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Detection of Circulating B Cells Producing Anti-GPIb Autoantibodies in Patients with Immune Thrombocytopenia

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Abstract

Background: We previously reported that an enzyme-linked immunospot (ELISPOT) assay for detecting anti-GPIIb/IIIa antibody-secreting B cells is a sensitive method for identifying patients with immune thrombocytopenia (ITP). Here we assessed the clinical significance of measuring circulating B cells producing antibodies to GPIb, another major platelet autoantigen.

Methods: Anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells were simultaneously measured using ELISPOT assays in 32 healthy controls and 226 consecutive thrombocytopenic patients, including 114 with primary ITP, 25 with systemic lupus erythematosus (SLE), 30 with liver cirrhosis, 39 with post-hematopoietic stem cell transplantation (post-HSCT), and 18 non-ITP controls (aplastic anemia and myelodysplastic syndrome).

Results: There were significantly more circulating anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in primary ITP, SLE, liver cirrhosis, and post-HSCT patients than in healthy controls ($P<0.05$ for all comparisons). For diagnosing primary ITP, the anti-GPIb ELISPOT assay had 43% sensitivity and 89% specificity, whereas the anti-GPIIb/IIIa ELISPOT assay had 86% sensitivity and 83% specificity. When two tests were combined, the sensitivity was slightly improved to 90% without a reduction in specificity. In primary ITP patients, the anti-GPIb antibody response was associated with a low platelet count, lack of *Helicobacter pylori* infection, positive anti-nuclear antibody, and poor therapeutic response to intravenous immunoglobulin.

Conclusion: The ELISPOT assay for detecting anti-GPIb antibody-secreting B cells is useful for identifying patients with ITP, but its utility for diagnosing ITP is inferior to the anti-GPIIb/IIIa ELISPOT assay. Nevertheless, detection of the anti-GPIb antibody response is useful for subtyping patients with primary ITP.

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Introduction

Immune thrombocytopenia (ITP) is an autoimmune disease in which accelerated platelet consumption and impaired platelet production are mediated primarily by IgG anti-platelet autoantibodies [1]. This condition is seen in patients with various diseases, including systemic lupus erythematosus (SLE) and human immunodeficiency virus infection. It can also occur without an underlying disease, which is known as primary ITP. The production of IgG autoantibodies to platelet surface glycoproteins, such as GPIIb/IIIa and GPIb, is the hallmark of the disease [2]. Several antigen-specific assays for detecting platelet-associated anti-GPIIb/IIIa and anti-GPIb antibodies are reported to be useful for identifying patients with ITP [3–5]. However, no laboratory test for detecting platelet antigen-specific antibodies is used widely in clinical settings, because these assays require complicated procedures such as platelet solubilization, the use of commercially unavailable monoclonal antibodies, and a relatively large blood sample.

We previously developed an enzyme-linked immunospot (ELISPOT) assay for detecting IgG anti-GPIIb/IIIa antibody-secreting B cells in the circulation and spleen of patients with primary ITP [6]. We subsequently showed that the detection of circulating anti-GPIIb/IIIa antibody-producing B cells is a sensitive, specific, and convenient method for evaluating the presence or absence of an anti-platelet autoantibody response [7]. The anti-GPIIb/IIIa antibody response is very common in patients with primary ITP as well as those with various forms of secondary ITP, including thrombocytopenia associated with SLE [8], liver cirrhosis with or without hypersplenism [9], and post-hematopoietic stem-cell transplantation (post-HSCT) [10]. These findings led us to propose preliminary diagnostic criteria for ITP based on a

combination of ITP-associated laboratory findings, including circulating anti-GPIIb/IIIa antibody-producing B cells, reticulated platelets, and thrombopoietin [11]. The ELISPOT assay has several advantages over assays that detect platelet antigen-specific antibodies, i.e., the results are not influenced by the binding of the antibodies to platelet surfaces and only a small blood sample (<3 mL) is required. However, the anti-GPIIb/IIIa antibody response was not detectable in a small proportion (~20%) of ITP patients, even if the sensitive ELISPOT assay was used. Thus, adding a concomitant measurement of B cells producing antibodies to another major platelet autoantigen, GPIb, may increase the assay's sensitivity to the anti-platelet autoantibody response in patients with ITP. In this study, we established an ELISPOT assay for detecting anti-GPIb antibody-producing B cells and evaluated its potential usefulness for the diagnosis, disease subtyping, and assessment of the anti-platelet autoantibody profiles in patients with primary ITP and a various forms of secondary ITP.

Materials and Methods

Subjects

This study included 114 consecutive patients with primary ITP whose peripheral blood samples had been sent to an autoimmune laboratory at Keio University Hospital between April 2003 and March 2005. Eighteen patients were also included in a multicenter prospective study for verification of our preliminary diagnostic criteria for ITP [11]. The inclusion criteria were: (i) clinical diagnosis of primary ITP; (ii) thrombocytopenia (platelet count $\leq 50\times10^9$/L); (iii) no previous treatment with corticosteroids or immunosuppressants; and (iv) availability of detailed clinical information for at least one year after the diagnosis. The clinical diagnosis of ITP was made by one of the authors (YI) on the basis of clinical history, physical examination, complete blood test, and bone marrow findings if available, according to the guidelines proposed by the American Society of Hematology [12]. The final diagnosis was re-evaluated, taking into account the clinical course of the disease over at least one year, especially the therapeutic responses to corticosteroids, splenectomy, and eradication of *Helicobacter pylori* (*H. pylori*). YI was blinded to the results of the anti-GPIIb/IIIa and anti-GPIb antibody-producing B cell assays, so the clinical diagnosis of primary ITP was not influenced by these laboratory findings. Patients with primary ITP were classified as having newly diagnosed, persistent, or chronic ITP, as described previously [13].

Additional thrombocytopenic patients with underlying diseases that could potentially cause secondary ITP or non-ITP thrombocytopenia were selected from consecutive patients whose peripheral blood samples had been sent to the autoimmune laboratory during the same period, based on the definitive diagnosis of underlying diseases/conditions and platelet count $\leq 50\times10^9$/L. SLE and liver cirrhosis were diagnosed according to the published criteria [14,15]. HSCT recipients were selected based on a lack of sustained anemia or leukopenia, and no apparent cause for thrombocytopenia, such as engraftment failure, recurrence of the underlying hematologic malignancy, microangiopathy, or drugs [10]. To minimize the potential influence of procedure-related complications, we selected patients who had survived for >100 days after HSCT. Patients with aplastic anemia or myelodysplastic syndrome (MDS) were also enrolled as a non-ITP disease control. Diagnosis of aplastic anemia and MDS was based principally on bone marrow findings and cytogenetic analysis [16,17]. Thirty-two healthy individuals were also included as a control. All samples were obtained after the subjects gave their written informed consent, as approved by the ethical committee of Keio University School of Medicine (Application number 2010-031-2).

Therapeutic Response

A therapeutic response to intravenous immunoglobulin (IVIG) was defined as a platelet count $>100\times10^9$/L at one week, respectively [8], while a response to *H. pylori* eradication or corticosteroids (>0.5 mg/kg prednisolone in combination with or without IVIG) was defined as a platelet count $>100\times10^9$/L at 24 weeks, respectively [18]. We used the published definition for therapeutic response to splenectomy [19], but complete and partial responses were combined. That is, a therapeutic response was defined as a platelet count of $\geq 50\times10^9$/L for 30 days or longer after splenectomy, with or without other treatment.

H. pylori Infection

H. pylori infection was evaluated with a ^{13}C urea breath test using a UBiT tablet (Otsuka Assay, Tokyo, Japan), the detection of serum IgG anti-*H. pylori* antibodies using a commercially available kit (Kyowa Medex Company, Tokyo, Japan), and the detection of *H. pylori* antigen in stool samples using ImmunoCard® HpST® (Meridian Bioscience, Cincinnati, OH). Patients positive for the urea breath test plus at least one additional test were regarded as *H. pylori*-positive [18].

Antinuclear Antibody (ANA)

ANA was measured by indirect immunofluorescence using commercially available HEp-2 slides (MBL, Nagoya, Japan) as the substrate. A positive result was determined as a significant signal using two different cut-off levels: serum samples diluted 1:40 and 1:160.

Measurement of IgG Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

B cells producing IgG anti-GPIIb/IIIa antibodies were measured using the ELISPOT assay as described previously [6,7]. Briefly, a polyvinylidene difluoride-bottomed 96-well microplate (Millipore, Bedford, MA) was activated by incubation with ethanol (>99.5%) at room temperature for 10 minutes. After extensive wash with phosphate-buffered saline (PBS) containing 0.5 mM $CaCl_2$ (PBS-Ca), the microplates were coated with affinity-purified human GPIIb/IIIa (purity >80%; Enzyme Research Laboratories, Swansea, UK) dissolved in PBS-Ca at a concentration of 30 µg/mL over night at 4°C. Then, the plates were washed three times with PBS-Ca, and were subsequently blocked with 1% bovine serum albumin (Sigma-Aldrich, St. Louis, MO) in PBS-Ca at room temperature for one hour. Peripheral blood mononuclear cells (PBMCs), isolated from heparinized peripheral blood by Lymphoprep (Fresenius Kabi Norge AS, Halden, Norway) density gradient centrifugation, were re-suspended in RPMI1640 containing 10% fetal bovine serum (Life Technologies, Carlsbad, CA), and were pipetted into the wells (10^5/well) and cultured at 37°C with 5% CO_2 for 4 hours. After washing away the cells with PBS-Ca containing 0.05% Tween 20, the membranes were incubated with alkaline phosphatase-conjugated goat anti-human IgG (ICN/Cappel, Aurora, OH) diluted at 1:1,000 in PBS-Ca at room temperature for 2 hours, followed by wash two time each with PBS-Ca with 0.05% Tween 20 and PBS-Ca. Finally, anti-GPIIb/IIIa antibodies that bound to the membrane were visualized as spots by incubation with nitro blue tetrazolium (Sigma-Aldrich; 300 µg/mL)/5-bromo-4-chloro-3-indolyl phosphate (Sigma-Aldrich; 60 µg/mL) in a buffer consisting of 100 mM Tris-HCl

(pH9.5), 100 mM NaCl, 50 mM $MgCl_2$ at room temperature for 20 minuts. B cells producing IgG anti-GPIb antibodies were also measured by ELISPOT assay, in which a recombinant GPIbα fragment was used instead of GPIIb/IIIa as the antigen. The recombinant GPIbα fragment, which covered the entire von Willebrand factor-binding site (residues 1 to 302), was expressed in Chinese hamster ovary cells [20]. For the anti-GPIb antibody ELISPOT assay, PBS was used instead of PBS-Ca in the entire protocol. The plates coated with bovine serum albumin in the blocking buffer in the absence of GPIIb/IIIa or GPIb were used as control for the ELISPOT assay. Each assay was conducted in 5 independent wells, and the results represented the mean of the 5 values. The frequency of circulating anti-GPIIb/IIIa or anti-GPIb antibody-producing B cells was calculated as the number per 10^5 PBMCs. The cut-off value for anti-GPIIb/IIIa antibody-producing cells was defined as 2.0 per 10^5 PBMCs [7]. The cut-off value for anti-GPIb antibody-producing cells was set at 5 standard deviations above the mean value from healthy controls.

