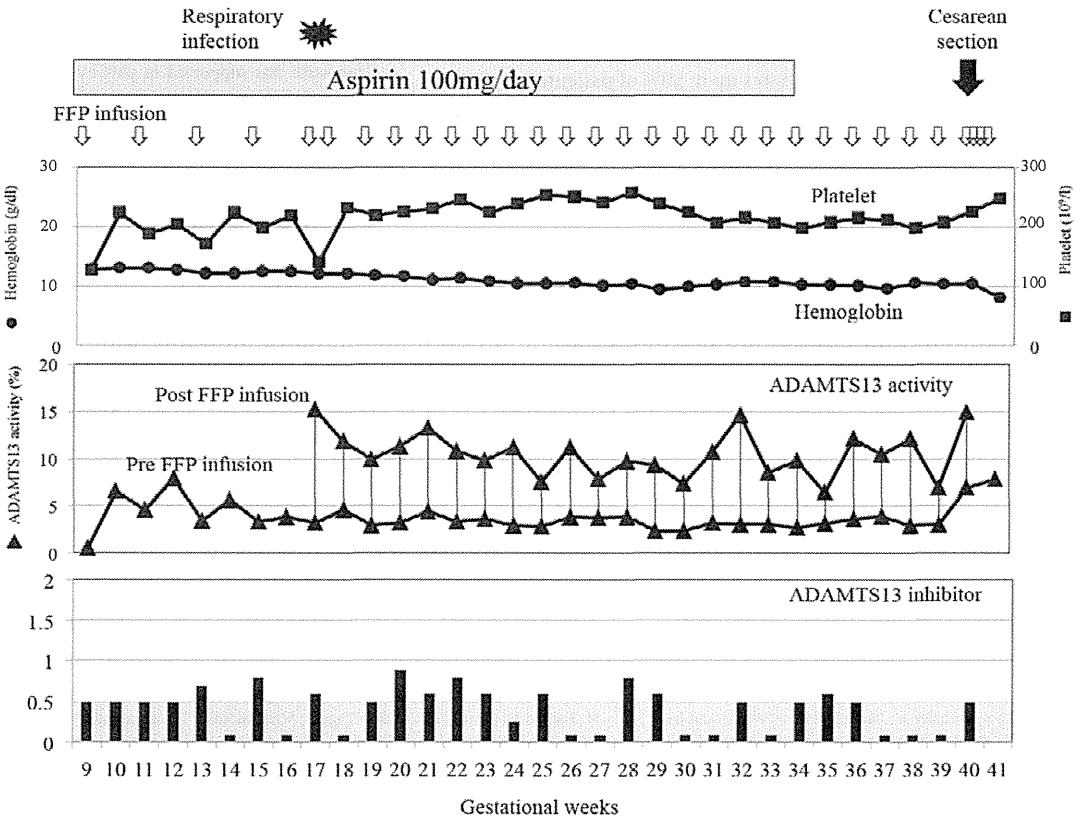

Fig. 2. Clinical course of USS-LL4 during pregnancy. FFP infusions were started at 5 ml/kg biweekly. At 17 weeks of gestation, she had an episode of respiratory infection. Her platelet count decreased from 200 to 140 × 10^9 /l. Subsequently, the interval between FFP infusions was shortened to 1 week. Plasma levels of ADAMTS13 activity before FFP infusion were 3–5% and those after FFP infusion were approximately 10%. Platelet counts were maintained over 200 × 10^9 /l. The patient took low-dose aspirin between 9 and 34 weeks of gestation. At 39 weeks, she gave birth to a healthy baby girl via cesarean section.

Discussion

We diagnosed patient described here with USS with low titer of ADAMTS13 antibodies based on the results of *ADAMTS13* gene analysis. When the patient first became pregnant at 28 years of age, we chose an elective termination due to the risk of developing TTP. However, in her second pregnancy at 30 years of age, we decided to continue the pregnancy with close monitoring of her condition and her fetus. Since plasma VWF levels increase with gestational age even in normal pregnancy [15], much more ADAMTS13 supplementation may be necessary in late gestation in USS patients. Thus, the therapeutic protocol for this patient involved dose escalation of FFP infusions, and the interval between infusions was gradually shortened with the progression of pregnancy, with frequent monitoring of ADAMTS13 activity levels.

In addition to FFP infusion, we used low-dose aspirin between 9 and 34 weeks of gestation. In our USS registry, one patient with USS (USS-L2) successfully gave birth to 4 babies (including twins) with taking low-dose aspirin during pregnancy [5]. Another patient successfully treated with FFP infusion and low-dose aspirin was reported by another group from our registry in Japan [6]. Antiplatelet agents such as aspirin, dipyridamole and ticlopidine, have been used in the acute treatment of acquired TTP. British treatment guidelines for TTP recommend low-dose aspirin during platelet recovery (platelet count >50 × 10^9/l) for patients with acquired TTP. Fetal loss in patients with USS is presumably caused by the disturbance of utero-placental circulation by platelet thrombi. Although there is only anecdotal evidence, low-dose aspirin in addition to FFP infusion may be effective in pregnant patients with USS.

In this patient, we identified the presence of both ADAMTS13 inhibitors and ADAMTS13 binding antibodies before pregnancy. Kentouche et al. [16] reported a similar patient in whom ADAMTS13 inhibitors were detected during pregnancy. However, binding ADAMTS13 antibodies were not detected by a commercially available assay (Technoclone) in stored samples in which ADAMTS13 inhibitors were detected. In contrast, both ADAMTS13 inhibitor and binding antibodies were detected in our patient (table 1).

We were concerned about increasing ADAMTS13 antibody titers with frequent antigen stimulation associated with FFP infusions. Thus, plasma levels of ADAMTS13 inhibitor and ADAMTS13 activity were analyzed each week before and after FFP infusion. However, increases in ADAMTS13 inhibitor were not observed as her pregnancy progressed. On the contrary, plasma levels of ADAMTS13 inhibitor decreased to marginal levels (<0.5–0.9 BU/ml) during pregnancy, and so far have not increased again after delivery. ADAMTS13 IgG antibodies also decreased to almost undetectable levels after delivery (table 1).

Regarding this interesting phenomenon, it is generally said that during pregnancy a mother has a natural intra-uterine allograft (fetus), which is regularly not rejected, indicating that immunological tolerance is up-regulated during this period [17]. In fact, it

has been reported that rheumatoid arthritis (RA) disease activity is often transiently lower during pregnancy [18]. However, unlike RA, in which disease activity flares up in 90% of patients within the first 3 months postpartum unless appropriate medications are given before delivery [19], both neutralizing and non-neutralizing antibodies against ADAMTS13 in our USS patient have not increased after delivery. Although we cannot fully explain this interesting phenomenon at present, it is possible that an immunological reset after delivery might be involved [16, 17]. So far, we have observed the patient for over 2 years after delivery, but much longer observation may shed a light on this difficult question.

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Disclosure Statement

YF is a member of clinical advisory boards for Baxter BioScience.

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Clinical Case Reports

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CASE REPORT

STEC:O111-HUS complicated by acute encephalopathy in a young girl was successfully treated with a set of hemodiafiltration, steroid pulse, and soluble thrombomodulin under plasma exchange

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Key Clinical Message

We report a 14-year-old girl, who developed shigatoxin-producing *E. coli* (STEC)-HUS complicated by encephalopathy. She was successfully treated with hemodiafiltration, high-dose methylprednisolone pulse therapy, and soluble recombinant thrombomodulin under plasma exchange. von Willebrand factor multimers analysis provides potential insights into how the administered therapies might facilitate successful treatment of STEC-HUS.

Keywords

Encephalopathy, *Escherichia coli* O111, hemolytic uremic syndrome, plasma exchange, recombinant soluble thrombomodulin, von Willebrand factor.

Introduction

Hemolytic uremic syndrome (HUS) is a life-threatening disease, characterized by microangiopathic hemolytic anemia, destructive thrombocytopenia, and renal failure [1]. Most HUS occurs in association with Shiga toxin-producing *Escherichia coli* (STEC) infection [2]. Patients with STEC-HUS generally recover with fluid therapy and hemodialysis. Mortality is high among STEC-HUS patients with encephalopathy, despite treatments including plasma exchange, steroid pulse, and more recently eculizumab [3]. In recent STEC outbreaks in the United States (STEC-O111) and Germany (STEC-O104) in 2008 and 2011, respectively [4, 5], STEC-HUS incidence and mortality were 16.7% and 3.8% and 22% and 3.7%, respectively.

In 2011, an outbreak of STEC-O111 and/or -O157 infection in Toyama, Japan occurred following raw meat ingestion in a barbecue restaurant chain. Overall, 181 patients were infected, of whom 34 developed STEC-HUS (18.8%) including 21 with encephalopathy (61.8%) and five deaths (14.7%; all with encephalopathy) [6–8]. Ten STEC-HUS patients were aged 1–14 years, including eight with encephalopathy [7]. Seven children including five

with encephalopathy recovered and three died [7]. We report clinical and laboratory findings for a 14-year-old girl in the Toyama series with STEC-HUS and encephalopathy.

Case Report

In April 2011, a 14-year-old girl ingested raw meat in a barbecue restaurant in Toyama, and then traveled to Osaka. Bloody diarrhea developed 5 days later. At a local hospital, levofloxacin was prescribed without improvement. Six days later after raw meat ingestion, she was transferred to Yodogawa Christian hospital. Almost simultaneously, multiple outbreaks of hemorrhagic enterocolitis due to STEC: O111 (producing both shigatoxin-1 and -2) were reported from several hospitals around Toyama. All affected patients had eaten raw meats in the same chain restaurants around Toyama. Admission laboratory findings included: white blood cell (WBC) [24,700/μl], red blood cell (RBC) [5.28 $\times$ 10^6/μL], hemoglobin (Hb) [16.7 g/dL], platelet [143 $\times$ 10^3/μL], C-reactive protein (CRP) [3.55 mg/dl], lactate dehydrogenase (LDH) [227 IU/L], blood urea nitrogen (BUN) [15.6 mg/dL], creatinine (Cr) [0.69 mg/dL], normal hemostatic tests, proteinuria, and no hematuria. Stool cultures showed normal flora, stool shigatoxin stool was negative, and both the antigens of STEC:O111 and O157 in stool were negative.

