopenia (TP+) and without thrombocytopenia (TP–), ITP patients, aplastic anemia patients, and healthy controls. The bold line in each column indicates the mean. The cutoff values for normal GCI, plasma TPO, and IgG anti-GPIIb-IIIa antibody-producing B cells are indicated by the broken line (2.3, 142 pg/ml, and $2/10^5$ PBMCs, respectively). Differences between two groups were evaluated using the Mann–Whitney *U*-test. Only statistically significant differences between SCT recipients with or without thrombocytopenia and other groups are shown. Right, correlations between platelet count and GCI, TPO, or IgG anti-GPIIb-IIIa antibody-producing B cells in all SCT recipients combined. A correlation coefficient was determined using the single regression model. A fitted line was obtained from the plots of all patients.

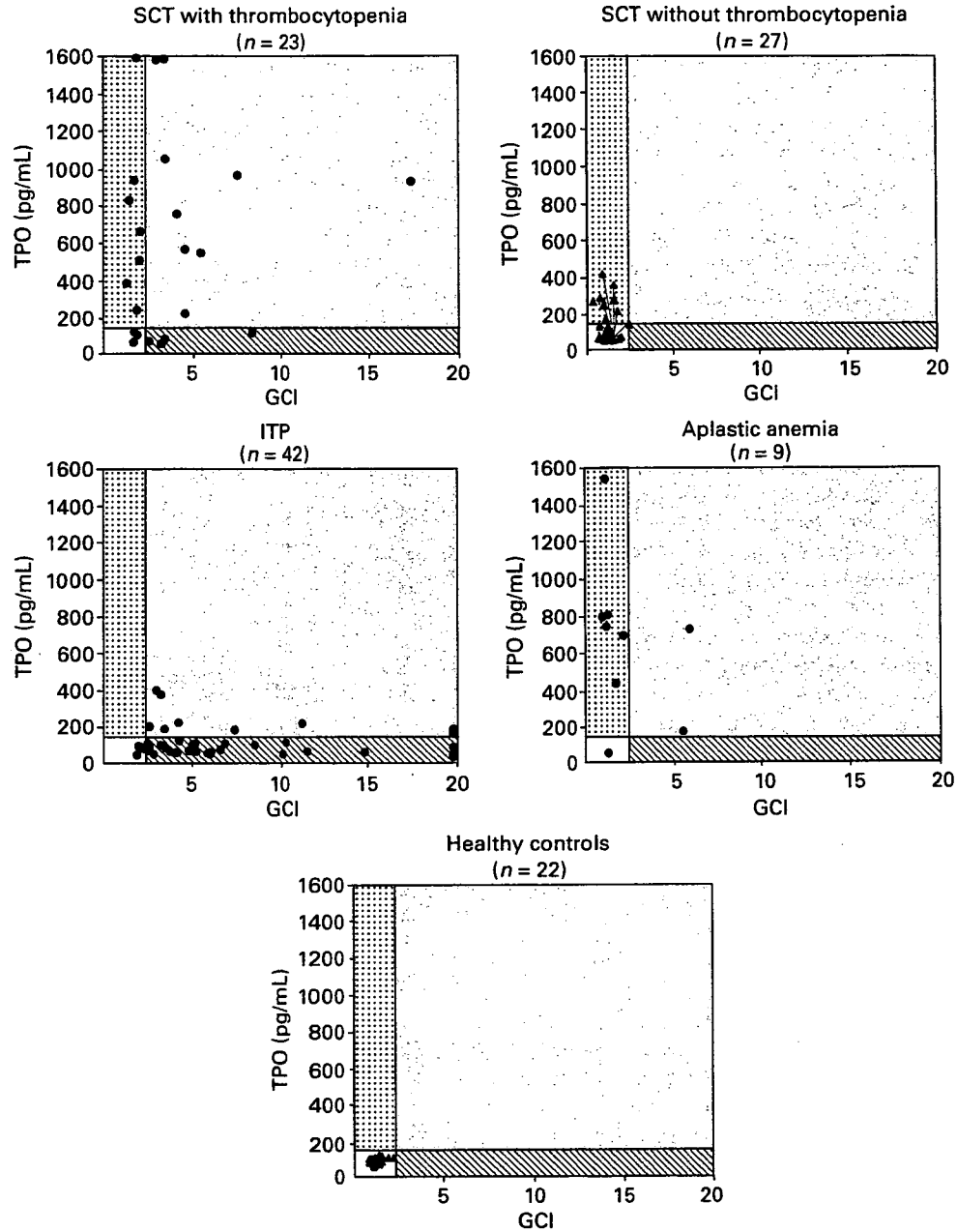


Figure 3 GCI and TPO profiles of SCT recipients with and without thrombocytopenia, ITP patients, aplastic anemia patients, and healthy controls. The subjects were divided into four groups based on being above or below the cutoffs for GCI (2.3) and TPO (142 pg/ml). Closed circles indicate subjects with thrombocytopenia and closed triangles indicate those without it.

GCI and TPO; and four (17%) with normal GCI and TPO. In contrast, 18 (67%) of the 27 SCT recipients without thrombocytopenia and all 22 healthy controls had normal GCI and TPO levels. The patients with ITP and those with aplastic anemia showed distinct patterns: 38 (91%) of the ITP patients had increased GCI, and eight (89%) of the aplastic anemia patients had increased TPO. The GCI and TPO distribution in the SCT recipients with thrombocytopenia was apparently different from that in the ITP patients, but was rather similar to the distribution in patients with aplastic anemia.

Discussion

In this study, the pathogenic processes of prolonged thrombocytopenia in SCT recipients were evaluated by measuring convenient markers for platelet turnover, platelet production, and antiplatelet antibody response. As a result, the GCI and TPO were identified as laboratory markers that were independently associated with thrombocytopenia in SCT recipients, indicating that prolonged thrombocytopenia after SCT results from complex mechanisms, including increased platelet turnover and impaired