Statistical Analysis

All continuous variables were expressed as the mean ± standard deviation (SD). Comparisons between two groups were tested for statistical significance using the Mann-Whitney test. Differences in frequency between two groups were compared using the Chi-square test or Fisher's exact test, when applicable. The correlation coefficient (r) was determined using a single-regression model.

Results

Patient Characteristics

This study enrolled a total of 226 thrombocytopenic patients. They were composed of 114 with primary ITP, 25 with SLE, 30 with liver cirrhosis, 39 with post-HSCT, and 18 non-ITP controls, including 4 with aplastic anemia and 14 with myelodysplastic syndrome. Table 1 summarizes the sex, age at examination, and platelet count of thrombocytopenia patients and healthy controls. Forty-eight patients (42%) with primary ITP were categorized as having newly diagnosed ITP, while the remaining patients had persistent or chronic ITP. The etiologies of liver cirrhosis included hepatitis B virus infection in 5, hepatitis C virus infection in 21, and alcohol in 4. Of the post-HSCT patients, 37 had received bone marrow transplantation while 2 had received peripheral blood stem cell transplantation. Compared with patients with primary ITP, patients with SLE were predominantly female ($P = 0.02$) and those with liver cirrhosis and MDS were older ($P = 0.001$ and $P = 0.03$, respectively). There was no difference in platelet count among the thrombocytopenic patient groups. The mean follow-up period in patients with primary ITP was 49±26 months.

Detection of IgG Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

Circulating B cells producing IgG anti-GPIIb/IIIa and anti-GPIb antibodies were simultaneously measured in patients with primary ITP, various thrombocytopenic conditions, and healthy controls (Figure 1). No clear spot was detected in the control plates coated with bovine serum albumin alone. There were significantly more circulating anti-GPIIb/IIIa antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT than in healthy controls (5.4±4.7, 6.0±6.4, 10.0±5.8, and 6.3±8.3 versus 0.3±0.4; $P<0.05$ for all comparisons). In contrast, there was no difference in anti-GPIIb/IIIa antibody-producing B cells between the non-ITP disease controls, including aplastic anemia and MDS, and healthy controls. Among ITP-related conditions, patients with liver cirrhosis had a greater frequency of anti-GPIIb/IIIa antibody-producing B cells than did those with primary ITP, SLE, or post-HSCT ($P<0.05$ for all comparisons). Similarly, there were significantly more anti-GPIb antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT than in healthy controls (3.0±3.3, 10.5±25.6, 4.8±5.2, and 3.4±6.2 versus 0.4±0.4; $P<0.01$ for all comparisons). Again, there was no difference between the non-ITP disease controls and healthy controls. The frequency of anti-GPIb antibody-producing B cells tended to be higher in SLE patients than in those with other ITP-related conditions, but the difference was not statistically significant.

The circulating frequencies of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells were correlated with each other in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT ($P<0.0003$ for all correlations) (Figure 2). Based on the slopes of the fitted lines obtained by the single regression model, the anti-GPIIb/IIIa antibody-producing cells exceeded the anti-GPIb antibody-producing B cells in patients with primary ITP, liver cirrhosis, and post-HSCT (slope <1), whereas the anti-GPIb antibody-producing B cells predominated in SLE patients (slope >1).

Diagnostic Utility of Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells

To evaluate the diagnostic utility of the anti-GPIIb/IIIa and anti-GPIb ELISPOT assays, the results of these tests were judged as positive or negative based on being above or below a defined cut-off level. We used 2.0 per 10^5 PBMCs as the cut-off value for anti-GPIIb/IIIa antibody-producing B cells, which was determined in our previous study [7] and 2.4 per 10^5 PBMCs for circulating anti-GPIb antibody-producing B cells, which was 5 standard deviations above the mean value obtained from healthy controls. The positive frequencies of circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells, and their combination in patients with primary ITP, various thrombocytopenic conditions, and healthy controls are summarized in Table 2. Anti-GPIIb/IIIa antibody-producing cells were detected in 86% of the patients with primary ITP, and in 76%, 97%, and 62% of the patients with SLE, liver cirrhosis, and post-HSCT, respectively. In contrast, the percentages of patients with a positive frequency of anti-GPIb antibody-producing B cells were lower (38–50%) than those of anti-GPIIb/IIIa antibody-producing cells. These antibody-producing cells were infrequently detected in patients with aplastic anemia or MDS. Of 16 patients with primary ITP who were negative for the anti-GPIIb/IIIa antibody-producing cells, 5 (31%) were positive for the anti-GPIb antibody-producing cells. Three (50%) out of 6 SLE patients with the negative anti-GPIIb/IIIa ELISPOT result were positive for the anti-GPIb ELISPOT assay, but none of the patients with liver cirrhosis or post-HSCT who showed the negative anti-GPIIb/IIIa ELISPOT result were positive for the anti-GPIb ELISPOT assay. When the results for anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells were combined, the positive frequency was slightly increased in patients with primary ITP and SLE, but not in those with liver cirrhosis and post-HSCT, because in the latter cases the anti-GPIb antibody-producing B cells always coexisted with anti-GPIIb/IIIa antibody-producing ones.

We then focused on the utility of anti-GPIIb/IIIa and anti-GPIb antibody-producing B cell measurement for the diagnosis of primary ITP. This analysis included 114 patients with primary ITP and 18 with non-ITP thrombocytopenia, including aplastic anemia and MDS. The anti-GPIIb/IIIa antibody-producing B cell measurement had a sensitivity of 86%, specificity of 83%, positive

Table 1. Demographic features and platelet count of subjects enrolled in this study.

	Number	Sex (% male)	Age at examination (years)	Platelet count (×10⁹/L)
Primary ITP	114	40%	49.6±17.1	28.1±11.6
SLE	25	12%	43.6±14.6	28.6±13.3
Liver cirrhosis	30	53%	63.3±9.1	35.2±11.5
Post-HSCT	39	59%	37.6±10.6	31.5±11.3
Aplastic anemia	4	25%	46.3±23.9	24.5±16.2
MDS	14	57%	60.4±17.5	26.9±13.3
Healthy controls	32	50%	44.1±12.2	252.4±56.8*

ND: not determined.
*Data were derived from 16 healthy donors.
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predictive value of 98%, and negative predictive value of 50%. In contrast, the sensitivity of the anti-GPIb antibody-producing cells was only 53%, while the specificity was 89% and positive and negative predictive values were 86% and 20%, respectively. When the two tests were combined, the sensitivity was slightly improved to 90% without effectively reducing the specificity or positive predictive value of the anti-GPIIb/IIIa ELISPOT assay alone (83%, 97%, respectively). When the same analysis was performed in patients with SLE, the sensitivity was improved from 76% in case of the anti-GPIIb/IIIa ELISPOT assay alone to 88% in case of combining the two tests.

Clinical Characteristics Associated with Anti-GPIIb/IIIa and Anti-GPIb Antibody-producing B Cells in Patients with Primary ITP

Patients with primary ITP were stratified into two groups based on the presence or absence of anti-GPIIb/IIIa or anti-GPIb antibody-producing B cells (Table 3). Newly diagnosed ITP was less common in patients with anti-GPIIb/IIIa antibody-producing B cells, than in those without. The positive anti-GPIb ELISPOT assay result was associated with a low platelet count, lack of *H. pylori* infection, and positive ANA, independent of the cut-off levels. Therapeutic responses to *H. pylori* eradication, IVIG, and splenectomy tended to be worse in patients with a positive anti-GPIb ELISPOT assay than in those without, but only the difference in the response to IVIG reached statistical significance. On the other hand, there were no differences in clinical

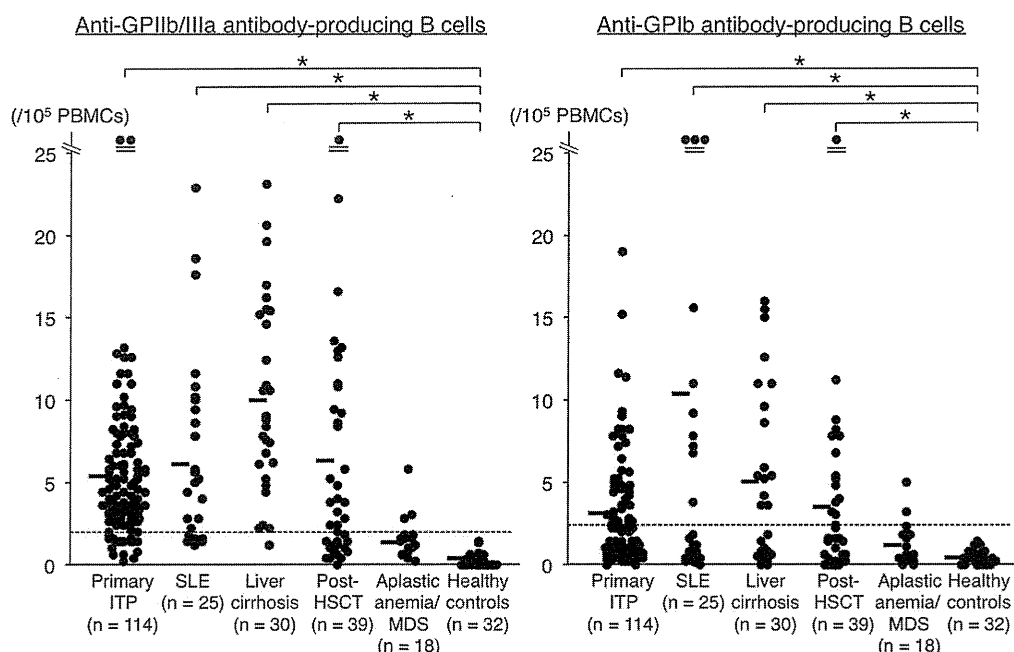

Figure 1. Anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells in the circulation of patients with various thrombocytopenic conditions and healthy controls. Cut-off values for anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells were 2.0 and 2.4 per 10^5 PBMCs, respectively. Bars indicate the mean, and asterisks indicate statistical significance ($P<0.05$).
doi:10.1371/journal.pone.0086943.g001