On day 3, the patient developed anemia (RBC [2.63 $\times$ 10^6/μL], Hb [8.2 g/dL], LDH [1148 IU/L], haptoglobin [8 mg/dl], and thrombocytopenia [12,000/μl], with an increase in BUN [26.6 mg/dL] and Cr [1.06 mg/dL] as shown in Figure 1). Schistocytes were seen in the peripheral blood smear. Plasma ADAMTS13 activity levels were 43% of normal. The patient became anuric and comatose (Glasgow Coma Scale [GCS] 14). Continuous hemodiafiltration was initiated with plasma exchange. On day 5, pleural effusions developed, respiratory function worsened, and consciousness deteriorated further. Intubation was performed. Brain magnetic resonance imaging showed high intensity areas in the bilateral thalamus and basal ganglia, and part of the pontine tegmentum on T2 FLAIR images (Fig. 1 Inset). Acute encephalopathy developed. STEC-HUS was diagnosed. High-dose methylprednisolone pulse therapy [500 mg/day] for days 5–7 was administered. On day 6, serum antibodies to STEC:O111 antigen were noted. On day 9, hemolysis worsened, whereas severe thrombocytopenia persisted. Plasma exchange was increased to twice daily. A second 3-day course of a high-dose methylprednisolone pulse therapy was administered. Gabexate mesilate, a synthetic anticoagulant was administered. Serum levels of fibrin/fibrinogen degradation product (FDP) and thrombin–antithrombin complex (TAT) increased to 120 μg/mL and 24.3 ng/mL, respectively. Soluble recombinant thrombomodulin (130 units/kg/day) was infused during days 9–14. Clinical and laboratory findings subsequently improved, including thrombocytopenia, hemolysis, and renal function (Fig. 1). Extubation occurred on day 22. Plasma exchange was tapered, and discontinued on day 24. After rehabilitation, the patient was discharged without appreciable sequelae on day 64.

Retrospective analyses of stored plasma samples were performed. Plasma samples from admission showed that levels of the following cytokines were not elevated: interleukin (IL)-6 [4 pg/mL (normal: <4)], IL-8 [59 pg/mL (normal: <2)], and tumor necrosis factor (TNF)α [12 pg/mL (normal: <15)]. In contrast, plasma samples from admission identified elevated levels of neopterin [98 nmol/L (normal: <5)], soluble form TNF receptor type I (sTNF-RI) [13,200 pg/mL (normal: 484–1407)], sTNF-RII [18,300 pg/mL (normal: 829–2262)], and tau protein [344 pg/mL (normal: undetectable)]. Plasma samples from day 3 identified reduced plasma ADAMTS13 activity (43%) levels and high levels of plasma VWF antigen levels (605% of normal).

Retrospective analysis of plasma VWF multimer patterns using citrated plasma samples (frozen at −80°C) was also performed (Fig. 2). During the acute phase, no high-to-intermediate sized VWF multimers were identified in samples taken three and 13 days prior to initiation of plasma exchange. After each plasma exchange, VWF multimer patterns were present, although high-sized VWF multimers continued to be absent. Plamsa exchange was performed once or twice daily until day 20, then tapered, and discontinued on day 24. UL-VWF multimers appeared in plasma at days 21 and 24, and disappeared at day 61 just before discharge. At discharge, plasma levels of VWF and ADAMTS13 had returned to almost normal ranges.

Discussion

We report a patient with STEC-HUS, mild-to-moderate reduction of plasma ADAMTS13 activity, and increased plasma levels of VWF antigen. Despite persistent thrombocytopenia in the acute phase, VWF multimers were degraded on one occasion and highly multimerized on a different occasion. Therapy with continuous hemodiafiltration, high-dose methylprednisolone pulse therapy and soluble recombinant thrombomodulin was successful and the patient was discharged without any deficits. In explaining our findings, several factors should be considered.

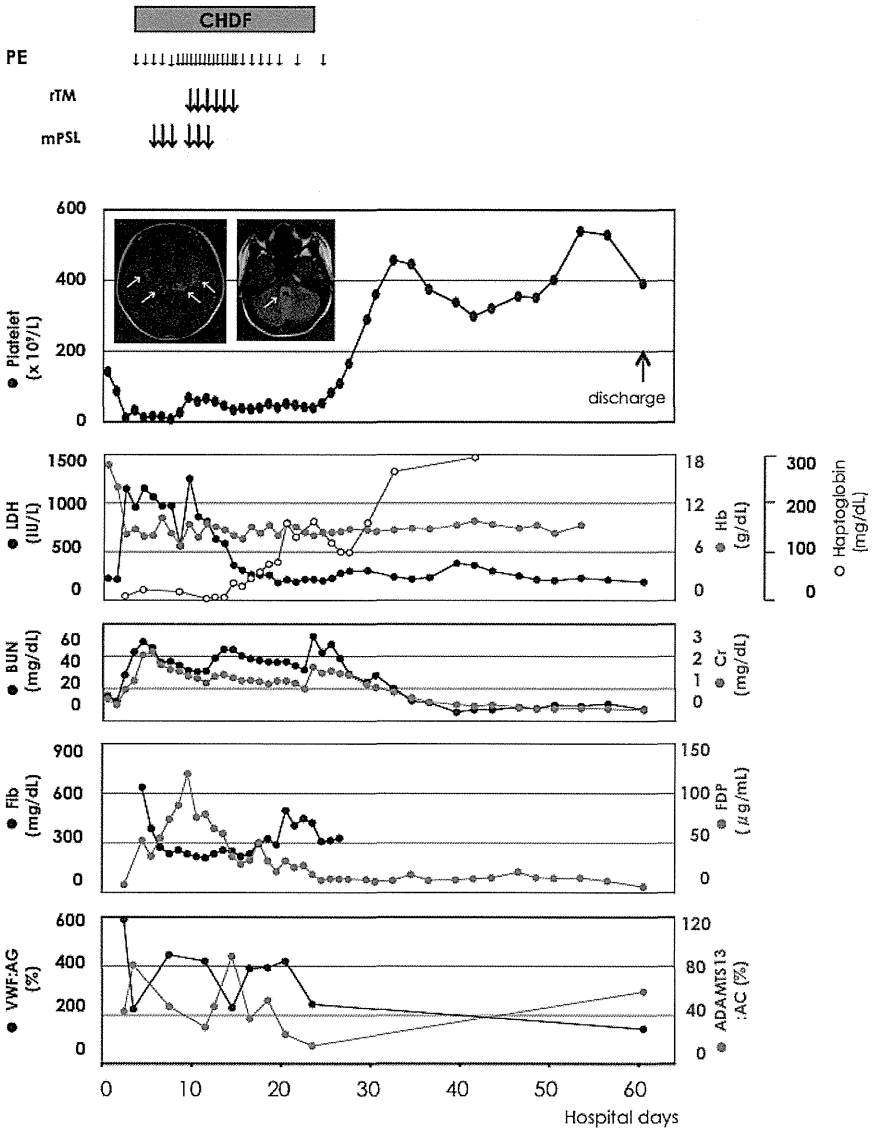
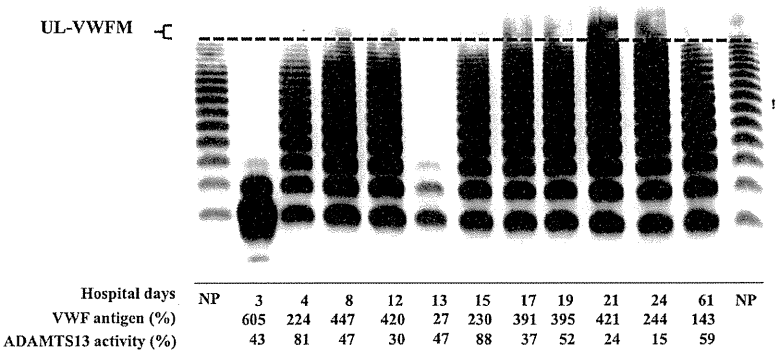


Figure 1. Clinical course in a 14-year-old girl with STEC-HUS complicated by acute encephalopathy after admission.

First, identification of UL-VWF multimers in this patient differs from the VWF pattern usually seen with STEC-HUS where the multimers are usually depleted. UL-VWFMs, stored in Weibel–Palade bodies (WPBs) of vascular endothelial cells, are released upon stimulation by inflammatory cytokines, such as IL-6, IL-8, and TNFα [9]. Likewise, UL-VWFMs are released into the circulation by injured vascular endothelial cells. On admission, plasma levels of cytokines including IL-8, neopterin, TNF-RI and RII, and tau protein were high, indicating vascular injury, inflammation, and neurological cell damages [6]. Also, the B-subunit of shigatoxin-1 and -2, both AB5-holotoxins, binds to globotrialosyl ceramide (Gb3) by which UL-VWFMs are released from Weibel–Palade bodies [10]. Shigatoxin binds to Gb3, internalizes, and blocks protein synthesis by attachment to ribosomal RNA. Shigatoxin also directly enhances platelet aggregation under high and low shear stress at very low concentrations [11]. Thus, in our patient, UL-VWFM, may have been released excessively from activated vascular endothelial cells, was involved in platelet thrombi formation, and then was consumed by proteases released from platelets and/or leucocytes.