thrombopoiesis. However, the frequency and magnitude of the increased TPO were comparable between SCT recipients with thrombocytopenia and patients with aplastic anemia, and the GCI and TPO status in SCT recipients with thrombocytopenia had a pattern similar to that seen in patients with aplastic anemia, suggesting that impaired thrombopoiesis played the predominant role.

Two-thirds of SCT recipients with prolonged thrombocytopenia had elevated TPO, at a level comparable to that of aplastic anemia patients. The level of circulating TPO is principally regulated by the level of its receptor c-Mpl, which is mainly expressed on megakaryocytes in the bone marrow.[24,25] Taken together with the trend towards a high frequency of megakaryocytic hypoplasia in SCT recipients with thrombocytopenia, these data suggest that incomplete recovery of platelet production is one of the major causes of prolonged thrombocytopenia in SCT recipients. In this regard, the number of hematopoietic stem cells transplanted is reported to be important for platelet recovery after SCT,[26] but there was no difference in the number between patients with and without thrombocytopenia in our series of patients.

A significant proportion of SCT recipients with prolonged thrombocytopenia showed an increased GCI, although some of them had increased TPO as well. The increased anti-GPIIb-IIIa antibody-producing B cells and platelet-associated anti-GPIIb-IIIa antibodies in SCT recipients strongly suggest the involvement of platelet-bound antibodies in the process of increased platelet turnover. There are several case series showing that thrombocytopenia observed after SCT can be treated successfully with corticosteroids, high-dose intravenous immunoglobulin, splenectomy, or androgen, all of which are used for treatment of ITP.[2,5,27–29] As the GPIIb-IIIa antigen used in our assays was purified from pooled platelets, the anti-GPIIb-IIIa antibodies could have been either auto- or alloantibodies. In this regard, thrombocytopenia caused by recipient-origin allo-antibodies has been reported in SCT recipients.[6,7] One report described that antibodies of recipient-origin can be sustained for up to 8 years, due to the relative radiation resistance of recipient plasma cells, although recipient antibodies tend to disappear late in the post transplantation period.[6] Alternatively, unbalanced lymphocyte reconstitution after SCT may lead to the oligoclonal proliferation of the autoreactive B-cell repertoire and the resultant production of auto-antibodies against platelets.[5]