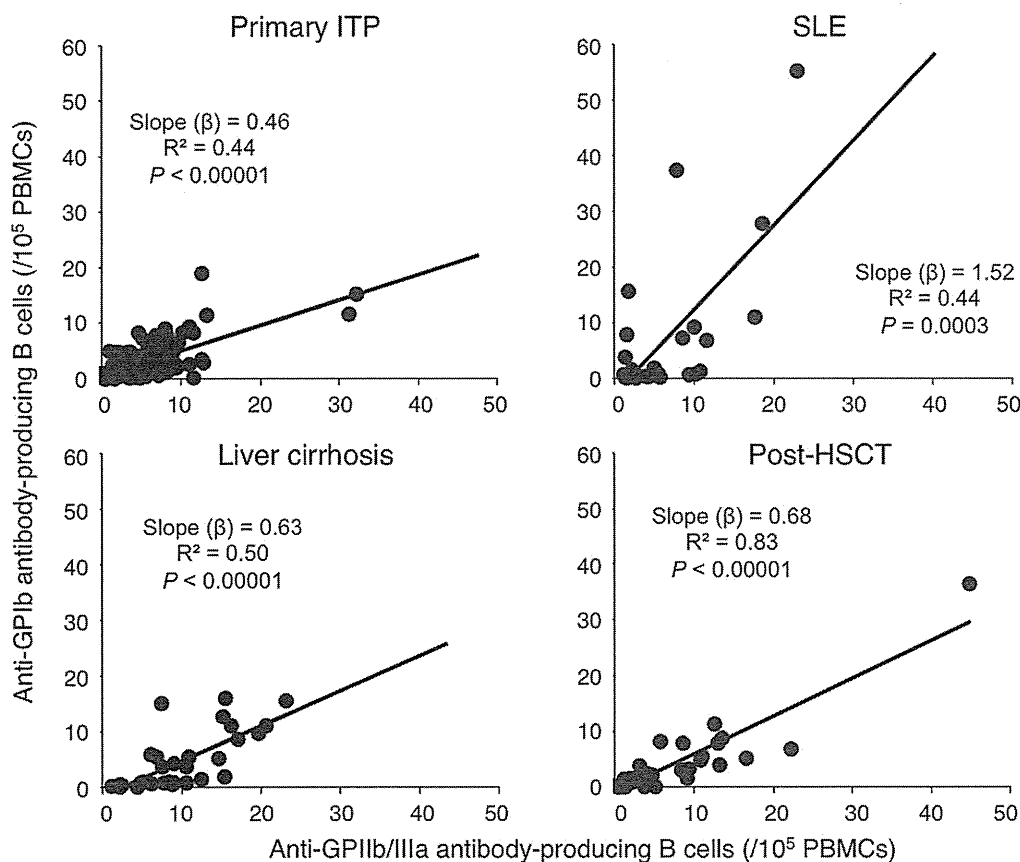

Figure 2. Correlations between circulating anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in patients with primary ITP, SLE, liver cirrhosis, and post-HSCT.
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characteristics except the ITP classification between patients with and without anti-GPIIb/IIIa antibody-producing B cells.

Discussion

In this study, we successfully developed an ELISPOT assay for detecting anti-GPIb antibody-secreting B cells, by applying the principles of our previously developed anti-GPIIb/IIIa antibody-producing B cell measurement. Anti-GPIb antibody-secreting B cells were detected in the circulation of patients with primary ITP as well as conditions that potentially cause secondary ITP, but were infrequently found in patients with aplastic anemia or MDS. Thus, the anti-GPIb ELISPOT assay is useful for identifying patients with ITP, but its sensitivity was much inferior to that of the anti-GPIIb/IIIa ELISPOT assay, indicating that detection of anti-GPIb antibody-producing cells could not replace the anti-GPIIb/IIIa assessment in ITP diagnosis. In addition, concomitant measurement of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells had limited utility: a slight increase in sensitivity only for primary ITP and SLE. These findings indicate that, rather

Table 2. Positive frequencies of circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells, and their combination in patients with primary ITP, various thrombocytopenic conditions, and healthy controls.

	Primary ITP (n = 114)	SLE (n = 25)	Liver cirrhosis (n = 30)	Post-HSCT(n = 39)	Aplastic anemia/ MDS (n = 18)	Healthy controls (n = 32)
Anti-GPIIb/IIIa antibody-producing B cells alone	86%	76%	97%	62%	17%	0%
Anti-GPIb antibody-producing B cells alone	43%	40%	50%	38%	11%	0%
Anti-GPIIb/IIIa antibody-producing B cells AND anti-GPIb antibody-producing B cells	38%	28%	50%	38%	11%	0%
Anti-GPIIb/IIIa antibody-producing B cells OR anti-GPIb antibody-producing B cells	90%	88%	97%	62%	17%	0%

SLE, liver cirrhosis, and post-HSCT are conditions potentially causing secondary ITP, whereas aplastic anemia and MDS are non-ITP disease controls.
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Table 3. Clinical findings in patients with primary ITP, stratified by the presence or absence of circulating anti-GPIb or anti-GPIIb/IIIa antibody-producing B cells.

	Anti-GPIIb/IIIa antibody-producing B cells			Anti-GPIb antibody-producing B cells		
	Present (n = 98)	Absent (n = 16)	P	Present (n = 49)	Absent (n = 65)	P
Sex (% female)	58%	69%	0.60	65%	55%	0.28
Age at examination (years)	50.3±17.5	45.4±14.1	0.29	50.0±17.6	49.3±16.9	0.85
Newly diagnosed ITP (%)	37%	75%	0.009	47%	38%	0.47
Platelet count (x 10^9/L)	27.5±11.5	31.7±12.1	0.19	19.8±9.4	34.4±8.8	<0.0001
H. pylori infection	28%	19%	0.66	14%	35%	0.01
Positive ANA (≥1:40)	26%	44%	0.23	51%	23%	0.002
Positive ANA (≥1:160)	17%	13%	0.83	24%	5%	0.004
Therapeutic response						
H. pylori eradication	62% (n = 26)	100% (n = 3)	0.50	33% (n = 6)	74% (n = 23)	0.16
IVIG	65% (n = 40)	56% (n = 9)	0.60	46% (n = 24)	80% (n = 25)	0.03
Corticosteroids	21% (n = 53)	17% (n = 12)	0.91	24% (n = 33)	15% (n = 33)	0.54
Splenectomy	76% (n = 37)	75% (n = 8)	0.97	64% (n = 22)	86% (n = 23)	0.14

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disappointingly, the additional measurement of anti-GPIb antibody-producing B cells on top of the anti-GPIIb/IIIa ELISPOT assay does not improve the diagnostic accuracy for patients suspected of having ITP. However, it may be worth measuring the anti-GPIb ELISPOT assay in patients who are suspected to have primary ITP or secondary ITP in association with SLE, but are negative for anti-GPIIb/IIIa antibody-producing cells, because there is a >30% chance for obtaining the positive result.

Several laboratories have reported antigen-specific assays, such as the monoclonal antibody-specific immobilization of platelet antigen (MAIPA) assay, for detecting autoantibodies to GPIIb/IIIa and GPIb, which are either bound to platelet surfaces or present in plasma, although an international study comparing these antigen-specific assays revealed that good inter-laboratory agreement was obtained only when the platelet-associated antibodies were measured [21]. In this regard, Warner and colleagues reported that an antigen-specific assay detecting platelet-associated anti-GPIIb/IIIa antibodies had a sensitivity of 57% and a specificity of 96% for the diagnosis of primary ITP, and the additional measurement of anti-GPIb antibodies increased the diagnostic sensitivity to 66%, and retained a specificity of 92% [3]. In another report using a prospective cohort of thrombocytopenic patients, platelet-associated anti-GPIIb/IIIa and anti-GPIb antibodies detected by direct MAIPA were present in 49% of 93 patients with ITP, including 74 with the primary form, and in only 22% of 54 patients with non-ITP thrombocytopenia [4]. The platelet-associated antibodies to GPIIb/IIIa (88%) were more frequently directed than to GPIb (52%), while 40% of patients had concomitant antibodies to both of these platelet glycoproteins. Finally, McMillan et al examined platelet-associated anti-GPIIb/IIIa and anti-GPIb antibodies in 282 patients with primary ITP, and found that the majority of patient samples contained platelet-associated antibodies recognizing GPIIb/IIIa alone (52%); fewer reacted to GPIb alone (12%) or to both complexes (15%) [5]. Our findings, obtained by measuring the anti-GPIIb/IIIa and anti-GPIb antibody-producing circulating B cells, were generally concordant with these results from assays detecting specific platelet-associated antibodies: GPIIb/IIIa antibodies were predominantly recognized, while anti-GPIb antibody measurement contributed minimally to the diagnosis of primary ITP. The ELISPOT assays appear to be more sensitive than the platelet-associated antigen-specific assays (90% versus 49–66%), but prospective studies comparing the ELISPOT assays with other anti-platelet autoantibody detection tests are necessary to confirm this.

The main reason for the low utility of anti-GPIb antibody-producing B cells for the diagnosis of ITP is that circulating anti-GPIIb/IIIa and anti-GPIb antibody-producing B cells coexist in the majority of patients with ITP. Only a small number of patients had anti-GPIb antibody-producing B cells alone. Therefore, in routine clinical settings, the anti-GPIIb/IIIa ELISPOT assay appears to be sufficient for the diagnosis of ITP. Interestingly, the levels of anti-GPIb and anti-GPIIb/IIIa antibody-producing B cells in the circulation were correlated with each other in ITP patients, irrespective of whether the diagnosis was primary ITP or one of the secondary forms. These findings indicate that the autoimmune response in the majority of ITP patients targets multiple platelet glycoproteins, which might be a consequence of "epitope spreading" [2]. In this regard, we have proposed a "pathogenic loop" model for the ongoing anti-platelet autoantibody response in ITP patients [22]. Namely, macrophages in the reticuloendothelial system (spleen in the majority of the patients) capture opsonized platelets, and activate autoreactive T helper cells that stimulate the B cells to proliferate [23], differentiate into plasma cells [24], and produce anti-platelet autoantibodies, which in turn bind to circulating platelets. The continuous destruction of platelets in the reticuloendothelial system would allow the processing and presentation of a whole panel of platelet antigens by macrophages, some of which could elicit additional autoreactive T cell responses, resulting in the production of autoantibodies against other platelet glycoproteins.

In primary ITP and various forms of secondary ITP, SLE was unique in having a predominant anti-GPIb antibody-producing B cell response. SLE is a systemic autoimmune disease characterized by a loss of tolerance to nuclear and other self-antigens, a production of pathogenic autoantibodies, and damage to multiple organ systems [25]. Taken together with the association between anti-GPIb antibody-producing B cells and the production of ANAs, even in patients with primary ITP, the anti-GPIb autoantibody response might be linked to systemic autoimmunity.