Second, our findings may explain how plasma exchange may have had therapeutic benefit in this patient. In par-



Hospital days	NP	3	4	8	12	13	15	17	19	21	24	61	NP
VWF antigen (%)		605	224	447	420	27	230	391	395	421	244	143	
ADAMTS13 activity (%)		43	81	47	30	47	88	37	52	24	15	59	

Figure 2. Change of VWF multimer patterns during the acute phase.

ticular, plasma exchange might work bifunctionally: one effect was to reduce concentrations of various cytokines, UL-VWFM, and shigatoxin, and the other effect was to supply normal VWFM (for hemostasis). During the acute phase of STEC-HUS, the STEC vigorously produces shigatoxin, which consistently activates platelets, even at low concentrations (pg/ml). So, plasma exchange alone for STEC-HUS is likely to be inefficient, unless shigatoxin function is blocked. Hence, in addition to basic supportive therapy for STEC-HUS such as dialysis and fluid therapy, cytokine adsorption is favorable, and high-dose methylprednisolone pulse therapy might suppress cytokine production [12].

Third, in comparison to previous reports, the occurrence of acute encephalopathy associated with STEC-HUS in Toyama was high, and the deceased cases had encephalopathy. This toxicity is attributable to brain edema, presumably due to increased vascular permeability and/or severe vascular endothelial cell injuries mediated by shigatoxin itself and cytokines, yet the mechanism is not fully understood [13]. Strains of STEC:O111 isolated in Toyama predominantly produced shigatoxin-2, which is more toxic than shigatoxin-1. However, a peculiar MRI finding on high intensity areas, often symmetrical in thalamus, basal ganglia, and pontine tegmentum, has not been favorably addressed [14].

Fourth, common therapeutic features on seven survived childhood patients in Toyama included continuous hemodiafiltration, high-dose methylprednisolone pulse therapy, and recombinant thrombomodulin. High-dose intravenous immunoglobulin infusion was administered to six of the seven survivors. Administration of recombinant thrombomodulin may have been particularly important, as this drug has been available in Japan as treatment for disseminated intravascular coagulation (DIC) since 2008 [15]. Recombinant thrombomodulin is a multifunctional protein. A lectin-like domain directly absorbs and neutralizes high mobility group box1 (HMGB1), which is a pro-inflammatory cytokine that acts as a lethality factor when endotoxin shock occurs [16]. Also, EGF-like domains 4–6 of the recombinant thrombomodulin can bind thrombin and inactive the catalytic activity of thrombin. The thombin–recombinant thrombomodulin complex can accelerate activation of protein C and thrombin activatable fibrinolytic inhibitor (TAFI) to activated protein C and TAFIa, respectively. In turn, activated protein C generates anticoagulant action via inactivation of Va and VIIIa and TAFIa suppresses complement activation via inactivation of C3a and C5a [15]. As the action of recombinant thrombomodulin on platelets remains unclear, we are unable to directly address how recombinant thrombomodulin can resolve STEC-HUS. There are at least two possibilities: one is direct inhibitory activity to platelet aggregation, and the second is to block fibrin clot formation over platelet thrombi, as suggested by significant increases of FDP and TAT during the clinical course before recombinant thrombomodulin is administered.

In conclusion, we report a novel therapy for STEC-HUS. VWF-dependent hemostatic defect that is generated in STEC-HUS appears to have been restored by plasma exchange. Hypercoagulability, presumably induced by shigatoxin or cytokine storms, appears to have been suppressed with high-dose methylprednisolone pulse therapy and recombinant thromobomodulin.

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Conflict of Interest

Nothing to declare.

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A rapid, fully automated and highly sensitive ADAMTS13 gold particle immunoassay using a routine biochemistry analyser

Thrombotic microangiopathies (TMAs) are pathological conditions, characterized by thrombocytopenia, microangiopathic haemolytic anaemia and organ failure due to platelet thrombi (Moake, 2002). Two typical phenotypes of TMAs are thrombotic thrombocytopenic purpura (TTP) and haemolytic uremic syndrome, both of which are life-threatening generalized diseases. TMAs, however, occur more frequently in association with a variety of underlying clinical conditions, including pregnancy, autoimmune diseases, malignancy and transplantation (Lämmle *et al*, 2005). Therefore, the differential diagnosis of TMAs is critically important and often urged in clinical practice. Among the TMAs, TTP is now well defined by severe deficiency of the activity of the von Willebrand factor (VWF)-cleaving protease, termed ADAMTS13 (a disintegrin and metalloprotease with thrombospondin type 1 motifs 13) (Sadler, 2008). However, currently existing assays for ADAMTS13 activity are tedious and time-consuming. To overcome these problems, we have developed a rapid, fully automated and highly sensitive assay for ADAMTS13 activity, termed ADAMTS13 act-GPI.

ADAMTS13 act-GPI is performed using gold particle immuno-agglutination with a biochemistry analyser, such as a Hitachi 7170 automatic clinical analyser (Hitachi, Tokyo, Japan), which is commonly found in routine laboratories. The principle of this novel assay, which consists of two reaction steps, is illustrated in Fig 1A. The first step involves the digestion of the substrate GST-VWF73-His (Kokame *et al*, 2003) by plasma ADAMTS13 for 4·93 min between Points 2 and 3. The second step involves the measurement of particle agglutination generated by immunoreactive lattice formation with the cleaved substrate (GST-VWF10) and two gold particles, coated with anti-GST monoclonal antibody (mAb) (IgG) or anti-N10 mAb (IgG) (Kato *et al*, 2006), respectively. Anti-N10 mAb specifically recognizes the Y1605 residue, which is exposed when the substrate VWF73 is cleaved by ADAMTS13. The degree of particle agglutination is continuously monitored by the change in absorbance (Fig 1B). The decrease in absorbance at 546/660 nm from 335·43 s to 590·04 s is represented as ΔAbs, and a calibration curve was obtained for ΔAbs and ADAMTS13 activity (Fig 1C). This novel assay can be completed in almost 10 min.

Two reagents are essential for ADAMTS13 act-GPI. One is the substrate GST-VWF73-His, which is dissolved at a concentration of 1·0 μg/ml in 10 mmol/l PIPES buffer (pH 5·5) containing 10 mmol/l $CaCl_2$, 5 mmol/l benzamidine, 1 mmol/l transamin, 0·4% argatroban, 0·01% Tween 20, 10% immobilized murine plasma and 3% chondroitin sulfate sodium. The other reagent is a mixture of two kinds of colloidal gold particles, coated with anti-GST mAb (IgG) and anti-N10 mAb (IgG), respectively, prepared according to the method of De Roe *et al* (1987) using gold particles with a mean diameter of 50 nm. The mAb-coated colloidal gold particles are suspended in 5 mmol/l HEPES buffer (pH 7·5) containing 30 g/l mannitol, 2 g/l EDTA-2Na and 0·08% Tween 80.

The detection limit for ADAMTS13 activity with ADAMTS13 act-GPI was determined to be 0·4% of normal, defined as three standard deviations (SDs) above the mean for samples with 0% activity (n = 8). Intra-assay reproducibility with three plasma samples (at 9·1%, 37·2% and 75·9% of plasma ADAMTS13 activity) determined by eight serial measurements was 1·5%, 0·5% and 0·4%, respectively. The inter-assay reproducibility of the same three samples determined on eight different days was 0·9%, 1·0% and 1·0%, respectively. Furthermore, no interference was found with the presence of unconjugated bilirubin (337·0 μmol/l), conjugated bilirubin (359·0 μmol/l), haemoglobin (4·88 g/l),