However, only half of the SCT recipients with thrombocytopenia and increased anti-GPIIb-IIIa antibody-producing B cells showed an increased GCI, and the anti-GPIIb-IIIa antibody response was also detected in nearly half of the SCT recipients without thrombocytopenia. These observations suggest that the anti-GPIIb-IIIa antibody response is not necessarily associated with thrombocytopenia induced by platelet destruction in the periphery. The inability of anti-GPIIb-IIIa antibodies to bind circulating platelets is one potential mechanism, but the anti-GPIIb-IIIa antibodies in SCT recipients were preferentially detected in platelet eluates rather than in plasma. The precise reason for the presence of antiplatelet antibodies independent of thrombocytopenia in SCT recipients is not clear, but it could be explained by an impaired capacity to process opsonized platelets by the reticuloendothelial system, which might be damaged by high-dose chemotherapy, radiotherapy, and/or GVHD. Therefore, SCT recipients are apparently prone to have an antiplatelet antibody response, but whether they develop thrombocytopenia or not may depend on additional factors, such as the reticuloendothelial function.

In summary, our findings indicate complex mechanisms of post transplant prolonged thrombocytopenia. Measurement of convenient serologic markers, GCI and TPO, may be useful to evaluate underlying processes of prolonged thrombocytopenia in SCT recipients.

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Platelets

Effects of a *Helicobacter pylori* eradication regimen on anti-platelet autoantibody response in infected and uninfected patients with idiopathic thrombocytopenic purpura

Thirty-seven patients with idiopathic thrombocytopenic purpura (ITP) were treated with a standard *Helicobacter pylori* eradication regimen irrespective of *H. pylori* infection. Our results indicate that platelet recovery results from the disappearance of *H. pylori* itself, and is mediated, in part, through suppression of anti-platelet autoantibody production.

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It has been proposed that *Helicobacter pylori* (*H. pylori*) infection is associated with idiopathic thrombocytopenic purpura (ITP), based on increased platelet counts after successful eradication of *H. pylori*.[1,2] However, the prevalence of *H. pylori* infection is similar in ITP patients and the general population,[3] suggesting that ITP is not necessarily linked to *H. pylori* infection. A critical question is how the *H. pylori* eradication regimen increases platelet counts. The most plausible explanation is that the disappearance of *H. pylori* has a therapeutic effect. However, a 1-week regimen of a combination of antibiotics should eradicate bacteria other than *H. pylori*, which may include a bacterium truly associated with the pathogenesis of ITP, or induce prominent changes in the normal flora. Moreover, immunomodulatory effects are reported for drugs used for *H. pylori* eradication. To evaluate these possibilities, we conducted an open-label, prospective study involving 37 consecutive patients with ITP (aged 24 to 73, 14 male) who satisfied the following criteria: thrombocytopenia persisting >6 months, normal or increased bone marrow megakaryocytes without morphologic evidence of dysplasia, no secondary immune or non-immune diseases that could account for thrombocytopenia, and a platelet count <50×10⁹/L at ≥3 measurements during the preceding 3 months. None of the patients satisfied the classification criteria for systemic lupus erythematosus,[4] but 13 (35%) had a low titer (≤ 80) of anti-nuclear antibodies. All patients were assessed for *H. pylori* infection and given amoxicillin (1.5g daily), clarithromycin (800 mg daily), and lansoprazole (60 mg daily) for 7 days, irrespective whether they did or did not have *H. pylori* infection.

All patients visited our hospital at 0, 1, 4, 8, 12, and 24 weeks, and all responders were followed for ≥56 weeks. The anti-GPIIb/IIIa autoantibody response was evaluated by detecting circulating B cells producing anti-GPIIb/IIIa antibodies.[5] The patients were allowed to continue other therapy (danazol, n=4), provided their dosages were maintained at a constant level until 24 weeks, except for prednisolone (≥10mg daily), which was allowed to be decreased or discontinued after platelet counts reached >100×10⁹/L.