Because a significant proportion of SLE patients had anti-GPIb antibody-producing B cells in the absence of anti-GPIIb/IIIa antibody-producing B cells, measurement of the anti-GPIb in addition to anti-GPIIb/IIIa antibody-producing B cells may have some merit for accurately identifying secondary ITP in patients with SLE and thrombocytopenia, although the number of patients analyzed in this study was too small to draw a firm conclusion.

Much effort has been made to identify clinical associations of individual anti-platelet glycoprotein antibodies, but the clinical significance of such antibodies remains uncertain. In patients with primary ITP, the presence of platelet-associated anti-GPIb antibodies was shown to be associated with a lower platelet count [26,27] and inadequate responses to corticosteroids [26] and IVIG [28]. Our results were consistent with these previous observations, including the low platelet count and poor responses to therapeutic interventions, especially to IVIG. In this regard, some monoclonal antibodies against GPIb are known to induce platelet activation, which may lead to accelerated platelet destruction independent of the Fcγ receptor-mediated process in ITP patients [29]. We additionally found correlations between anti-GPIb antibody-producing B cells and a low prevalence of *H. pylori* infection or a high frequency of positive ANA. These findings indicate that there may be a relatively homogeneous subset of primary ITP cases defined by the anti-GPIb antibody response, and characterized by severe thrombocytopenia, the absence of *H. pylori* infection, a positive ANA, and a poor therapeutic response.

In summary, our ELISPOT assay for detecting anti-GPIb antibody-secreting B cells is useful for identifying patients with ITP, but its utility for diagnosing ITP is apparently inferior to the anti-GPIIb/IIIa ELISPOT assay. Nevertheless, detection of the anti-GPIb antibody response is useful for subtyping patients with primary ITP and predicting the therapeutic response.

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Author Contributions

Conceived and designed the experiments: MK YI. Performed the experiments: MK YO YI. Analyzed the data: MK YI. Wrote the paper: MK YO YI.

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Case of maternal and fetal deaths due to severe congenital thrombotic thrombocytopenic purpura (Upshaw–Schulman syndrome) during pregnancy

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Abstract

Upshaw–Schulman syndrome (USS) involves a congenital deficiency of von Willebrand factor-cleaving metalloprotease (ADAMTS13) activity due to gene mutations. Female patients develop overt thrombotic thrombocytopenic purpura (TTP) caused by a decline of ADAMTS13 activity in pregnancy. A 23-year-old nulliparous Japanese woman died due to severe, rapid progression of TTP with intrauterine fetal death at 20 weeks of gestation after its onset, even though she underwent intensive treatment which included plasma exchange. She had a history of idiopathic thrombocytopenic purpura at the age of 3 years. The patient's ADAMTS13 activity was of very low level. It should be borne in mind that there is the possibility of rapidly progressive fulminant USS during pregnancy.

Key words: ADAMTS13, intrauterine fetal death, maternal death, pregnancy, thrombotic thrombocytopenic purpura, Upshaw–Schulman syndrome.

Introduction

Thrombotic thrombocytopenic purpura (TTP) is characterized by thrombocytopenia, hemolytic anemia, fever, renal dysfunction and neurological dysfunctions.[1] Congenital TTP (Upshaw–Schulman syndrome, USS) involves a congenital deficiency of von Willebrand factor (VWF) cleaving metalloprotease (ADAMTS13) activity caused by ADAMTS13 gene mutations.[2–4] 'When ADAMTS13 activity is deficient, unusually large VWF multimers accumulate in the circulation that can cause platelet thrombi under high shear stress of the microcirculation'.[5] We present herein a very rare case of congenital TTP with both maternal and fetal death in the second trimester of pregnancy.

Case Report

A 23-year-old pregnant Japanese woman, gravida 1, para 0, had epigastric pain and hematuria at 20 weeks and 3 days of gestation. She was treated for gastritis in a local private clinic, but the severe languid feeling and vomiting showed aggravation. She was referred to our university hospital at 20 weeks and 5 days. She had a history of severe thrombocytopenia at the age of 3 years, which was diagnosed as idiopathic thrombocytopenic purpura (ITP).

There was a decline in her consciousness. Moreover, she showed tonic and clonic convulsions. Sonographic examination revealed intrauterine fetal death (IUFD) on her visit. Cerebral hemorrhage and infarction were

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Table 1 Clinical data and laboratory findings

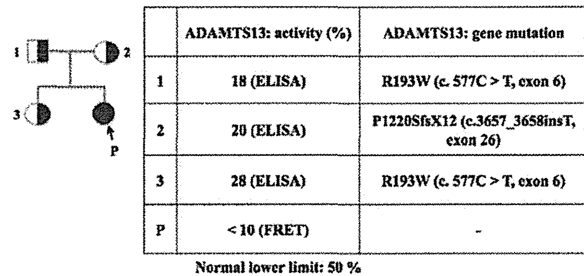
BP (mmHg)	110/80		BIL-T (mg/dL)	3.5	(0.1–1.2)
PR (b.p.m.)	90		Pro	4+	(–)
Fever (°C)	37.4		LDH (U/L)	3384	(110–220)
Purpura	No		BUN (mg/dL)	41	(7.0–20.0)
Jaundice	No		Cr (mg/dL)	1.37	(0.50–1.00)
Oliguria	+		FDP (μg/dL)	29.5	(0.0–5.0)
NS sign	Coma		D-dimmer (μg/dL)	8.7	(0.0–1.0)
Hb (g/L)	54	(110–150)	APTT (s)	28.0	(27.0–40.0)
Plt ($\times 10^9$/L)	16	(150–350)	PT-INR	1.77	(0.85–1.15)
ALT (U/L)	93	(5–40)	Distorted RBC	+	(–)
AST (U/L)	15	(10–35)	P-Hb (mg%)	13.4	

Data in parentheses indicate ranges. ALT, alanine transaminase; APTT, activated partial thromboplastin time; AST, aspartate transaminase; BIL-T, total bilirubin; BP, blood pressure; BUN, blood urea nitrogen; Cr, creatinine; FDP, fibrin/fibrinogen degradation products; Hb, hemoglobin; LDH, lactate dehydrogenase; NS, neulogis system; P-Hb, plasma-free hemoglobin; Plt, platelet; PR, pulse rate; Pro, proteinuria; PT-INR, prothrombin time – international normalized ratio; RBC, red blood cell.

not observed with computed tomography, magnetic resonance imaging and magnetic resonance angiography. Clinical data and laboratory findings on admission are shown in Table 1. Acute disseminated intravascular coagulation such as HELLP syndrome and dead fetus syndrome was ruled out based on the data and clinical symptoms. We diagnosed her with TTP because of severe hemolytic anemia, thrombocytopenia, fever, renal dysfunction and neurological deficits (Table 1).

She was treated with fresh frozen plasma (FFP) transfusion (2880 mL) 5 h after admission, continuous hemodiafiltration with FFP (2440 mL) 9 h after admission and plasma exchange with FFP (4800 mL) 25 h after admission. Induced abortion with dilatation of the cervical canal and artificial rupture of membrane was also initiated. However, she died 32 h after admission without delivery. Consent for autopsy could not be obtained from the family.

The plasma level of ADAMTS13 activity of the patient was 10% or less, and those of her family were moderately deficient (Fig. 1). The ADAMTS13 mutation was p.R193W (c.577C>T, exon 6) in her father and p.P1220Sfs*12 (c.3657_3658insT, exon 26) in her mother. Her sister had the ADAMTS13 mutation from the father (p.R193W, c.577C>T, exon 6) (Fig. 1). They were heterozygous carriers of the mutations. However, the ADAMTS13 gene sequence analysis ended in failure. Therefore, we inferred from the familial genetic analysis that the patient was a compound heterozygote of p.R193W (c.577C>T, exon 6) and p.P1220Sfs*12 (c.3657_3658insT, exon 26). The family of our patient was proven to be a new family line of USS by genetic analysis.



	ADAMTS13: activity (%)	ADAMTS13: gene mutation
1	18 (ELISA)	R193W (c. 577C > T, exon 6)
2	20 (ELISA)	P1220SfsX12 (c.3657_3658insT, exon 26)
3	28 (ELISA)	R193W (c. 577C > T, exon 6)
P	< 10 (FRET)	-

Normal lower limit: 50 %

Figure 1 The plasma level of ADAMTS13 activity and genetic analysis of ADAMTS13 gene. Her parents and sister were heterozygous carriers of ADAMTS13 mutation. 1, father; 2, mother; 3, older sister; ELISA, enzyme-linked immunosorbent assay; FRET, fluorescence resonance energy transfer method; P, patient.

Discussion

Certain clinical symptoms of USS may be absent during childhood in many cases. Therefore, many female patients are not diagnosed accurately before their first pregnancy. However, it is a significant feature of USS that many patients are treated for ITP in childhood. Twenty-nine of 37 USS patients had a history of thrombocytopenia during their childhood, and six of the nine with their first pregnancies had episodes misdiagnosed as ITP.[5] Our patient also received a diagnosis of ITP at 3 years of age, and underwent platelet transfusion.

Plasma ADAMTS13 activity decreases due to the progression of pregnancy,[6] and plasma levels of von Willebrand factor antigen increase markedly during normal pregnancy.[7] Fujimura *et al.*[5] pointed out that the rapidly increased plasma level of unusually large VWF

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multimers due to a defect of the VWF cleaving enzyme plays a critical role in precipitating TTP in pregnant women with USS. They showed that pregnancy consistently induced thrombocytopenia during the second–third trimester, as occurred in the present case.

In the previous and present 12 patients with USS during pregnancy, thrombocytopenia was confirmed at the first pregnancy from 12–28 weeks (mean, 22 weeks).[5,8,9] It was diagnosed in one of the 12 patients based on an episode in the older sister, and was treated early. In the other 11 patients, the fetal or neonatal infants' prognoses were extremely poor, and a favorable outcome was reported in only one of the 11. Another three cases that were diagnosed with USS during previous pregnancies or due to an episode involving an older sister's pregnancy received FFP or Octaplas transfusion during pregnancy from an early stage. Their newborn baby prognoses were favorable. Regarding the mothers' prognoses, the symptoms of TTP were serious except for in three cases, in which USS was diagnosed before becoming pregnant. However, it improved immediately on treatment for TTP after delivery or miscarriage. Only one case died at 3 months after discharge from the hospital. In the present case, the patient died a few days after symptom appearance during pregnancy. We could not induce abortion because of the rapid, severe progression. The fetus remaining *in utero* after IUFD may be one of the causes of a poor prognosis. There may be a number of cases in which USS is not proved when there is maternal death due to rapidly severe TTP with pregnancy. It is necessary to diagnose precisely by assay of ADAMTS13 activity and gene analysis when the possibility of USS is suspected based on the clinical course and natural history of TTP with pregnancy. Moreover, we must be aware of the possibility of rapidly progressive fulminant USS with pregnancy.