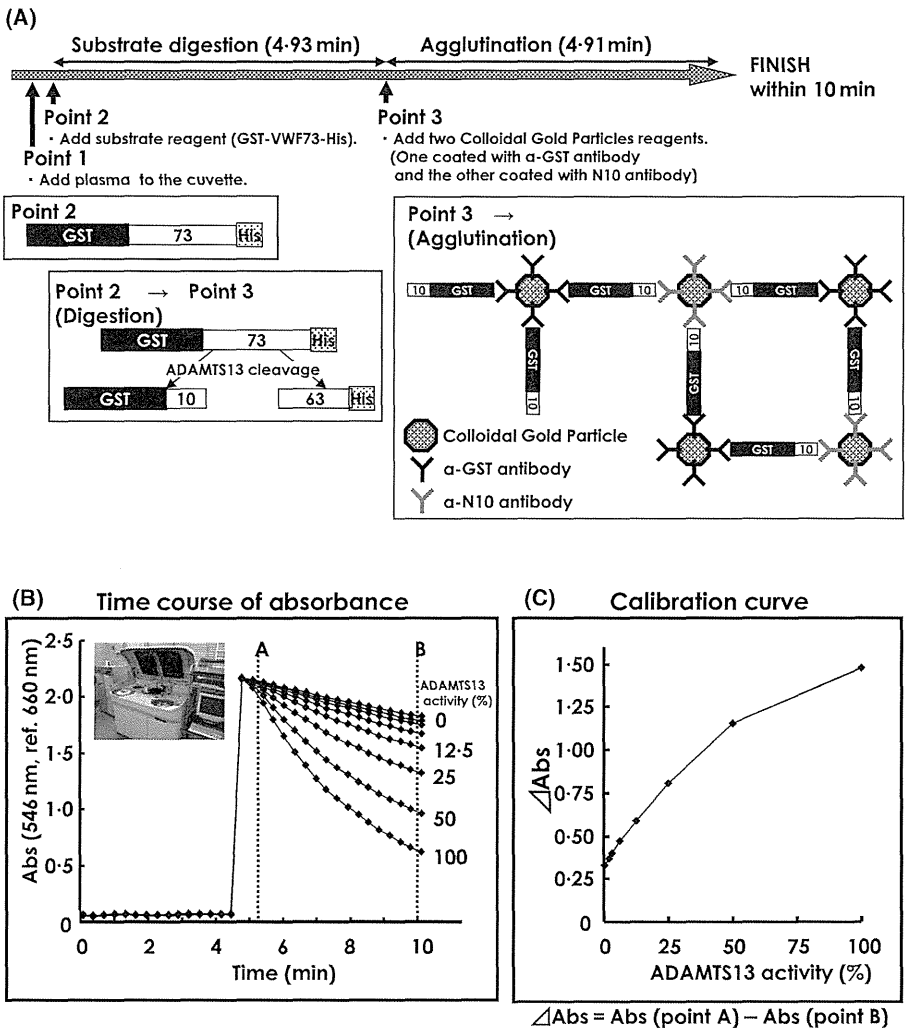

Fig 1. Determination of ADAMTS13 activity using novel ADAMTS13 act-GPI. (A) Schema illustrating the principle of the assay. There is only one manual step, putting a centrifuged tube of blood in the same manner that plasma is usually prepared as a sample into the analyser. The time-table and reactions in ADAMTS13 act-GPI are as follows. At Point 1, 5 μl of the sample is transferred to an assay cuvette. At Point 2, 90 μl of the substrate reagent, containing GST-VWF73-His, is added and stirred. Subsequently, the reaction mixture is incubated for 4·93 min at 37°C for the enzymatic reaction. After enzymatic digestion, at Point 3, 90 μl of the reagent containing colloidal gold particles coated with anti-GST and anti-N10 are added and stirred. The reaction between the particles and GST-VWF10, generated by ADAMTS13 activity in the sample, results in agglutination. This assay can be completed within approximately 10 min. (B) The time course of changes in absorbance. The time course with calibrants that have 100%, 50%, 25%, 12·5%, 6·25%, 3·125%, 1·5625% and 0% ADAMTS13 activity are depicted. The change in the ratio of absorbance values for 660 and 546 nm (secondary and primary wavelengths, respectively) from 335·43 s (dotted line A) to 590·04 s (dotted line B) was calculated and expressed as ΔAbs. The calibrants were prepared with pooled normal plasma sequentially diluted by pooled normal plasma that was heat inactivated. Inset: a photograph of the analyser. (C) A representative standard curve of ADAMTS13 activity.

chyle as a turbidity of sample (1550 formazin turbidity units; FTU), or rheumatoid factor (500 IU/ml) in the plasma samples, which were spiked with substances included in Interference Check A and RF plus (Sysmex, Kobe, Japan) (data shown in Fig S1). The specificity of this assay was confirmed by measuring plasma samples that were pre-incubated with two anti-ADAMTS13 mAbs, a neutralizing mAb (A10) and a non-neutralizing mAb (C7) (Yagi *et al*, 2007). Furthermore, EDTA at a final concentration of 10 mmol/l completely abolished ADAMTS13 activity by chelating metal ions (data shown in supporting information, Fig S1).

Comparative studies of ADAMTS13 activity were performed using three methods: ADAMTS13 act-GPI, ADAMTS13 act-enzyme-linked immunosorbent assay (ELISA), and a modified FRETS-VWF73 assay (Kokame *et al*, 2005; Kremer Hovinga *et al*, 2006). For this purpose, the initial determination of ADAMTS13 activity and its inhibitor (neutralizing antibodies) was performed using a chromogenic

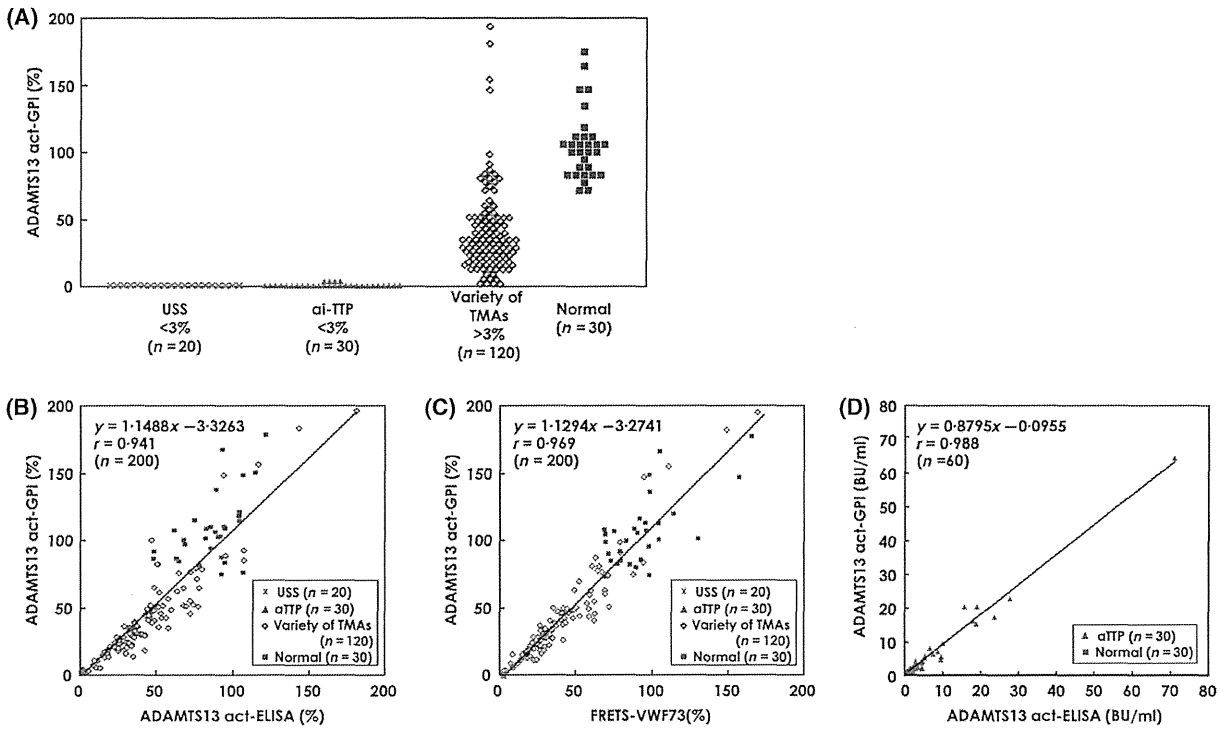

Fig 2. (A) Plasma ADAMTS13 activity levels determined by ADAMTS13 act-GPI. All the samples used in this study were 0·32% citrated plasma stored at −80°C until use. Plasma was obtained from the following four groups: 20 genotyped patients with congenital thrombotic thrombocytopenic purpura (TTP) (Upshaw-Schulman syndrome, USS), 30 patients with primary acquired TTP (aTTP) with severe deficiency of ADAMTS13 activity and positive for ADAMTS13 inhibitors (>1·0 Bethesda U/ml), 120 patients with a variety of suspected TMAs, based on clinical and laboratory information, with more than 3% ADAMTS13 activity by ADAMTS13 act-ELISA, and 30 normal individuals aged 20–40 years (15 females and 15 males). (B) The correlation in plasma ADAMTS13 activity levels between ADAMTS13 act-ELISA and ADAMTS13 act-GPI. (C) The correlation in plasma ADAMTS13 activity levels between FRETS-VWF73 and ADAMTS13 act-GPI. (D) The correlation in titres of plasma ADAMTS13 inhibitor determined using ADMTS13 act-ELISA and ADAMTS13 act-GTI in 30 aTTP patients and 30 healthy individuals (see text for details).

ADAMTS13 act-ELISA kit (Kainos Laboratories, Tokyo, Japan). Plasma samples used for this study were obtained from the following four groups: 20 genotyped patients with congenital TTP (Upshaw-Schulman syndrome, USS) (Fujimura *et al*, 2011), 30 patients with primary acquired TTP (aTTP) with severe deficient ADAMTS13 activity and positive ADAMTS13 inhibitors (>1·0 Bethesda U/ml), 120 patients with a variety of TMAs based on clinical and laboratory data, with more than 3% ADAMTS13 activity by ADAMTS13 act-ELISA, and 30 normal individuals aged 20–40 years (15 females and 15 males). The calibrants were prepared by pooled normal plasma (prepared from 30 males and 30 females aged 20–40 years) sequentially diluted with heat inactivated pooled normal plasma.

Plasma levels of ADAMTS13 activity determined by ADAMTS13 act-GPI in the aforementioned four categories are shown in Fig 2A. The correlation in plasma ADAMTS13 activity levels between ADAMTS13 act-ELISA (x-axis) and ADAMTS13 act-GPI (y-axis) is shown in Fig 2B, with the regression line ($y = 1{\cdot}1488x - 3{\cdot}3263$) and the correlation coefficient ($r = 0{\cdot}941$). Further, the correlation in plasma ADAMTS13 activity levels between FRETS-VWF73 (x-axis) and ADAMTS13 act-GPI (y-axis) is shown in Fig 2C, with the regression line ($y = 1{\cdot}129x - 3{\cdot}2741$) and r (0·969). The plasma ADAMT13 activity levels, determined by three methods in four groups, are shown in Table S1. In Fig 2D, the correlation in plasma ADAMTS13 inhibitor titres determined by ADMTS13 act-ELISA (x-axis) and ADAMTS13 act-GTI (y-axis) is shown in 30 aTTP patients and 30 healthy individuals, with the regression line ($y = 0{\cdot}8795x - 0{\cdot}0955$) and r (0·988).

Although these three ADAMTS13 activity assays operate on totally different principles, the values obtained using these assays were highly correlated. We propose that ADAMTS13 act-GPI could be useful in clinical practice because automation has the advantages of rapidity, high-throughput performance and decreasing human contact with infectious materials.

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Author contribution

SK, ST and YF designed the research study; SK, MT and AI performed experiments; MM collected plasma samples; SK analysed data; MT supported construction of assay procedure; SK and YF wrote the manuscript. All authors reviewed the manuscript.

Conflicts of interest

The authors have some conflicts of interest relevant to this manuscript submitted to *British Journal of Haematology*.

Employment: Seiji Kato and Mutsumi Tanaka are employees of Alfresa Pharma Corporation. Tomohiro Samori is an advisor of Japan Clinical Laboratories, Inc.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig S1. Effect of interfering substances, anti-ADAMTS13 monoclonal antibody and EDTA on the ADAMTS13 act-GPI.

Table S1. Plasma ADAMTS13 levels activity determined by three methods in four groups.