The study protocol conformed to the ethical principles of the World Medical Association Declaration of Helsinki as reflected in a priori approval from the Institutional Review Board. Twenty-six patients (70%) who had positive results in a 13°C urea breath test plus serum anti-*H. pylori* antibodies or stool *H. pylori* antigen were regarded as *H. pylori*-positive, whereas 11 patients negative for all three tests were *H. pylori*-negative. Eradication was successful in all *H. pylori*-positive patients according to a negative urea breath test at 12 weeks. When a therapeutic response was defined as a platelet count >100×10⁹/L at 24 weeks, 16 *H. pylori*-positive patients (62%) were responders, while none of the *H. pylori*-

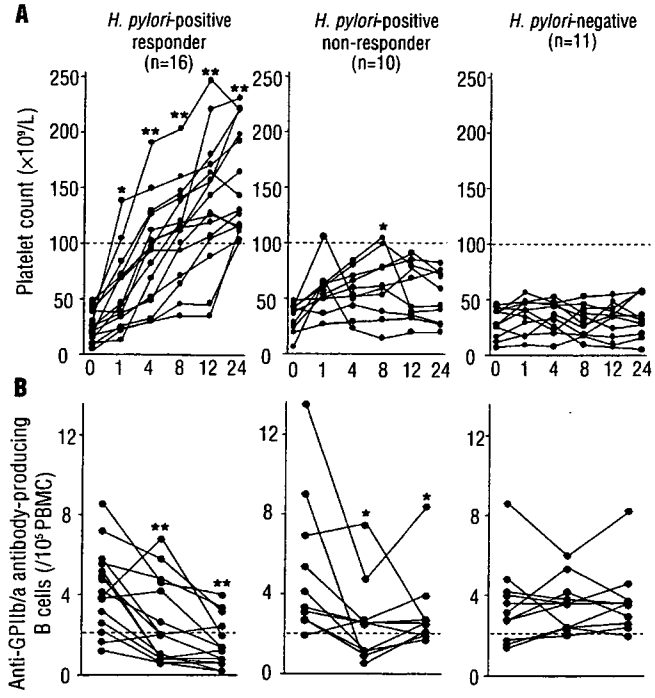

Figure 1. Serial platelet counts (A) and number of circulating anti-GPIIb/IIIa antibody-producing B cells (B) before and after *H. pylori* eradication regimen in *H. pylori*-positive ITP responders (n=16) and non-responders (n=10), and *H. pylori*-negative ITP patients (n=11). Changes in the absolute values at different time points from the baseline value taken at week 0 were compared by repeated measures analysis of variance. When the *p* value for this overall comparison was statistically significant, post-hoc pairwise comparisons were performed using Dunnett's test. *<0.05 and **<0.01 compared with week 0. Broken lines indicate the cut-offs for the platelet response (100×10⁹/L; A) and the number of circulating anti-GPIIb/IIIa antibody-producing B cells (2/10⁵ peripheral blood mononuclear cells; B. At week 0, a positive anti-GPIIb/IIIa antibody response was detected in 14 H. pylori-positive responders (88%), nine *H. pylori*-positive non-responders (90%), and eight *H. pylori*-negative patients (73%).

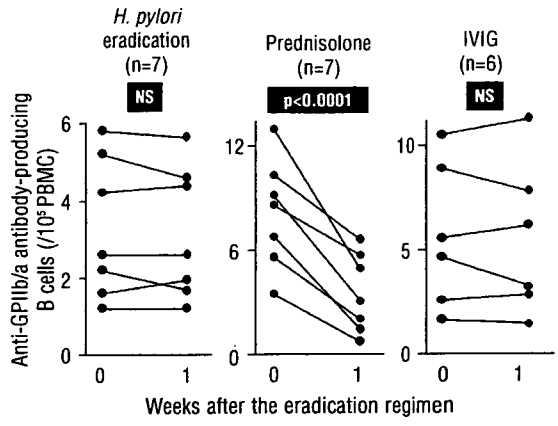

Figure 2. The number of circulating anti-GPIIb/IIIa antibody-producing B cells in ITP patients who responded to *H. pylori* eradication regimen (n=7), high-dose prednisolone (n=7), or IVIG (n=6) before and 1 week after the therapeutic regimen. The prednisolone group received 1 mg/kg prednisolone for at least 1 week while the IVIG group received 400 mg/kg human immunoglobulins for 3 days. Absolute values at weeks 0 and 1 were compared by a paired t-test. NS: not significant.

negative patients was a responder (p=0.0006). The platelet response lasted for ≥56 weeks in all responders. Serial platelet counts in *H. pylori*-positive responders and non-responders, and *H. pylori*-negative patients are shown in Figure 1A. Almost no fluctuation was observed in the *H. pylori*-negative patients. In 13 *H. pylori*-positive patients (50%), platelet counts increased two-fold or more at 1 week irrespective of the platelet response at 24 weeks. Anti-GPIIb/IIIa antibody-producing B cells were significantly decreased at 12 and 24 weeks in *H. pylori*-positive responders (p<0.0001) and, to a lesser extent, in non-responders (p=0.02), but not in *H. pylori*-negative patients (Figure 1B). There was no difference in frequencies of responders between anti-nuclear antibody-positive and negative *H. pylori*-positive ITP patients (50% versus 65%).