In conclusion, we should examine ADAMTS13 activity based on the possibility of USS, when we confirm that pregnant women have a history of thrombocytopenia, a low platelet count in early pregnancy and unclear thrombocytopenia after the second trimester of pregnancy.

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特　集
造血器疾患 ―最新の治療戦略（血小板・凝固・線溶系疾患）―

血栓性血小板減少性紫斑病

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Key words : TTP, ADAMTS13, VWF, USS

はじめに

1924 年，米国の Moschcowitz[1] によって肺を除く全身諸臓器の細動脈に出来たヒアリン膜血栓症として最初に報告された重篤疾患は，1966 年に Amorosi & Ultmann[2] による自他験例の計 271 例の解析にて，細血管障害性溶血性貧血，破壊性血小板減少，血小板血栓による臓器機能障害（特に腎機能不全），発熱，そして動揺性精神神経障害の 5 徴候（pentad）を特徴とする事が示され，血栓性血小板減少性紫斑病（thrombotic thrombocytopenic purpura, TTP）と命名された。

一方，血管内皮細胞で産生される von Willebrand 因子（VWF）は巨大分子構造を持つ止血因子であるが，2001 年にこの VWF の特異的切断酵素である ADAMTS13 (a disintegrin-like and metalloproteinase with thrombospondin type 1 motifs 13) が同定された[3~8]。今日では TTP 症例の 2/3 以上が，この ADAMTS13 活性欠損により，血中に未消化の超巨大 VWF 多重体（unusually large VWF multimers, UL-VWFM）が蓄積し，これにより細血管内で生ずる高ずり応力下に過剰な血小板凝集が生じ，全身性重篤疾患が引き起こされる事が広く認識されるようになった。この結論に至る迄の約 90 年間の歴史を振り返ると，その後半期の渦中にいた筆者等は改めて基礎と臨床の研究者間の緊密な情報交換の重要性を痛感するのである。本項では，この TTP の病態解明への集約過程と治療について紹介し，最後に現在直面している問題点について述べる。

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表裏一体の VWD-TTP 研究歴史

von Willebrand 病（VWD）は 1924 年フィンランド人医師 Erik Adolf von Willebrand によって同国 Bothnia 湾内の Åland 島で，血族結婚を繰り返す家系で男女両性に出現する先天性出血性素因として最初に報告された[9]。この背景から VWD 研究の初期は北欧中心に発展し，1950 年代にスウェーデンの Inga Marie Nilsson 等は[10] VWD 患者では凝固 VIII 因子活性が低下している事，VWD 患者に VIII 因子活性を欠く血友病 A 血漿を輸血すると，VIII 因子活性が出現し，しかもその血中半減期が著しく長いという発見をした。1964 年に米国の Pool & Shannon[11] がクリオ沈殿の分離技術を確立し，これには VIII 因子活性が豊富に含まれる事を示し，この製剤が血友病 A の治療に用いられるようになった。1970 年前後にアガロース ゲルクロマトグラフィーでクリオ沈殿から VIII 因子活性を持つ蛋白が高純度に精製された。この分離方法にてオランダの van Mourik 等は[12] 精製 VIII 因子分画には VWD に見られる血小板粘着能低下を補正する作用がある事を見出した。また，米国の Zimmerman 等は[13]，精製 VIII 因子関連蛋白に対する家兎免疫沈降抗体の作成を通じて血友病 A と VWD は免疫学的に鑑別しうる事を示した。しかし，これら VIII 因子と血小板粘着能補正活性は常にその挙動を共にする事から[14]，これらは同一分子上に存在すると考えられた。以後，VIII 因子活性を示す蛋白は第 VIII 因子関連抗原（VIII-related antigen: VIIIR:AG）と呼ばれるようになった。結果的にはこの表記は誤りで，VIIIR:AG と称されたものは VWF 抗原であった。1985 年前後に VIII と VWF の両蛋白質の cDNA クローニングが行われ，両者が異なった蛋白である事が明瞭となり，VIIIR:AG の名称は 1986 年に国際血栓止血学会の国際標準化委員会

(Scientific and Standardization Committee, SSC) で正式に廃止された[15~20]。

van Mourik らは[21] 1974 年に VIIIR:AG について，一つの不可思議な現象を報告していた。それは，精製した VIIIR:AG は巨大分子構造を持つ事が示唆され，ポリアクリルアミドゲル電気泳動ではゲル先端からわずかにしか内部に移動しないが，このサンプルを低イオン強度緩衝液（具体的には低 NaCl 濃度環境）に一昼夜透析しておくと低分子化し，「ゲル内を早く進む蛋白バンド（fast-moving component: FMC）と，遅く進むバンド（slow-moving component: SMC）の二つ（Fig. 1）が出現し，しかも各々のバンドは抗 VIIIR:AG 抗体と一本の免疫沈降線を形成するが，お互いのバンドは融合しない事から免疫原性は異なる」というものであった。この後，同研究グループからはこの現象を説明できる新たな研究の進捗はなかった。

1980 年，Scripps 研究所の Ruggeri & Zimmerman[22] により SDS アガロースゲル電気泳動による VWF 蛋白の解析法が確立され，正常血漿中の VWF は分子量 500 kD～15,000 kD の多岐にわたる多重体（multimer, M）構造を持つ事が示された。一方，個々の VWF バンドは 1 本の主バンドとその上下に各々 1 本のサテライトバンドからなる三連符構造を示す事から，健常人に於いても *in vivo* proteolysis がある事，また type 2 VWD では高分子量（high molecular weight, HMW)-VWFM が欠失している事から，かかる患者では前記の proteolysis が亢進していると考えられるようになった。実際，1987 年に同研究所の Berkowitz 等は[23] 抗 VWF モノクローナル抗体を用いて，還元条件下での SDS ポリアクリルアミドゲル電気泳動後の western blot にて本来の VWF サブユニット分子量 250 kD に対して，type 2A VWD 患者血漿中には分解を受け低分子化した 176 kD と 140 kD のサブユニットバンドがコントロールに比して，より多く存在する事を報告している。さらに同ラボの Dent 等は[24] type 2A-VWF を精製し，患者に見られる特異的 VWF サブユニット断片の解析にて，その切断部位は成熟 VWF サブユニットを構成する 2050 アミノ酸残基の Tyr842-Met843（全長 cDNA レベルのアミノ酸残基では

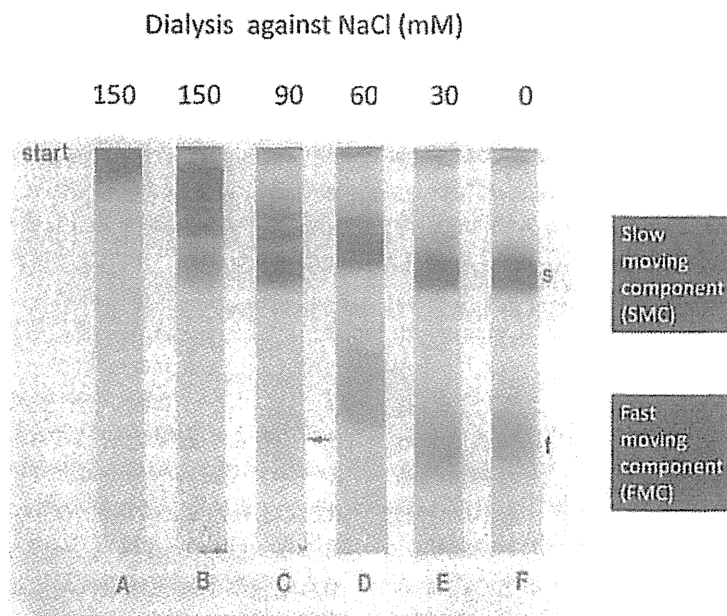

Fig. 1 Large-pore polyacrylamide gel electrophoresis of the "purified factor VIII-related antigen (VIIIR:AG)" (later shown as VWF). Human cryoprecipitate was separated by agarose gel chromatography, and the "purified VIII:RG" (VWF) was eluted at the void volume. Because of the high molecular weight nature of "purified VIIIR:AG" (VWF), it did not enter the gel. However, after dialysis against the low ionic strength buffer (pH7.4) over night, the "purified VIIIR: AG" (VWF) turned to two protein bands, namely slow-moving component (SMC) and fast-moving component (FMC). This phenomenon was a clue to discover ADAMTS13.[21]

Tyr1605-Met1606に相当）結合である事を見出した。しかしながら，このペプチド結合切断がVWF-CP（ADAMTS13）という酵素によってなされている事を示したのは，後述のように1996年のFurlan等[25]，そしてTsai等[26]である。彼らはVWF-CP（ADAMTS13）活性測定法を確立して，TTPの多くの症例ではこの活性が著減しているというブレークスルーを見出した。即ち，VWF機能が弱すぎると出血症状が表面に出てVWDとなり，逆に強すぎると血栓症状が現れてTTPとなる。換言すると、VWD-TTPは両面貨幣（two-sided coin）の間柄にある。これより，真のVWF-CP研究はこの年から始まったと考えられる。前記のvan Mourik等の観察は，彼等が精製したVIIIR:AGはその質量の99%がVWFで，真のVIII因子は1%に満たない程度であった。そして，このVWFに微量ながらVWF-CP/ADAMTS13が混在（接着）しており，これが低イオン強度下で酵素機能を発現したと説明できる。

ADAMTS13活性測定法の進歩

HMW-VWFMは抗生物質リストセチン存在下に強い血小板凝集を惹起する。一方，HMW-VWFMは固相化コラーゲンへの結合能も高く，さらに細コイル灌流，cone plate，そしてparallel plate flow chamberなどで，高ずり応力環境下に暴露すると，ADAMTS13による分解反応を受けて低分子化し，酵素反応が完結すると，VWFはN末端とC末端の2種類のダイマーに転じる事になる。これより，初期のADAMTS13活性測定法には様々なものが考案されたが，酵素消化後の測定原理では，1）リストセチン血小板凝集法[27]，2）VWFサブユニットのN末端とC末端を認識する抗VWF抗体を用いたenzyme-linked immunosorbent assay（ELISA），3）コラーゲン結合能ELISA[28]，4）SDS-アガロース電気泳動[29]，5）SDS-ポリアクリルアミド電気泳動[30]，等に分類される。今日，HMW-VWFMは高ずり応力下では速やかにADAMTS13で分解される事が知られているが，この高ずり応力下での測定系では高感度定量が難しい。それ故，静止系での測定が主に行われているが，この場合，基質は精製HMW-VWFMを用いているため，長時間の反応を要する事と，またVWFが消化され易いように1.5 mol/l濃度の尿素やグアニジン塩酸などの蛋白変性剤を加える必要があった。