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Functional analysis of truncated forms of ETV6

The ETV6 (TEL) transcription factor has been shown to play a wide role in haematopoiesis, influencing the development of multiple lineages, while chromosomal translocations involving fusions of the *ETV6* gene occur frequently in haematological malignancies (Rasighaemi *et al*, 2014). Recurrent mutations of *ETV6* have been reported in cases of acute myeloid leukaemia (AML) (Barjesteh Van Waalwijk Van Doorn-Khosravani *et al*, 2005; Silva *et al*, 2008), childhood B cell acute lymphoblastic leukaemia (B-ALL) (Zhang *et al*, 2011) and early immature adult T-cell ALL (T-ALL) (Van Vlierberghe *et al*, 2011), along with alternative splicing of *ETV6* in myelodysplastic syndrome (MDS) (Sasaki *et al*, 2004). These

Poor responder to plasma exchange therapy in acquired thrombotic thrombocytopenic purpura is associated with ADAMTS13 inhibitor boosting: visualization of an ADAMTS13 inhibitor complex and its proteolytic clearance from plasma

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BACKGROUND: Plasma exchange (PE) is the first-line treatment for primary acquired thrombotic thrombocytopenic purpura (aTTP) with severe deficiency of ADAMTS13 activity (ADAMTS13:AC). Some patients are poor responders to PE, raising concern over multiple pathogenetic pathways.

STUDY DESIGN AND METHODS: Based on 52 aTTP patients in our national cohort study, we monitored plasma levels of ADAMTS13, clinical and laboratory findings, and outcomes. In a representative poor responder to PE, we examined an ADAMTS13 inhibitor (ADAMTS13:INH) complex in plasma milieu, by means of a large-pore isoelectric focusing (IEF) analysis.

RESULTS: Of 52 aTTP patients, 20 were good responders and 32 were poor responders. In the latter group, plasma ADAMTS13:AC levels never increased to more than 10% of normal during 14 days after PE initiation. Mean (±SD) plasma ADAMTS13:INH titers (Bethesda unit/mL) were 5.7 (±4.5) before PE, but decreased to 1.4 (±0.8) on the fourth PE day and then remarkably increased to 14.8 (±10.0) on the 10th PE day, termed "inhibitor boosting," and then slowly decreased to undetectable level over 1 month. On admission, none of the routinely available clinical and laboratory markers differentiated these two groups. However, elevated pre-PE levels of ADAMTS13:INH were correlated with a poor response. We visualized an ADAMTS13:INH (immunoglobulin G) complex in a patient plasma by an IEF analysis and found proteolytic fragment of ADAMTS13 antigen by a two-dimensional IEF and sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis.

CONCLUSION: Findings from this cohort of aTTP patients demonstrated that inhibitor boosting often occurs in aTTP patients in Japan. Poor responders could be predicted by elevated pre-PE ADAMTS13:INH levels on admission, but not by routinely collected clinical or laboratory data.

Thrombotic thrombocytopenic purpura (TTP), a life-threatening generalized disorder originally characterized by a pentad of thrombocytopenia, microangiopathic hemolytic anemia, renal dysfunction, neurologic signs, and fever,[1] is now primarily defined by severe deficiency of von Willebrand factor (VWF)-cleaving protease, termed ADAMTS13 (a

ABBREVIATIONS: ADAMTS13:AC = ADAMTS13 activity; ADAMTS13:AG = ADAMTS13:antigen; ADAMTS13:INH = ADAMTS13 inhibitor; aTTP = primary acquired thrombotic thrombocytopenic purpura; IEF = isoelectric focusing; PE = plasma exchange; UL = unusually large; VWFM = von Willebrand factor multimer.

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disintegrin-like and metalloprotease with thrombospondin Type 1 motifs 13) accompanied by thrombocytopenia.[2-4] The ADAMTS13 specifically cleaves unusually large VWF multimer (UL-VWFM) with hyperaggregability of platelets (PLTs)[5] and down regulates VWF function. Deficiency of ADAMTS13 activity (ADAMTS13:AC) is caused by either gene mutations in or acquired autoantibodies to this enzyme,[6] occasionally without a known cause, termed primary acquired TTP (aTTP).[7]

Plasma exchange (PE) has long been the first-line treatment for aTTP, often with an adjunct of corticosteroid and corticosteroid pulse therapy,[8] PE removes ADAMTS13 inhibitor (ADAMTS13:INH), UL-VWFM, and inflammatory cytokines that mediate UL-VWFM release from vascular endothelial cells and replenishes ADAMTS13 and regular-sized VWFM required for normal hemostasis. Patients with aTTP frequently take several days to weeks of PE before PLT counts recover. However, one population of aTTP patients often shows an initial increase in the PLT count that is paradoxically followed by secondary thrombocytopenia. These patients have been categorized as having "PE-refractory aTTP," but the pathogenesis of secondary thrombocytopenia is unclear. Böhm and colleagues[9] reported an increase in ADAMTS13:INH titers after PE, but no systematic studies on this potentially important issue have been conducted. In addition, 30% to 50% of aTTP patients will subsequently relapse, although these relapses occur at a time point that is much longer than the secondary thrombocytopenia described herein.[6,10] Due to concerns over both short-term thrombocytopenic events and long-term aTTP relapse, the anti-CD20 rituximab, which removes inhibitory immunoglobulin (Ig)G-producing B lymphocytes from circulation,[11,12] has been used recently to treat aTTP patients in conjunction with PE and corticosteroid, in an effort to reduce IgG of ADAMTS13:INH with the goal of improving short-term responses as well as decreasing the rate of relapse.

We have recently shown that ADAMTS13 antigen (ADAMTS13:AG) in the plasma milieu consisted of three groups of bands with different isoelectric points (pI); Band I (pI 4.9 to 5.6) represents ADAMTS13 not bound to VWF, Band II (pI 5.8 to 6.7) remains unaddressed, and Band III (pI 7.0 or 7.5) corresponds to ADAMTS13 bound to larger VWFM.[13] Further, during 1998 to 2012 we identified 52 patients with new onset of aTTP, whose plasma samples were followed for serial ADAMTS13 level measurements more than three times within 2 weeks after initiation of PE. These patients were classified into two groups based on the pre-PE levels of plasma ADAMTS13:AC measured on the 14th day after PE initiation; 20 good ADAMTS13:AC responders (ADAMTS13:AC $\geq$10%) and 32 poor ADAMTS13:AC responders (ADAMTS13:AC < 10%). We found that poor ADAMTS13:AC responders were uniformly associated with a tremendous increase of ADAMTS13:INH titers (IgG1 subclass) on the 10th PE day, termed "inhibitor boosting." Further, using an isoelectric focusing (IEF) gel we visualized for the first time a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), that underwent proteolytic cleavage followed by clearance from circulation. These studies may help to understand how treatment shortly after PE with corticosteroid and rituximab infusion works in aTTP patients who otherwise may develop secondary thrombocytopenia due to inhibitor boosting.

MATERIALS AND METHODS

Fifty-two aTTP patients enrolled in this national cohort study

Since 1998, our laboratory at Nara Medical University has served as a nationwide referral center for thrombotic microangiopathy that analyzes ADAMTS13 as requested by clients across Japan. Using a chromogenic assay for ADAMTS13:AC,[14] between January 2004 and March 2012, we were able to identify 184 new patients (89 males and 95 females) with aTTP, based on the following criteria: severe deficiency of plasma ADAMTS13:AC (<10% of normal) with positive ADAMTS13:INH (>1.0 Bethesda unit [BU]/mL), but without the underlying diseases. Of 184 aTTP patients, 52 were able to have follow-up determination of plasma ADAMTS13:AC levels more than three times at 3- to 5-day intervals within 14 days after PE initiation and were enrolled in this study (see Tables S1and S2, available as supporting information in the online version of this paper).

Patient outcome definitions

The study definitions were the same as those reported by Kremer Hovinga and colleagues[15] and Froissart and colleagues.[16] Remission was defined as a complete resolution of laboratory and clinical data after cessation of PE. Durable remission was defined as laboratory and clinical resolution of aTTP evaluated at least 30 days after the first day of PLT count recovery (>150 $\times$ 10^9/L; this period included the time on maintenance PE). Relapse was considered a new episode of TTP if a patient had achieved a durable remission and subsequently experienced a laboratory relapse. Death from TTP was defined as any death that occurred within 30 days after a diagnosis of TTP.

Assays for plasma levels of ADAMTS13:AC, ADAMTS13:INH, and ADAMTS13:AG

Plasma levels of ADAMTS13:AC were measured by chromogenic ADAMTS13-act-enzyme-linked immunosorbent assay (ELISA)[14] with a detection limit of 0.5% of normal, and the ADMTS13:INH titers were determined by means of the Bethesda method[17] using heat-inactivated patient plasmas, as previously described. One BU is defined as the amount necessary to reduce ADAMTS13:AC to 50% of control levels, and the value of at least 1.0 BU/mL was

assumed to be positive. Further, plasma levels of ADAMTS13:AG were measured using sandwich ELISA.[18]

Titers of anti-ADAMTS13 IgG, IgM, and IgA autoantibodies and IgG1-4 subclasses

Assays were performed as previously described,[19] with the following modifications. First, microtiter plates (Nunc Immuno Maxisorp, Nunc A/S, Roskilde, Denmark) were coated with 100 μL of human recombinant ADAMTS13 (2 μg/mL) dissolved in a coating buffer (carbonate-bicarbonate buffer, pH 9.6, Sigma C-3041, Sigma-Aldrich, St Louis, MO]) and left overnight at 4°C. On the next day, 250 μL of blocking buffer (Pierce protein-free T20, Thermo Fisher Scientific, Inc., Rockford, IL) was added to the plates and incubated for 2 hours at room temperature. Then, 100 μL of each diluted test sample was added to each well and left for 3 hours at room temperature. After incubation, 100 μL of diluted horseradish peroxidase–conjugated goat anti-human IgG, IgG1-4, IgM, or IgA was added, and the plates were incubated for 2 hours at room temperature. Finally, 100 μL of 3,3′,5,5′-tetramethylbenzidine-H_2O_2 solution (3,3′,5,5′-tetramethylbenzidine substrate kit, Thermo Fisher Scientific Inc.) was added, and the absorbance was measured at 450 nm with a reference filter of 620 nm on a microplate reader (Thermo Fisher Scientific, Inc.). Between each step, the plates were washed with phosphate-buffered saline (PBS, pH 7.2) four times. Titers were calculated as a ratio of the sample optical density (OD) at each dilution to the normal human plasma OD at each dilution. Ratios were determined for each individual dilution and compared to the corresponding cutoff values, and the results are expressed as the titer. The titer of a sample corresponded to the last dilution at which the ratio was above the cutoff level.[19]