Despite a rapid platelet increase by a 1 week, there was no reduction in anti-GPIIb/IIIa antibody-producing B cells at this time point in seven *H. pylori*-positive responders (Figure 2). For reference, we additionally measured anti-GPIIb/IIIa antibody-producing B cells in a separate cohort of ITP patients who responded to high-dose prednisolone (n=7) or intravenous immunoglobulin (IVIG) (n=6). The antibody-producing B cells were significantly reduced at 1 week in patients treated with prednisolone (p<0.0001), but not in patients treated with IVIG.

In this prospective study, the efficacy of the *H. pylori* eradication regimen in *H. pylori*-infected ITP patients was in agreement with previous studies,[1-3] but platelet recovery was observed in none of *H. pylori*-negative patients. Taken together with previous case series of *H. pylori*-negative patients who failed to respond to the *H. pylori* eradication regimen,[6-8] it is likely that platelet recovery results from the eradication of *H. pylori* itself, rather than from other *H. pylori*-independent mechanisms. Whether the *H. pylori* eradication regimen influences the anti-platelet autoantibody response is still under debate.[9,10] Our study clearly showed that anti-GPIIb/IIIa autoantibody response was suppressed at 12 and 24 weeks after successful *H. pylori* eradication, suggesting involvement of *H. pylori* infection in the process of anti-platelet autoantibody production. However, a rapid platelet increase at 1 week might be mediated by other mechanisms, potentially similar to the actions of IVIG. These different actions of *H. pylori* eradication suggest that multiple processes are responsible for platelet recovery in ITP patients.

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Key words: idiopathic thrombocytopenic purpura, autoantibody, Helicobacter pylori, platelet.

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Helicobacter pylori and Immune Thrombocytopenic Purpura: Unsolved Questions and Controversies

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Abstract

Immune thrombocytopenic purpura (ITP) is an autoimmune disease mediated by anti-platelet autoantibodies. Recent accumulating evidence indicates that eradication of *Helicobacter pylori* (*H pylori*) is effective in increasing platelet count in nearly half of ITP patients infected with this bacterium. The *H pylori* eradication therapy for adult ITP is becoming very popular in Japan and is now chosen as an initial treatment in *H pylori*–infected patients. The lack of efficacy of the *H pylori* eradication regimen in *H pylori*–negative ITP patients clearly indicates that platelet recovery results from the disappearance of *H pylori* itself. Despite extensive efforts, clinical features characteristic to *H pylori*–related ITP and factors predicting the response after the *H pylori* eradication therapy have not been identified. Great variability in the efficacy of the *H pylori* eradication therapy in ITP patients exists among countries: a higher response rate has been found in Japan and Italy than in the United States and non-Italian European countries. Some children infected with *H pylori* show the platelet response after successful eradication of *H pylori*, but the *H pylori* eradication therapy is ineffective in patients with secondary ITP. The pathogenesis of ITP associated with *H pylori* remains obscure; the mechanisms are not simple and may involve multiple steps, including cross-reactivity between *H pylori* antigen and platelets, and suppression of the reticuloendothelial system. Further studies to evaluate the mechanisms responsible for the platelet response in ITP patients after successful eradication of *H pylori* may be useful in clarifying the pathogenesis of ITP and developing new therapeutic strategies for ITP.
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Key words: Immune thrombocytopenic purpura; *Helicobacter pylori*; Autoantibody; Molecular mimicry

1. Introduction

Immune thrombocytopenic purpura (ITP) is an autoimmune disease mediated by anti-platelet autoantibodies that bind to circulating platelets, resulting in Fcγ receptor–mediated platelet destruction by the reticuloendothelial system [1]. A major target of the anti-platelet autoantibodies is GPIIb/IIIa [2], a platelet membrane receptor for fibrinogen and other ligands. Our recent studies on T-cells autoreactive to GPIIb/IIIa have revealed that the pathogenic process of ITP can be explained by a continuous loop in which B-cells produce anti-platelet autoantibodies, splenic macrophages phagocytose opsonized platelets and present GPIIb/IIIa-derived antigenic peptides, and GPIIb/IIIa-reactive T-cells are activated and exert their helper activity [3].