1996年にスイスのFurlan等は[25] VWF-CP活性測定法の確立を報告し，TTPではこれが著減している事を示した。この知見はブレークスルーであったが，測定原理の着想は前記van Mourik等[21]の報告から得たもので，実施は，精製VWF，正常血漿希釈または患者血漿，終濃度1.5 mol/l尿素，10 mM Pefabloc（セリンプロテアーゼ阻害剤）を混和し，低イオン強度（0 mol/l NaCl）緩衝液で37℃下に透析しながら24時間酵素消化反応を行うと云うものであった。この反応物はその後VWFM解析を行なって判定すると云う時間浪費性のものであった。一方，ほぼ同時期にアメリカのTsai等は酵素消化を1.5 mol/lグアニジン塩酸下に行い，反応物をSDSポリアクリルアミドゲル電気泳動で解析するすると云う方法を報告している。以後，Furlan等[29]とTsai等[30]の二つの研究グループの論文が1998年のNEJMに同時掲載され，TTPが世界の注目を引くようになった。

Cruz等[31]は，ADAMTS13での切断部位はVWF-A2ドメイン内（Tyr1605-Met1606）で，またこの結合の切断を受け易いtype 2 VWDの遺伝子変異がこのドメイン内にある事から，まずHis標識VWF-A2ドメイン（Gy1481-Arg1668）の遺伝子発現蛋白を作成し，この基質が静止系で蛋白変性剤非存在下にADAMTS13で切断される事を見いだし，ELISAによる酵素活性測定系を確立した。Kokame等[32]はVWF-A2ドメイン内での基質狭小化を計り，最終的にAsp1596-Arg1668の73アミノ酸残基が最小基質となる事を示し，このGln1599に蛍光基Nmaを，またAsn1610に消光基Dnpを導入することにより，FRETS-VWF73という蛍光測定法を確立した[33]。本法は，今日ADAMTS13活性測定のgold standard法として世界的に普及している。しかし，本法は被検血漿中の高濃度ビリルビンやヘモグロビンの影響を受ける事が指摘されてきた。これより，最近，蛍光強度を増加させ，前記物質の干渉作用を無くし，また生理的pH条件下で測定可能なFRETS-rVWF71と名付けられた改良型測定法がMuia等[34]によって報告された。

筆者らはルーチンラボでの利便性を考え，chromogenic ADAMTS13 act-ELISAを開発した[35]。これはADAMTS13切断で生じるVWF73の断端アミノ酸残基Tyr1605を特異的に認識するマウスモノクロナール抗体N10（IgG）を得たことにより達成された。本法の測定感度は<0.5%（正常100%）と鋭敏で，前記阻害物質の影響を受けない。一方，他の高感度測定法としてGST-VWF73-Hisを基質に用い，SELDI-TOF-MASで測定する方法[36]が報告されているが，器材が大掛かりである事から汎用性については問題が残る。最近，国内外でADAMTS13活性測定の臨床現場でのリアルタイム測定を視野に入れ，全自動測定法が開発されつつある。一つは化学発光法，他は金コロイド凝集法で，これら2方法は共に前記N10抗体を利用したものである。測定時間はいずれも10分前後で，且つ極めて高感度と伝えられており，今後の進展が待たれる。本邦ではADAMTS13活性とそのインヒビター測定は現在，薬事承認に向けて準備されつつある。

本邦における血栓性微小血管障害症（TMA）患者登録と解析状況の update

筆者らは1998年にFurlan等のVWFM法によるVWF-CP/ADAMTS13活性測定法を本邦で最初に立ち上げ[37]，この後，全国の医療施設から依頼された検体についても，その測定を受諾する事になった。2001年以降は測定検体数が飛躍的に増え，当ラボのホームページ〈http://www.naramed-u.ac.jp/~trans/〉を開設し，検体受け入れをシステム化した。また2005年4月からは前記の新規開発したchromogenic ADAMTS13 act-ELISAによる活性測定を取り入れたため，平日に検体を受け取ればその日の内に結果を返却できるシステムも構築できた。

原則としてADAMTS13活性が著減（通常＜5%）し，同インヒビターが陰性で，後述の患者背景と合わせて先天性TTP（下記USS）と診断できたものは，倫理委員会規定に従って国立循環器病研究センターで*ADAMTS13*遺伝子解析を実施している。一方，ADAMTS13活性が著減しておらず，また同インヒビターも陰性であるが，TMA発作の反復性，家族性が認められるものは従来，原因不詳の先天性TMA（congenial TMA with the etiology unknown）と分類していたが，本年，徳島大学小児科の香美先生や東京大学腎臓・内分泌内科の南学先生が中心となり，非典型溶血性尿毒症症候群（atypical HUS, aHUS）の診断基準が作成された[38]。これによると，我々が原因不詳の先天性TMAと分類していた患者の殆どが先天性aHUSのカテゴリーに入る事が示された。先天性aHUSは補体や補体調節因子の遺伝子異常にて生じ，遺伝形式は通常，常染色体優性である。これに対し，先天性TTP（USS）の遺伝形式は常染色体劣性なので，最近の患者数はaHUSがUSSを遥かにしのぐ状況となっている。過去15年間に本邦の医療機関からVWF-CP/ADAMTS13活性測定を依頼され，実施した検体数は膨大で，集積されたTMA患者数は2012年12月末で1,149例であった（Fig. 2）。これらの内訳をTable 1に示す。

先天性TTP（Upshaw-Schulman syndrome，USS）：診断と治療の問題点

小児血液専門医にとって，USSのhallmarkとは，「新生児期にCoombs試験陰性の重症黄疸があり，これは交換輸血で救命されるが，その後も貧血と血小板減少が慢性に経過し，これらの症状が血漿輸血にて劇的に改善

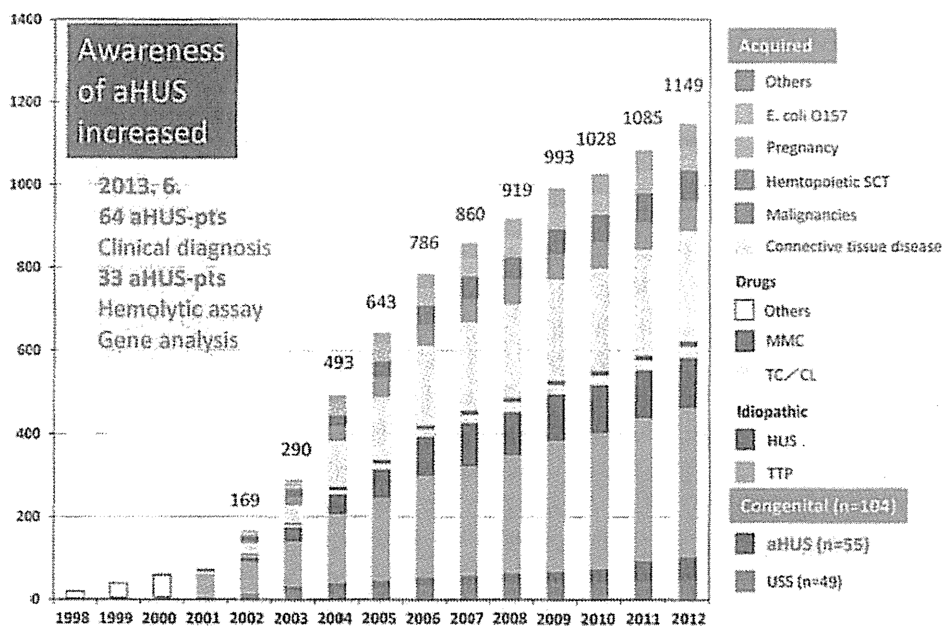

Fig. 2 Cumulative number of TMA patients in NMU registry.
Since 1998, our laboratory of Nara Medical university (NMU) has been functioning as a TMA referral center in Japan through analyzing VWF-cleaving protease/ADAMTS13 activity. Until the end of 2012, the cumulative number reached to 1149, which included 104 patients of congenital TMA (49 with USS and 55 with aHUS). Abbreviations: SCT, stem cell transplantation; MMC, mitomycin C, TC/CL, ticlopidine/clopidogrel.

Table 1 Plasma levels of ADAMTS13 activity and ADAMTS13 inhibitor in 1149 patients with TMA, registered to our laboratory of NMU between 1998 and 2012.

	Congenital TMAs		Acquired TMAs											Total
			Idiopathic		Secondary									
					Drug-induced (n=42)									
	Upshaw-Schulman syndrome (USS) (n=49)	aHUS (n=55)	Thrombotic thrombocytopenic purpura (TTP) (n=361)	Hemolytic-uremic syndroime (HUS) (n=119)	Ticlopidine (n=25) / Clopidogrel (n=2)	Mitomycin C (n=12)	Others (n=3)	Conective tissue diseases and Autoimmune diseases (CTDs/ADs) (n=263)	Malignancies (n=73)	Hematopoietic stem celltransplantation (HSCT) (n=75)	Pregnancy (n=19)	Stx-E. coliinfection (n=37)	Others (Liver cirrhosis, etc) (n=56)	(n=1149)
ADAMTS13:AC (%)	(n=48)	(n=55)	(n=361)	(n=119)	(n=25/n=2)	(n=12)	(n=3)	(n=263)	(n=73)	(n=75)	(n=19)	(n=37)	(n=56)	(n=1148)
<3	46	0	269	0	23	0	3	57	8	0	7	0	19	432
3~<25	2	2	70	22	2	2	0	76	24	23	5	6	17	251
25~<50	0	14	19	58	1	6	0	79	25	34	4	21	7	268
≧50	0	39	3	39	1	4	0	51	16	18	3	10	13	197
ADAMTS13:INH (U/ml)	(n=48)	(n=43)	(n=313)	(n=50)	(n=24/n=2)	(n=9)	(n=3)	(n=206)	(n=32)	(n=26)	(n=9)	(n=22)	(n=30)	(n=817)
≧2	0	0	139	0	18	0	0	31	5	0	4	0	12	209
0.5~<2	0	7	140	3	6	1	3	84	10	5	2	3	10	274
<0.5	48	36	34	47	2	8	0	91	17	21	3	19	8	334

( ) Sample number determined

する疾患」であった。この歴史の詳細については拙著で報告しているので割愛する[39]。実際，USS の病態解明には多くの研究者の貢献があったが，Upshaw と Schulman の両名の名前が今日も残っているのは，この両者のみが 1960 年と 1978 年に，溶血性貧血と慢性血小板減少を示すそれぞれの患者に対して，血小板輸血ではなく血漿輸血により，血小板数が著しく増加する事を報告した事による[40, 41]。当時は血漿中に血小板産生刺激因子（platelet stimulating factor）が存在する事を仮想していたが，この考えは，後年 thrombopoietin（TPO）が同定され，USS との関連は完全に否定された。