IEF using an agarose-acrylamide composite gel

In some experiments, ADAMTS13 in patient plasma was analyzed by IEF using a large-pore agarose-acrylamide composite gel as recently described in detail.[13] After IEF, the isolated proteins were electrophoretically transferred to nitrocellulose membranes, and then the blotted proteins were immunoreacted with anti-ADAMTS13 monoclonal antibody (WH2-11-1, epitope residing on the fourth thrombospondin Type 1 domain of ADAMTS13)[20] and visualized using a chemiluminescent detection kit (Perkin-Elmer Life Science, Boston, MA). With this method, ADAMTS13:AG from normal plasma consistently shows the following three groups of bands: a major Band I representing unbound ADAMTS13 with a pI of 4.9-5.6, as in the case of purified plasma-derived ADAMTS13, as well as Band II at pI 5.8-6.7 and Band III at pI 7.0/7.5. The composition of Band II is not well defined yet, but Band III was shown to be a complex of ADAMTS13:AG and high-molecular-weight VWFM.[13]

Therapeutic regimen

The basic therapeutic regimen for our cohort patients with aTTP consisted of PE, corticosteroids, and immunosuppressants such as rituximab (see Tables S1 and S2). All patients were initially treated with PE with fresh-frozen plasma at a dose of 40 to 60 mL/kg body weight once per day and mostly for the first 3 to 5 consecutive days. However, the subsequent regimen of PE therapy was variable based primarily on laboratory findings of patients (PLT count and serum lactate dehydrogenase [LDH]) and presence of neurological findings.

PE therapy was usually accompanied with corticosteroid therapy. In our cohort, 30 patients received high-dose methylprednisolone (0.5-1 g/day) pulse therapy, of whom 23 patients received it commonly for 3 consecutive days for 1 to 3 days after PE and seven patients received it beyond this period. An additional 13 patients who did not receive corticosteroid pulse therapy received per os administration of predonisolone (0.5-1 mg/kg/day). One patient did not have corticosteroid therapy. For the remaining eight patients, we were unable to retrieve information on corticosteroid therapy from the physicians-in-charge.

For immune suppressants, rituximab infusion was most frequently used (18/52 patients with aTTP). The rituximab dose was 375 mg/m^2 weekly for 4 weeks, although one patient (Patient 34) died soon after the first rituximab infusion and one patient (Patient 26) received five infusions of rituximab. Patient 28 (female) was an exception, who received a second course of rituximab therapy for 75 to 96 days (a total of eight rituximab infusions), as the patient had persistence of high titers of ADAMTS13:INH (peak of 42.6 BU/mL). An additional 10 patients received vincristine (1-2 mg/body per day) one to four doses administered 1 week apart, and four patients received one or two cycles of cyclophosphamide pulse therapy (500 mg/body/day). Therapies administered to three additional patients included intravenous immunoglobulin (one patient), one dose of cyclosporine (one patient), and aspirin (one patient).

Statistical analysis

Laboratory data are expressed as the mean ± SD. Comparisons between good ADAMTS13:AC responders and poor ADAMTS13:AC responders were analyzed using the Mann-Whitney U test or chi-square test. All analyses were carried out using with computer software (StatView, SAS Institute, Inc., Cary, NC). A p value of less than 0.05 was considered significant.

RESULTS

Two patient groups of aTTP: good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE

Follow-up clinical and laboratory findings were available for all 52 aTTP patients (between 7 and 2607 days after

TABLE 1. Comparison of laboratory markers: good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE therapy

	ADAMTS13:AC (%) on 14th day after PE		
Variable	≥10% (good responders)	< 10% (poor responders)	p value
Number	20	32	
Age on admission (years)	53 (1-81)	48 (13-84)	0.44
Sex			
Female	13 (65%)	19 (59%)	0.91
Male	7 (35%)	13 (41%)	
PE (times) total	7.5 (3-15)	14.3 (4-31)	<0.01
For 14 days	6.8 (3-11)	9.9 (4-14)	<0.01
After 14 days	0.7 (0-6)	4.5 (0-20)	<0.01
Rituximab therapy	3	15	<0.05
Steroid and steroid pulse therapy	18	30	0.62

the first admission). These follow-up data are summarized in Tables S1 and S2. The 52 aTTP patients were classified into two groups based on comparison of plasma levels of ADAMTS13:AC measured 14 days after initiation of PE: 20 aTTP patients are characterized as good ADAMTS13:AC responders (ADAMTS13:AC ≥10%) and 32 aTTP patients are characterized as poor ADAMTS13:AC responders (ADAMTS13:AC < 10%). Characteristics of these two aTTP patient groups are shown in Table 1. Most of the two patient groups received corticosteroids. Eighteen aTTP patients received rituximab, which was administered to poor ADAMTS13:AC responders more frequently than good ADAMTS13:AC responders ($p < 0.05$).

In good ADAMTS13:AC responders (Fig. 1, left), plasma levels of ADAMTS13:AC (mean ± SD) increased to 15.0 ± 10.9% on the third day of PE, with a slight dip on the 10th day of PE, and then did not decrease below 10% within 14 days after PE initiation. As for plasma ADAMTS13:INH titer in good ADAMTS13:AC responders, mean ± SD ADAMTS13:INH levels were 2.5 ± 1.5 BU/mL before PE, decreasing to 0.8 ± 0.4 BU/mL on the fourth PE day, and then increasing to almost pre-PE levels (2.7 ± 4.4 BU/mL) on the 10th PE day, followed by a consistent decrease to undetectable ADAMTS13:INH levels (<0.5 BU/mL) on the 14th PE day. PLT recovery began 1 to 2 days later and mean PLT count increased to 180 × 10^9/L on the 6th PE day, followed by a slight dip, and the mean PLT counts increased again to more than 150 × 10^9/L on the 14th PE day.

In poor ADAMTS13:AC responders (Fig. 1, right), plasma levels of ADAMTS13:AC never increased to more than 10% of normal as measured at the 14th day after PE initiation. Further, mean ± SD plasma ADAMTS13:INH titers were 5.9 ± 4.5 BU/mL before PE initiation, but decreased to 1.4 ± 0.7 BU/mL on the 4th PE day, and then increased to 14.8 ± 0.7 BU/mL on the 10th PE day. This is termed inhibitor boosting. Mean ADAMTS13:INH titers subsequently decreased to undetectable levels (<0.5 BU/mL) over a 4-week period after PE initiation. Among these aTTP patients, recovery of PLT counts was slow, and the PLT count was not more than 150 × 10^9/L in any of these patients by the 22nd PE day.

Sequential analysis of ADAMTS13:INH immunoglobulin class and IgG subclasses

A previous report[19] indicated clinical significance of the IgG4 subclass of ADAMTS13:INH (IgG) rather than others, and therefore we here sequentially measured the titers of immunoglobulins and IgG subclasses in six aTTP patients, including two good ADAMTS13:AC responders (Patients 1 and 3) and four poor ADAMTS13:AC responders (Patients 26, 36, 39, and 40; Fig. 2).

Both good ADAMTS13:AC responder patients had IgG antibodies (IgG1 in both patients, IgG2 and IgG4 classes in one patient) and one had IgA antibodies. Over time, IgG and IgA antibody titers correlated with ADAMTS13:INH titers. Further, all four patients in poor ADAMTS13:AC responders had IgG antibodies (IgG1 in four patients, IgG2 in two patients, and IgG4 in two patients) and one also had IgA antibodies, but none had IgM antibodies. Over time, IgG and IgA antibody titers correlated with ADAMTS13:INH titers. Thus, in both patient groups the ADAMTS13:INH titers were consistently correlated with the IgG1 titers, and inconsistently with the titers of other IgG subclasses.

Visualization of ADAMTS13:AG and its complex with ADAMTS13:INH (IgG) in plasma milieu during treatment

Plasma ADAMTS13:INH titers in aTTP patients correlated with titers of the IgG1 subclass. Further, despite severe deficiency of ADAMTS13:AC (<0.5% of normal) in all of our aTTP patients, these patients generally had low but variable levels of plasma ADAMTS13:AG (<0.1% to 84.8% of normal; Tables S1 and S2).

Most of the aTTP patients on admission had IEF patterns that differed from those of normal individuals (Fig.