Helicobacter pylori (*H pylori*) is a gram-negative bacillus, which colonizes the mucous layer of the human stomach and has been implicated in gastrointestinal disorders, including gastric and duodenal ulcers, chronic atrophic gastritis, and gastric cancer [4]. In 1988, a group in Italy first reported an increased platelet count in ITP patients after the successful eradication of *H pylori* [5]. This observation has been confirmed by many other studies, and eradication of *H pylori* is accompanied by a platelet response in approximately half of adult patients with ITP (reviewed in [6-8]). The *H pylori* eradication regimen consisted of a standard triple therapy with a proton pump inhibitor (PPI), clarithromycin, and either amoxicillin or metronidazole for 1 or 2 weeks. The majority of

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studies excluded patients with severe thrombocytopenia who were at high risk of bleeding, but several case series reported the efficacy in patients with refractory ITP who had severe thrombocytopenia resistant to multiple therapeutic regimens, including splenectomy [9-11]. In addition, accumulating evidence indicates that the platelet response lasts a year or longer and cases of relapse are quite few [9,10]. Because of the relatively high efficacy of the *H pylori* eradication therapy in *H pylori*–infected ITP patients, *H pylori* infection is believed to be associated with the pathogenic process of ITP. Certain microorganisms, including human immunodeficiency virus (HIV) and hepatitis C virus, are known to contribute to the development of ITP, and these conditions are often classified as distinct subsets of ITP [12,13]. Thus, some investigators now propose that ITP patients who are infected with *H pylori* and respond to the eradication therapy should be categorized as a distinct subgroup of ITP termed *H pylori*–related or *H pylori*–associated ITP [7,8]. However, prevalence of *H pylori* infection in ITP patients increases with age independent of sex and is similar to the prevalence in the general population [9], suggesting that ITP is not necessarily linked to *H pylori* infection. Interestingly, one case report described onset of severe thrombocytopenia following the standard *H pylori* eradication therapy [14], although it is unclear whether this patient's thrombocytopenia was mediated by anti-platelet autoantibodies. It is now apparent that *H pylori* eradication therapy is often accompanied by a platelet increase in some patients with ITP, but there are still many critical issues regarding the role of *H pylori* in the pathogenesis of ITP. The relationship between *H pylori* infection and ITP would open a new research field, with important implications for both pathogenesis and patient care. This review focuses on unsolved questions and controversies in the relationship between *H pylori* infection and ITP, and summarizes our current understandings.

2. Efficacy of the *H pylori* Eradication Therapy in Uninfected ITP Patients

One of the critical questions is how the *H pylori* eradication regimen increases the platelet count in ITP patients. The most plausible explanation is that disappearance of *H pylori* has a therapeutic effect. However, Fujimura et al reported that a platelet response was observed in 15 (33%) of 46 *H pylori*–infected patients with ITP who failed the eradication therapy [9]. A combination of antibiotics used in a standard triple therapy should eradicate bacteria other than *H pylori*, which may include a bacterium truly associated with the pathogenesis of ITP. In addition, prominent changes in the normal flora may modulate the host immune system [15]. If this were true, other antibiotics would be as effective as the standard triple therapy independent of ability to eradicate *H pylori*. Moreover, an immunomodulatory effect has been reported for drugs used for the *H pylori* eradication therapy, such as macrolides [16] and PPI [17]. In this regard, it has been reported that some ITP patients carrying *H pylori* respond to PPI monotherapy [18,19]. To evaluate these possibilities, we recently conducted a prospective study in which ITP patients were treated with a standard triple therapy irrespective of the presence or absence of *H pylori* infection [20]. Seventeen (65%) of 26 *H pylori*–positive ITP patients showed a significant platelet response, but platelet count did not change at all during the following 24 weeks in 11 *H pylori*–negative patients. This finding is consistent with previous case series showing the lack of efficacy of the *H pylori* eradication regimen in *H pylori*–negative ITP patients [21-23]. These studies clearly demonstrate that platelet recovery after *H pylori* eradication results from the disappearance of *H pylori* itself, rather than from other *H pylori*–independent mechanisms. Therefore, it is likely that the increased platelet count in patients who failed the *H pylori* eradication or in those who received PPI monotherapy could have been mediated through a reduction in the quantity of *H pylori* and/or a bacteriostatic effect of the regimen. A clear linkage of platelet recovery to the disappearance of *H pylori* after the eradication regimen suggests a direct role for *H pylori* infection in the pathogenesis of ITP. More importantly, in clinical settings, the *H pylori* eradication regimen should be used to treat ITP patients who are confirmed to have *H pylori* infection.