筆者等は 1998 年以降，本邦 USS 患者 3 家系の VWF-CP 活性を調べ，患者は活性が著減し，同両親は活性が半減を示すが無症状であることから，本疾患が常染色体劣性遺伝形式であることを報告したが[42]，その 6 ヶ月後 Levy 等[7]により *ADAMTS13* 遺伝子がクローニングされ，USS が *ADAMTS13* 変異遺伝子の劣性遺伝による事が示された。現在，世界で USS 患者は約 150 名同定されているが，筆者等は本邦で 2012 年末迄に 49 名の同患者を同定した。USS 患者で同定された *ADAMTS13* 遺伝子変異を Fig. 3 に示す[43]。注目すべきは欧米で発見された変異と本邦とでは全く異なる点である。また，本邦患者の自然史（natural history）を要約すると Table 2 のようになる。ここで特筆すべきは，前記の USS hallmark である交換輸血を必要とするような新生児重症黄疸を示す early-onset type（早期発症型）は僅か 39％の患者にしか見られない点と，USS の遺伝は常染色体劣性なので，患者数は本来，男女ほぼ同数と想定されるが，登録症例は圧倒的に女性優位である。これは USS 女性の場合，妊娠により TTP 発作が誘導されるので，妊娠適齢期である 20～40 才間に発見され易いことによる。一方，USS 男性にはこの年齢層で正確に診断されている例はなく，40～45 才を超えて初めて TTP 発作を起こして診断されている例が散見される。かかる例は late-onset type（晩期発症型）と表現されている。

Taguchi 等[44]が 2012 年に報告した男性症例を late-onset type の典型例として Fig. 4 に紹介する。症例は 1931 年生で，来院時は 63 才であった。男性はこの年令で初めて TTP 発作を起こして来院したが，直後には血漿交換療法の対応が出来ず，同日は血漿輸注だけを行った。しかし翌日に臨床症状と検査値の双方に著しい改善がみられ，以後は TTP 発作時に血漿輸注のみで治療していた。しかし加齢と共に TTP 発作回数も増加し，77 才時（2007 年）には小脳梗塞を起こした。この時点で

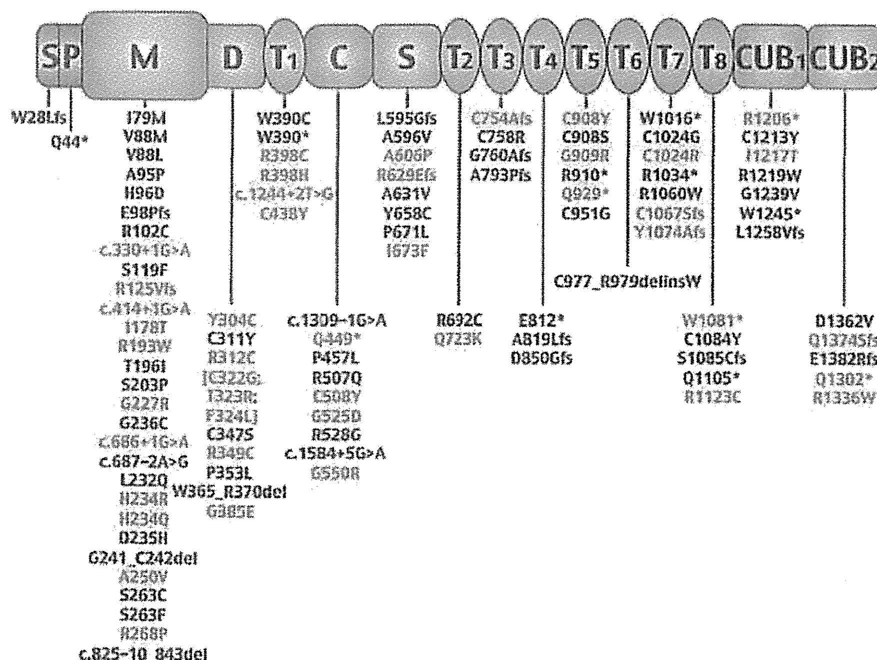

Fig. 3 *ADAMTS13* gene mutations responsible for congenital TTP (Upshaw-Schulman syndrome, USS).
The description of protein sequence mutation follows the recommendation of the Human Genome Society (WWW.hgvs.org/mutnomen/recs-prot.html). Mutations in red were identified in Japanese patients[43].

Table 2 Summary of natural history in 49 patients with Upshaw-Schulman syndrome in Japan.

Family:
- 43 families
- Female predominance (31 F and 18 M)
- Up to 79 years of age

EXBT to severe newborn jaundice: 19 pts/49 (39%)
Hemodialysis for CKD: 5 pts (3 pts were dead)

Aggravating factors:
- Severe infections such as FLU
- Pregnancy: 23 occasions in 13 female pts (1 pt was diagnosed after death)
- DDAVP
- interferon
- heavily drinking alcohol
- Aging

ADAMTS13 gene analysis in 47 pts: 9 homo. and 38 compound hetero.

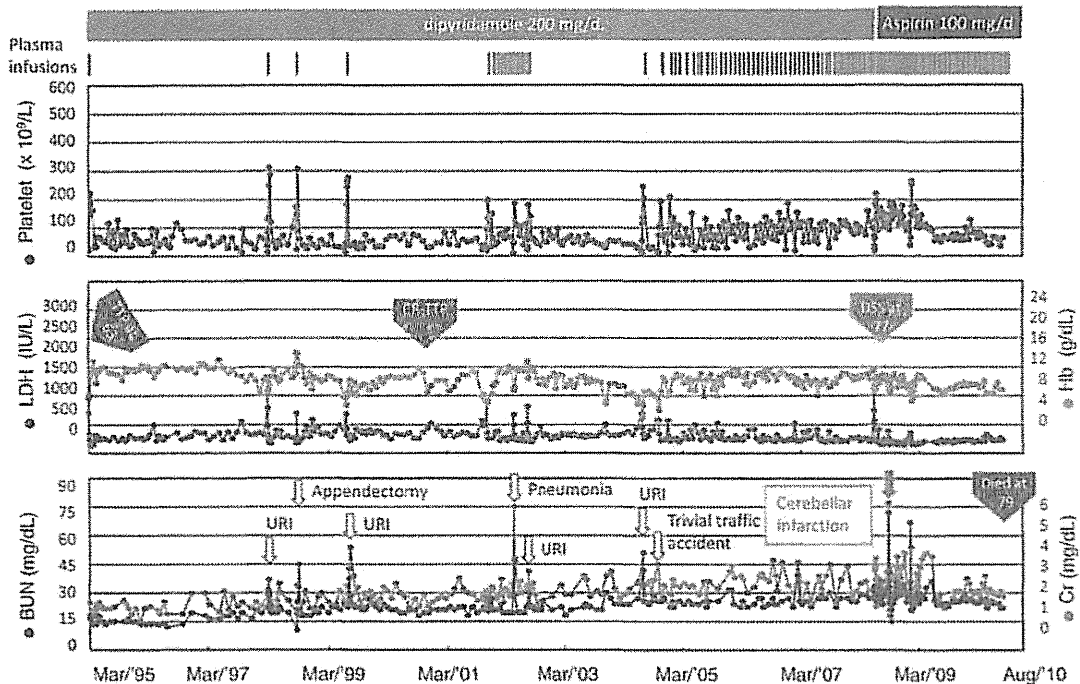

Fig. 4 Clinical course of a representative case of Upshaw-Schulman syndrome (USS) with the late-onset phenotype. This Japanese male patient first developed a TTP-bout at the age of 63 years and was admitted to a local hospital, where plasma exchange was not ready available, and so he was treated with plasma infusion, which dramatically improved his clinical conditions on the following day. Since then, he had been treated with plasma infusion on demand at TTP-bouts, but its frequency became quite often with aging. At age of 77 years, he had cerebellar infarct, and on that occasion ADAMTS13 was analysed, that disclosed a moderate deficiency of ADAMTS13 activity (2.4-3.4% of the normal) and a homozygous *ADAMTS13* gene mutation of p.C1024R. His parents were indeed the first cousins. As for his natural history, he worked as a business man until the age of 60 years and received health examination annually, but he never had appreciable abnormalities in both labolatory and clinical findings[41].

ADAMTS13 解析を実施し，ADAMTS13 活性は 2.4〜3.4%と著減し，また *ADAMTS13* 遺伝子解析では p.C1024R のホモ接合型変異が同定された。これより，家族歴を詳細に再調査すると患者の御両親はいとこ婚であった。本患者は会社員として定年迄勤務し，その間毎年定期健康診断を受けていたが，特に異常を指摘された事はなかったという。本患者はこの後 79 才で小脳梗塞の関連後遺症で逝去されたが，本症例が典型的な late-onset phenotype の経過模様を示した理由として，(1)男性，(2)ADAMTS13 活性は 2.4〜3.4%と僅かながら検出される例であった，の 2 点があげられる。これは TTP 発作の契機は前記のように，血管内皮細胞からの UL-VWFM 放出が過剰に起こる時と考えられており，女性では妊娠，特に妊娠後期がこれにあてはまるが，男女を問わず，40 才を超えると VWF 量は青年期のほぼ 1.5 倍に増えてくる事が知られているので，男性ではこの年齢層（40〜60 才）で TTP 発作が発来し，USS 体質が発見され易いと説明される。

【治療】 USS 患者の約半数は診断確定後，約 2 週間に 1 回の頻度で定期的 FFP 輸注（5〜10 ml/kg BW）を受けている。これは ADAMTS13 活性と抗原の血中半減期が略 2.5 日である事と，輸血後に血小板数は 7〜10 日目でピークを迎えると云うデータに基づいている。懸念される ADAMTS13 インヒビター発生の報告例は現在のところ皆無であるが，我々のデータでは[45] 2011 年に検索した USS 患者 43 例中 7 例（16.2%）に IgG 型の非中和抗体が検出されているので，今後，中和抗体の発生についても慎重に見守ってゆく必要性があると考えている。一方，日本赤十字社の血液製剤については 1988 年以降から HCV 検査が導入されているが，それ以前から血漿輸注療法を受けている USS 患者では HCV 既感染者もいる。現在，遺伝子発現 ADAMTS13 製剤の Phase I 臨床治験が欧米で開始されたと伝えられているので，その成果と同製剤の速やかな臨床への導入が期待されている。