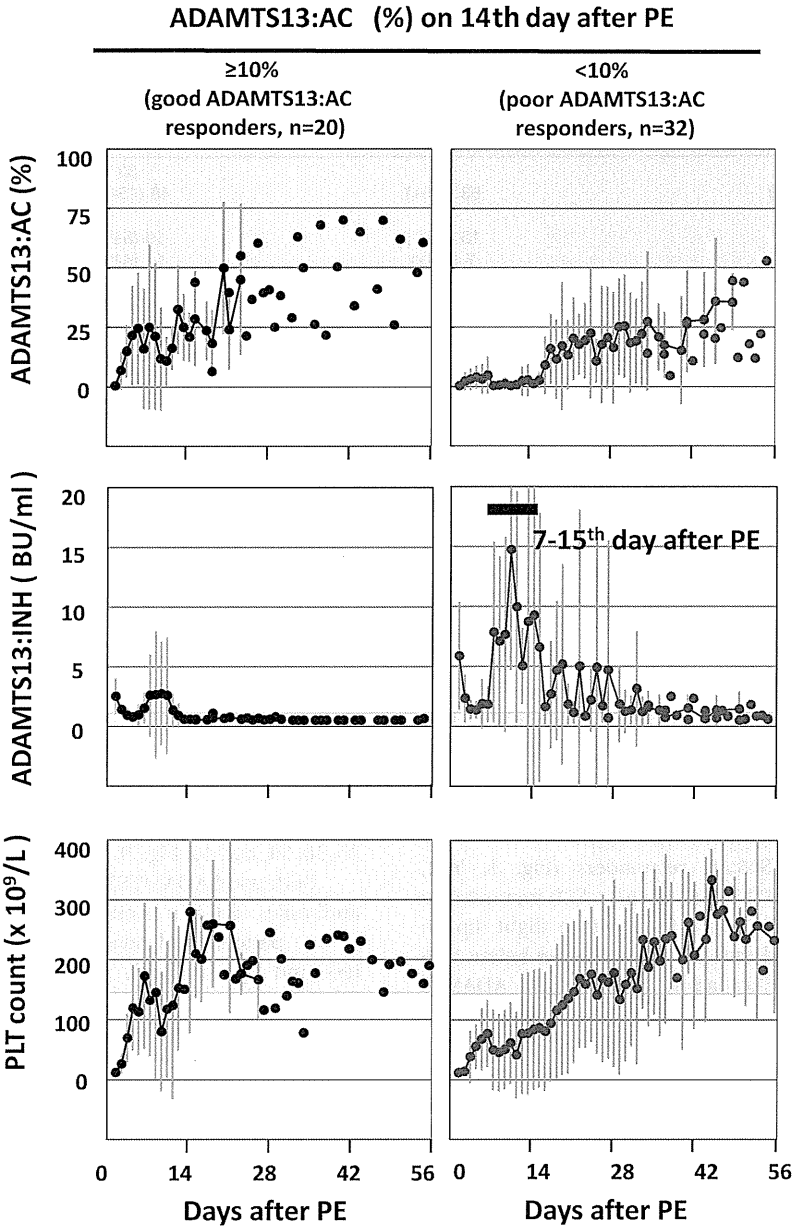

Fig. 1. Changes of plasma levels of ADAMTS13:AC and ADAMTS13:INH and PLT counts in 20 good ADAMTS13:AC responders (left) and 32 poor ADAMTS13:AC responders to PE (right). Shaded areas show normal ranges, and the vertical bars indicate values of mean ± SD. In good ADAMTS13:AC responders (left), plasma levels of ADAMTS13:AC increased to 15.0 ± 10.9% on the third PE day and then did not decrease below 10% within 14 days after PE initiation. Plasma ADAMTS13:INH titers decreased on the fourth PE day and then returned to almost pre-PE levels on 10th PE day, followed by a consistent decrease to the undetectable level on the 14th PE day. PLT counts increased to normal levels on the sixth PE day, followed by a slight dip, and then returned to normal on the 14th PE day. In poor ADAMTS13:AC responders (right), plasma levels of ADAMTS13:AC never increased to more than 10% for 14 days after PE initiation. Further, plasma ADAMTS13:INH titer decreased from 5.9 ± 4.5 BU/mL before PE initiation to 1.4 ± 0.7 BU/mL on the fourth PE day and then remarkably increased to 14.8 ± 0.7 BU/mL on the 10th PE day, termed inhibitor boosting, followed by a slow decrease to undetectable level more than 1 month after PE initiation. A recovery of PLT counts was very slow, and the count reached more than 150 × 10^9/L on the 22nd PE day.

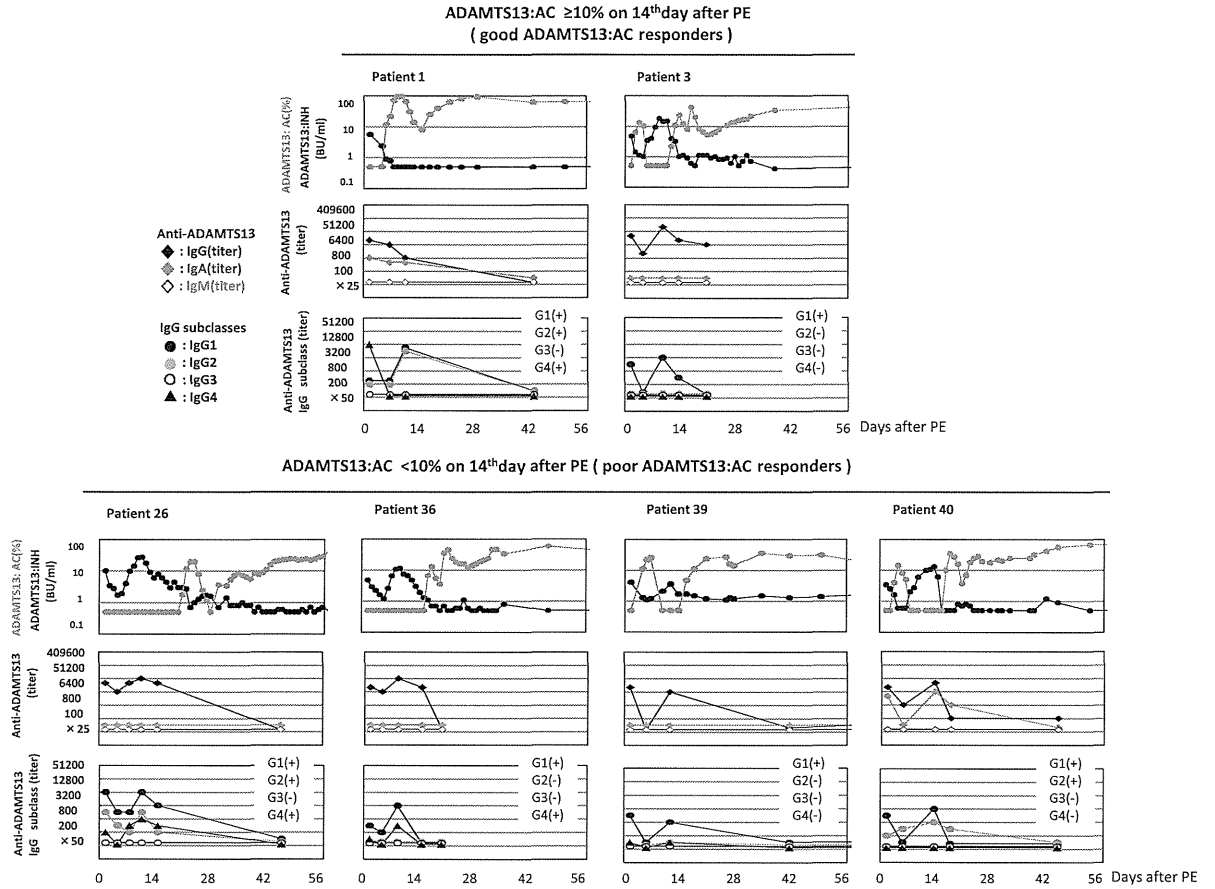

Fig. 2. Sequential analysis of ADAMTS13:INH immunoglobulin class and IgG subclasses. We sequentially analyzed the titers of immunoglobulins and IgG subclasses in six aTTP patients, including two good ADAMTS13:AC responders (Patients 1 and 3) and four poor ADAMTS13:AC responders (Patients 26, 36, 39, and 40). Both patients who were good ADAMTS13:AC responders had IgG antibodies (IgG1 in both patients, IgG2 and IgG4 classes in one patient) and one had IgA antibodies. All four patients who were poor ADAMTS13:AC responders had IgG antibodies (IgG1 in four patients, IgG2 in two patients, and IgG4 in two patients) and one had IgA antibodies. In both patient groups, the ADAMTS13:INH titers were consistently correlated with the IgG1 titers and inconsistently with the titers of other IgG subclasses.

S1, available as supporting information in the online version of this paper). Notably, among the 52 aTTP patients, three poor ADAMTS13:AC responders (Patients 22, 26, and 47) had undetectably low plasma level of ADAMTS13:AG (<0.1% of normal). We report laboratory information herein for Patient 26 (Fig. 3).

Before PE, Patient 26 had no visible band of ADAMTS13:AG on IEF gel, and 3 to 5 days after initiation of PE, a new ADAMTS13:AG band was detected at pI 6.0 to 7.3 (Band A). However, on Day 10, the ADAMTS13:AG band was not detected in accord with inhibitor boosting (Fig. 3B). During this period, plasma levels of ADAMTS13:AC were consistently below 0.5% of normal, suggesting that Band A was a complex of ADAMTS13:AG and ADAMTS13:INH (IgG). Band A was no longer detected after passing the patient's plasma through a protein G column to adsorb IgG (data not shown). After extensive treatment with PE, corticosteroid, and rituximab, ADAMTS13:AG patterns almost normalized on Day 100 after PE initiation, along with improvements of other aTTP-related clinical and laboratory findings. In fact, aTTP remission was achieved at this point. Furthermore, Band B with pI of more than 9.3 was identified as the degraded fragment of ADAMTS13:AG as shown by the presence of one ADAMTS13-related protein band (125 kDa) by two-dimensional analysis, using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions (Fig. 3C).