3. Clinical Characteristics of *H pylori*–Related ITP

On the assumption that *H pylori*–related ITP is a distinct clinical entity, many authors have sought characteristic features of such subset. The *H pylori*–infected ITP patients are shown to be significantly older than *H pylori*–uninfected patients [9], but this finding is predictable because the prevalence of *H pylori* infection in the general population increases with age [4]. In contrast, many studies failed to detect significant differences in other characteristics, including sex, platelet count, and therapeutic responses. One study from Turkey showed that platelet count after the standard corticosteroid treatment in *H pylori*–positive patients was significantly lower than that in *H pylori*–negative patients [24], but the number of patients analyzed was very small. Differences in genetic factors and serum cytokine levels were also examined in *H pylori*–infected and *H pylori*–uninfected ITP patients. Veneri et al assessed frequencies of the human leukocyte antigen (HLA)-DRB1 and DQB1 alleles in Italian ITP patients and found that *H pylori*–positive patients had a lower frequency of DRB1*03 and higher frequencies of DRB1*11, DRB1*14, and DQB1*03 compared with *H pylori*–negative patients [25]. This suggests importance of the genetic background in the *H pylori*–related ITP, but the association between *H pylori* infection and HLA class II alleles in ITP patients should be confirmed in other ethnic groups with different HLA allele distribution. On the other hand, serum levels of interleukin (IL)-2, IL-4, and IL-6 were not different in *H pylori*–positive and *H pylori*–negative ITP patients [26]. Serum levels of chemokines, including monocyte chemoattractant protein-1 (MCP-1), regulated upon activation normally T-cell expressed and secreted (RANTES), and epithelial cell–derived neutrophil attractant–78 (ENA-78), were significantly higher in *H pylori*–positive than in *H pylori*–negative patients [27], although increased levels in these chemokines are also observed in non-ITP individuals with *H pylori*–related gastrointestinal disorders. In summary, clinical, genetic, and immunologic features characteristic to *H pylori*–related ITP have not been identified, calling into question the theory that *H pylori*–related ITP is a distinct entity.

4. Factors Predicting the Response to the *H pylori* Eradication Therapy

Many studies have been carried out to identify parameters that predict the platelet response to the eradication therapy in *H pylori*–infected ITP patients. When a series of background characteristics in ITP patients who responded to the *H pylori* eradication therapy were compared to those who did not, the duration of ITP was shorter in responders than in nonresponders [9]. Other clinical features, such as age, sex, platelet count, and previous therapies including corticosteroids and splenectomy, were not useful to predict the platelet response. Veneri et al divided ITP patients into 3 groups (untreated, relapsed, and refractory) on the basis of previous therapies and their outcomes, and compared the platelet response rates after the *H pylori* eradication therapy [28]. Three (75%) of 4 patients in the refractory group showed the platelet response and were able to stop immunosuppressive therapy, and this rate tended to be higher than that in the other groups. In contrast, Ando et al reported a better response rate in corticosteroid-naive patients than in the patients previously treated with corticosteroids [29]. In another study, HLA-DQB1*03 haplotypes were shown to be associated with a higher probability of the platelet response [25], while Hashino et al failed to detect differences in serum levels of IL-2, IL-4, and IL-6 between responders and nonresponders [26]. Unfortunately, the numbers of patients analyzed in these studies are too small to draw conclusions. Despite extensive efforts, factors predictive of the platelet response after the *H pylori* eradication therapy have not been identified to date. Future studies should be aimed at identifying genetic backgrounds and bacterial factors that account for the variability in the response to the *H pylori* eradication therapy.