後天性 TTP：診断と治療の問題点

TTP 患者の 90%以上は後天性 TTP で，この診断は，ADAMTS13 活性著減と ADAMTS13 に対する活性中和抗体（IgG 型インヒビター）あるいは非中和抗体（主に IgG 型）が陽性の結果で診断される群と，ADAMTS13 活性は略正常であっても古典的 5 徴候（pentad）でなされる群の 2 種類がある。筆者等は前者を仮に定型 TTP，後者を非定型 TTP と呼んでいるが，非定型 TTP は非典型溶血性尿毒症症候群（atypical hemolytic uremic syndrome, aHUS）との鑑別が困難で，また aHUS には先天性と後天性の双方があるため，後天性 TTP で定型と非定型の患者割合は報告者によって大きく異なる。筆者らが 1998〜2012 年の間に奈良医大輸血部で登録した 1,149 名の TMA 患者の解析結果では TTP は 361 名で TMA 全体の 31.4%（361/1,149）であり，このうち 74.5%（269/361）は定型 TTP であった。TTP の発生頻度については ADAMTS13 発見以前に，人口 100 万人当たり 4.7 人という数字が出されているが，現在ではこれよりも遥かに高い頻度と推定される。実際，今日では血小板減少と貧血があり，ADAMTS13 活性著減が確認されれば，これで定型 TTP と診断されている。後天性・定型 TTP では再発率が高い事，また非定型 TTP ではこれが少ない事も近年報告されている。一方，後天性の定型及び非定型 TTP のいずれもが，一次性（特発性）に生じるものと，基礎疾患，薬物，移植等に関連して二次性に生じるものがある。また，後天性・定型 TTP に発生する IgG 型 ADAMTS13 インヒビターのエピトープは共通して Spacer ドメイン内にある。このドメインは VWF 切断時に，この基質を捕捉するに重要な部位と考えられており，Pos 等[46,47]はこのエピトープの詳細な解析にて Spacer ドメイン内の 5 アミノ酸残基（Arg568-Phe592-Arg660-Tyr661-Tyr665）が特に重要である事を示した。

【治療】(1)血漿交換（plasma exchange, PE）：後天性定型的 TTP に対する first-line 治療は PE である。PE の効用については，① ADAMTS13 インヒビター除去，② UL-VWFM の除去，③ ADAMTS13 の補充，④止血に必要な正常サイズの VWFM の補充，そして⑤炎症性サイトカインの除去，などで説明されている[48]。実施は 1 回 40〜60 ml/kg（1 日当たり循環血液〔血漿〕量の 1〜1.5 容）を輸注する。開始して 3 日間は 1 日当たり循環血液〔血漿〕量の 1.5 容/回で連日行う。殆どの症例でステロイド使用やステロイドパルス療法（後述）が併用される。PE の効果は血小板数，神経症状，LDH などの溶血モニターを観察しながら判断し，反応が悪ければ 5 日間連日行う事もしばしばある。現行 PE に対する保険適応は週 3 回，1ヶ月に 12 回，期間は 3ヶ月以内という制限がある。しかし，後述のように難治性・後天性 TTP においては様々な免疫療法と PE の併用が長期に渡って求められる事がしばしばあり，保険適応の期間と回数については，早急に見直しが必要と考えている。**(2)ステロイド療法**：多くの症例で，ステロイドパルス療法として PE 開始初期から実施されている。その方法は，PE 終了直後にメチルプレドニゾロン 1,000 mg を生理食塩水 100 ml に溶解し，1 日 1 回約 1 時間かけて点滴静注する。これを 3 日間連続して行う。4 日目からはプレドニン錠（5 mg）を 1 mg/kg，分 1 で投与開始する。その後，臨床症状を見ながら急速に減量する。**(3)抗血小板薬**：PE にて血小板数が回復し始めた時期（英国ガイドラインでは 50,000/ul 以上）に使用が考慮され，1)

低用量アスピリン（100 mg）1錠 分1（保険適用外），の使用が推奨されている。その他として，2）ペルサンチン（100 mg）3錠 分3（保険適用外）があるが，出血時間延長が懸念される。最近，出血副作用が少ない為に，TTP治療の新しい抗血小板療法剤として注目されているのが，3）シロスタゾール（100 mg）2錠 分2（保険適用外）である。しかし，これら全ての抗血小板剤については，その効果検証の課題が残っている。**(4)難治性TTPに対する治療**：PE治療に抵抗性を示す症例に対し，保険適応外であるが，1）シクロスポリン経口療法（保険適用外）〔処方例〕ネオーラルカプセル6 mg/kg 分3（保険適用外），2）シクロホスファミド経口療法〔処

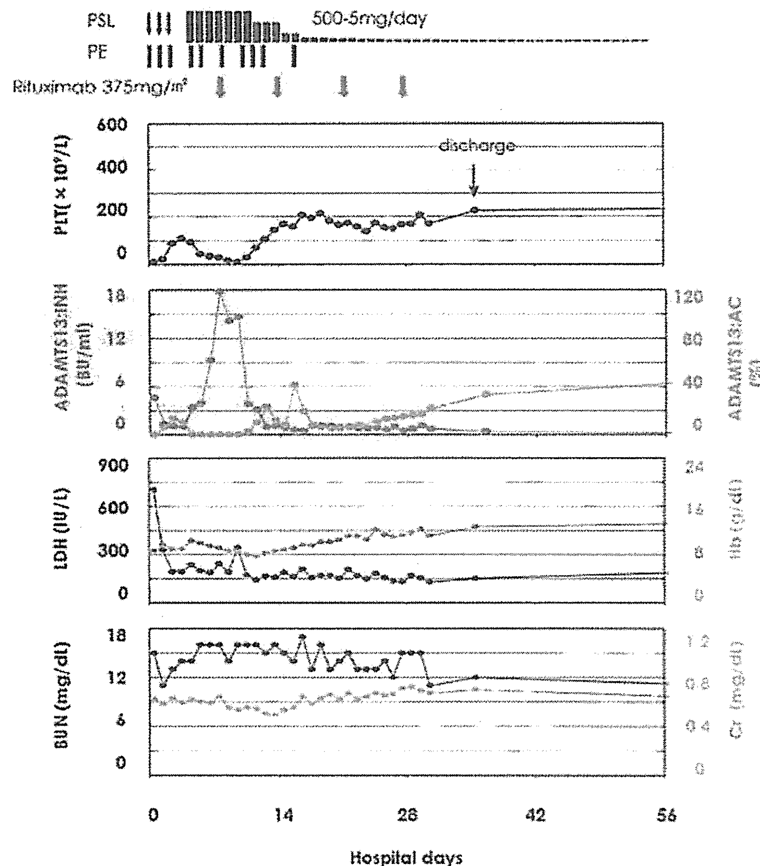

Fig. 5 The clinical course of ai-TTP patient with ADAMTS13 inhibitor boosting by plasma exchange.
A 67-year female was admitted to our hospital with complaints of thrombocytopenia (platelet count $10 \times 10^9/l$), hemolytic anemia (hemoglobin 8.6 g/dl and lactate dehydrogenase 703 IU/l), unconsciousness, and fever (38°C), without any known etiology. At that time, plasma levels of ADAMTS13 activity and inhibitor were <0.5% (of the normal), and 4.7Bethesda U/ml, respectively. Plasma exchange was performed for the consecutive 3 days with a steroid pulse therapy (methylpredonisolone 1 g/day×3) that resulted in an increase of ADAMTS13 activity (13%) and/or platelet count ($100 \times 10^9/l$). However, soon after this, both platelet count and ADAMTS13 activity dropped again, and the inhibitor titer was re-increased up to 18 Bethesda U/ml (inhibitor boosting). Rituximab was then administered from the day 7 after plasma exchange initiation, with a dose of 375 mg/m² per week for 4 times. Then, the clinical and laboratory findings rapidly improved to remission.

方例〕エンドキサン錠（50 mg）2錠 分2（保険適用外），3）ビンクリスチン（VCR）注 初回1～2 mg 静注。1週間後1 mg 追加静注（保険適用外），4）リツキシマブ注1回375 mg/m²，1週1回，点滴静注，4～8回（保険適用外）（後に詳述），5）脾摘などがある。**(5)次世代のTTP治療薬**：最近，欧米では recombinant（r）ADAMTS13 製剤の Phase I 治験が開始された。一方，ADAMTS13 は UL-VWFM の構造・機能モジュレーターで，作用物質は UL-VWFM である。UL-VWFM 依存性高ずり応力惹起血小板凝集においては，VWF-血小板膜糖蛋白 GPIb 軸の反応制御が最も効果的な抗血栓作用を示すと考えられている。このような分子標的療法として，AWJ-2 に代表される VWF-A1 ドメイン認識キメラ抗体，南米ラマで産生された nanobody（AKX-0681，Abylynx NV 社，Belgium），VWF-A1 ドメインアプタマー（ARC 1779，Archmix 社，Boston）等がある。これらの多くが，海外では臨床治験対象と考えられている。

【リツキシマブと Inhibitor boosting】 リツキシマブは本来B細胞性リンパ腫の治療薬であるが，本剤が後天性・定型 TTP に対しても極めて有効である[49]との結果が2002年以降，多数報告されている。これより，2012年にフランスの TMA レファレンスセンターグループによって PE と併用したリツキシマブ治療効果について，22例の TTP に対して前方視的研究がなされた[50]。この時のリツキシマブ投与量は 375 mg/m² で，回数は計4回と従来法に準じているが，投与日は TTP 診断日をXとすると，（X, X+3, X+7, X+14）日と最初の2週間以内に4回投与する形になっている。結果は，1）血小板数の回復が速やかで，寛解に至るまでの期間が短縮した，2）寛解後1年以内の再発が皆無であった，3）この1年期間中，ADAMTS13 活性はリツキシマブ使用群でより高値，また同結合 IgG 抗体はより低値との結果が示された。しかし予想に反して，4）PE の血漿使用量については両群で差を認めず，また，5）末梢のBリンパ球枯渇期間9ヶ月はB-リンパ腫での使用と同じであった。これを裏付けるように，実際，この期間中の再発は皆無であった。しかし，6）寛解後1年を超えると再発例が14%（3/21，1例は早期死亡）に見られた。

我々は最近，難治性 TTP の一表現型として，PE 後に IgG 型の ADAMTS13 インヒビター力価が急上昇（inhibitor boosting）するものがある事を確認した（Fig. 5）。自験例の後天性・定型 TTP では約40%がこの型を示した。この inhibitor boosting が確認されると，通常の PE 単独療法やステロイドパルス療法の併用も殆ど効果がなくなる。かかる例には PE とリツキシマブの併用は必須と考えている。我々が経験した典型例では，PE 治療前に高いインヒビター力価（>2 Bethesda U/ml）を示した TTP 症例は，PE 治療後，数日間は反応して血小板数も増大するが，1週間以内に血小板数の再低下が見られ，この時点で inhibitor boosting が確認された。かかる例にはリツキシマブの効用はより顕著であった。このような事実を受け，ごく最近，後天性定型的 TTP に対するリツキサンの医師主導型治験（代表：埼玉医科大学総合診療内科　宮川義隆）の実施が厚生労働省から認可された。該当症例は ADAMTS13 活性著減例で，PE 連続5日間実施するも臨床症状の改善のないもの，あるいは PE 治療前の ADAMTS13 インヒビター力価が2 Bethesda U/ml 以上と高いものとの条件がある。興味のある方は宮川先生までご連絡下さい。

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