Although remission was initially achieved in Patient 26, the patient subsequently experienced a relapse 2 years

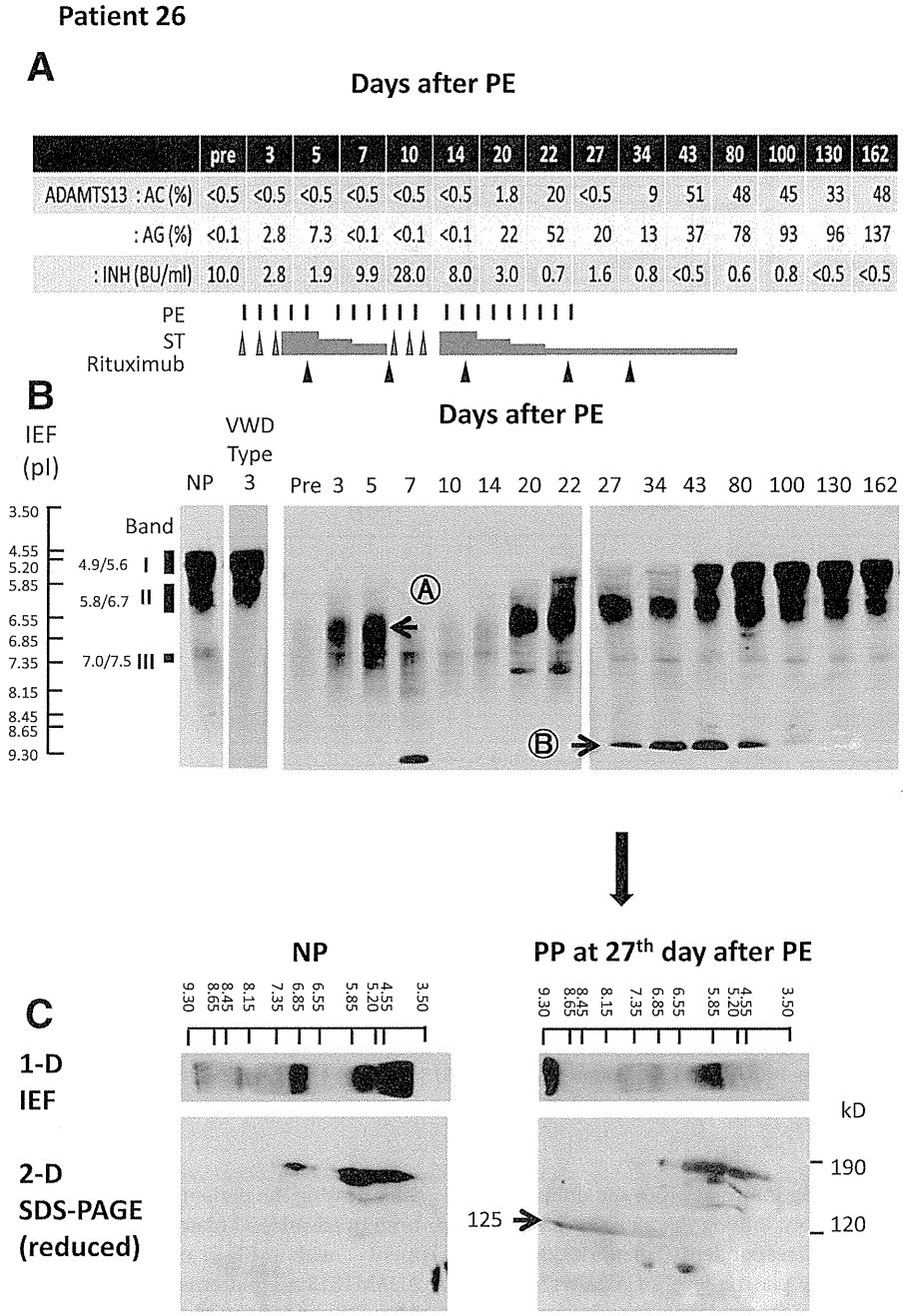

Fig. 3. Changes of ADAMTS13 variables and IEF analysis of plasma ADAMTS13:AG in patients with inhibitor boosting. Changes of ADAMTS13:AC, ADAMTS13:AG, and ADAMTS13:INH in addition to the therapy of patient with inhibitor boosting are shown in A. Before PE, this patient had no visible band of ADAMTS13:AG on IEF gel, and 3 to 5 days after initiation of PE, a new ADAMTS13 band at pI 6.0 to 7.3 (Band A) appeared, but on Day 10 it almost totally disappeared again in accord with inhibitor boosting as shown in B. After extensive treatment with PE, corticosteroid, and rituximab, ADAMTS13:AG patterns almost normalized on Day 100 after PE initiation. Band B with pI of more than 9.3 in B was identified as the degraded fragment of ADAMTS13:AG, shown by the presence of one ADAMTS13-related protein band (125 kDa) by two-dimensional analysis using SDS-PAGE under reducing conditions in C. VWD = von Willebrand disease; NP = normal plasma; PP = patient plasma.

TABLE 2. Comparison of laboratory markers between good ADAMTS13:AC responders and poor ADAMTS13:AC responders to PE therapy

	On admission			On 14th day after PE		
Marker	≥10% (good responders)	<10% (poor responders)	p value	≥10% (good responders)	<10% (poor responders)	p value
ADAMTS13						
:AC (%)	<0.5	<0.5		31.7 (12.0-68.0)	1.9 (<0.5-12.4)	<0.01
:AG (%)	12.3 (0.2-84.8)	7.9 (<0.1-37.9)	0.39	90.3 (36.0->100)	42.1 (0.1->100)	<0.01
:INH (BU/mL)	2.5 (1.0-5.6)	5.7 (1.1-20.0)	<0.01	0.54 (<0.5-0.9)	6.9 (0.7-51.0)	<0.01
Neurologic symptoms						
With	15 (15/20)	29 (29/32)		0 (0/20)	14 (14/32)	
Without	5 (5/20)	3 (3/32)		20 (20/20)	13 (13/32)	
PLTs ($\times 10^9$/L)	12.3 (3-36)	11.6 (3-54)	0.78	225.1 (70-772)	84.6 (3-330)	<0.01
LDH (IU/mL)	1696 (494-7768)	1262 (478-3126)	0.30	290 (165-891)	536 (161-1941)	<0.05
Hb (g/dL)	7.3 (4.2-14.3)	7.3 (4.9-10.1)	0.99	9.1 (7.0-11.9)	8.3 (5.1-12.6)	0.11
Blood urea nitrogen (mg/dL)	23.1 (9.5-58.7)	26.5 (8.8-122.0)	0.47	16.4 (3.7-32.0)	26.1 (9.3-83.0)	<0.05
Creatinine (mg/dL)	0.88 (0.31-1.73)	1.12 (0.39-7.70)	0.33	0.65 (0.20-0.97)	0.95 (0.43-4.30)	0.10

later, that was featured by thrombocytopenia and an ADAMTS:13 INH titer of 4.6 BU/mL. This relapse was successfully treated with PE and corticosteroids.

DISCUSSION

Using a large cohort of a TTP registry,[21] we conducted a retrospective analysis of 52 patients in Japan with aTTP. These patients were classified into two groups: 20 good ADAMTS13:AC responders and 32 poor ADAMTS13:AC responders, based on plasma levels of ADAMTS13:AC measured on the 14th day after PE initiation. aTTP patients who had ADAMTS13:AC levels less than 10% of normal on the 14th day after PE initiation were operationally considered to be "PE-refractory TTP." We also characterized the nature of ADAMTS13:INH antibodies and identified a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), using a large-pore IEF gel analysis.[13] In interpreting our findings, several factors should be considered.

First, measured pre-PE levels of ADAMTS13:INH appeared to have prognostic implications for measured ADAMTS13:AC levels after PE. As shown in Table 2, on admission, none of the clinical and laboratory markers except for the pre-PE levels of ADAMTS13:INH could predict good ADAMTS13:AC responders versus poor ADAMTS13:AC responders.

Second, Ferrari and coworkers[19] reported that IgG4 was the most prevalent IgG subclass in their sample of patients with aTTP (90%, 52/58), followed by IgG1 (52%), IgG2 (50%), and IgG3 (33%). They further showed that patients with high IgG4 and undetectable IgG1 levels were more prone to relapse than those with low IgG4 levels and detectable IgG1; they proposed that IgG4 could be a potentially useful indicator of patients at risk of relapse. In our serial study of two good ADAMTS13:AC responders (without inhibitor boosting) and four poor ADAMTS13:AC responders (with inhibitor boosting), ADAMTS13:INH titers were most strongly correlated with the IgG class, especially the IgG1 subclass. The difference in IgG subclass findings between the study by Ferrari and colleagues[19,22] from Europe and our study from Japan is unclear, but predisposing genetic factors might be involved.

Third, we identified a complex of ADAMTS13:AG and ADAMTS13:INH (IgG) in patient plasma of poor ADAMTS13:AC responders, using a large-pore IEF gel analysis. This finding has not been reported previously, because most of aTTP patients have a low but significant plasma level of ADAMTS13:AG (as shown in Tables S1 and S2). We reported three patients who were poor ADAMTS13:AC responders (Patients 22, 26, and 47) who had low levels of plasma ADAMTS13:AG (<0.1% of normal) before PE. Using the patient post-PE plasma level for Patient 26, we reported that Band A was a complex of ADAMTS13:AG and ADAMTS13:INH (IgG), and Band B was the fragment of ADAMTS13:AG, as evidenced by a two-dimensional IEF and SDS-PAGE analysis (Fig. 3). These results indicate that the ADAMTS13:AG-ADAMTS13:INH complex undergoes proteolytic modification, by which ADAMTS13:AG disappears from circulation.

We conclude that ADAMTS13:INH boosting is a frequent occurrence noted among aTTP patients, primarily occurring among aTTP patients who were poor ADAMTS13:AC responders. Our results also identified that high ADAMTS13:INH titers (approx. > 5 BU/mL) measured before PE might be a good indicator to predict poor ADAMTS13:AC responders, for whom earlier administration of intensive immunosuppressive therapy such as rituximab appeared to facilitate reduction of the time and volume of PE. Further, durable remission of aTTP in patients who had measurable levels of ADAMTS13:AC may be more consistently identified by a new laboratory marker consisting of normal ADAMTS13:AG IEF patterns in the plasma milieu, but this does not guarantee freedom from relapse in the long term.

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CONFLICT OF INTEREST

FS and BP are employees of Baxter Bioscience. The other authors have disclosed no conflicts of interest.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Isoelectric focusing (IEF) analysis of plasma ADAMTS13 antigen on admission in both patients of good ADAMTS13:AC responders and poor ADAMTS13:AC responders. ADAMTS13:AG exists in plasma