5. Variability in the Efficacy of the *H pylori* Eradication Therapy among Countries

There is great variability in the efficacy of the *H pylori* eradication therapy in ITP patients in previous reports [6-8]. Cohorts from Japan and Italy had response rates of 39% to 100% in *H pylori*–infected ITP patients. A recent nationwide survey conducted in Japan showed that 130 (63%) of 207 ITP patients carrying *H pylori* achieved partial or complete platelet recovery after successful *H pylori* eradication [9]. However, studies from Spain, France, and the United States have documented little or no platelet response to the *H pylori* eradication therapy even in *H pylori*–infected ITP patients [30-32]. Moreover, recent studies conducted in Serbia [33] and Turkey [34] showed a relatively low response rate (26% and 40%, respectively). These variable response rates could be explained, in part, by the different eligibility criteria for enrollment and the different definitions of platelet response among studies, but the high response rate in studies from Japan and Italy compared with studies from the other countries was consistent. In fact, the *H pylori* eradication therapy for adult ITP is becoming very popular in Japan and is now chosen as an initial treatment in *H pylori*–infected ITP patients [8]. In contrast, in the United States and many European countries, the *H pylori* eradication therapy is still only one of the options for patients with refractory ITP or those with ITP and less severe thrombocytopenia [35,36]. The *H pylori* infection rate varies between countries and even between races within the same country, and it depends on socioeconomic, geographical, and age-related factors [4]. The middle-aged population in developing countries indicates prevalence rates approaching 80%, while rates less than 50% are seen in many industrialized countries except for Japan and Italy, where the rate is nearly 70%. These rates may indicate a positive correlation between the prevalence rates for *H pylori* infection in general population and the efficacy of the *H pylori* eradication therapy in ITP patients, although the platelet response rate was only 13% in a Spanish cohort while the prevalence of *H pylori* infection was 71% [30]. The reason for such variability among countries is not clear but could be explained by differences in genetic backgrounds or epidemic *H pylori* strains between populations.

6. Efficacy of the *H pylori* Eradication Therapy in Childhood ITP

ITP manifests in chronic and acute forms. Adults mainly develop chronic ITP with an insidious onset, whereas children usually develop acute ITP with a complete recovery within 6 months, suggesting different pathogenic mechanisms in childhood and adult ITP [1]. Because the prevalence of *H pylori* infection is low in children, only a few pediatric studies have been performed to evaluate a role of *H pylori* infection in chronic ITP. In fact, a Finnish study failed to detect *H pylori* infection in any of 17 children with chronic ITP [37]. In contrast, a study conducted in Taiwan showed that 9 (41%) of 22 Chinese children with chronic ITP were infected with *H pylori* [38]. Another Japanese study reported that only 2 (20%) of 10 children with chronic ITP had *H pylori* infection [39]. In the Taiwan study, 5 (56%) of 9 *H pylori*–infected ITP children showed an increase in platelet counts following the eradication therapy [38], and several children who received the *H pylori* eradication therapy and showed a complete platelet recovery have been reported [39,40]. The prevalence of *H pylori* infection in children with chronic ITP is generally low and different among countries, but we should recognize that some children infected with *H pylori* have a chance to recover after successful eradication of *H pylori*.

7. Efficacy of the *H pylori* Eradication Therapy in Secondary ITP

ITP may be secondary to various illnesses, including lymphoproliferative, autoimmune, and infectious diseases [1], but efficacy of the *H pylori* eradication therapy has been examined in only a few cases of secondary ITP. The platelet response was not found in *H pylori*–infected patients with secondary ITP, including systemic lupus erythematosus (SLE) and Evan's syndrome, despite successful eradication of the bacterium [32,41]. In our experience, platelet recovery was not observed in 6 *H pylori*–infected SLE patients with thrombocytopenia even after successful eradication of *H pylori*. Interestingly, Sawalha et al reported an association between absence of *H pylori* infection and SLE in African-American women, suggesting a possible protective role for *H pylori*