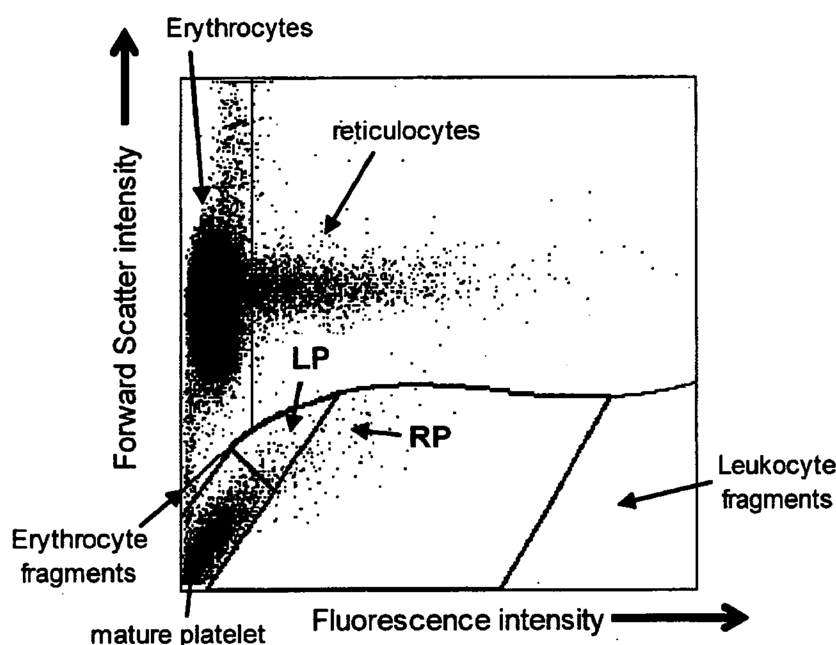
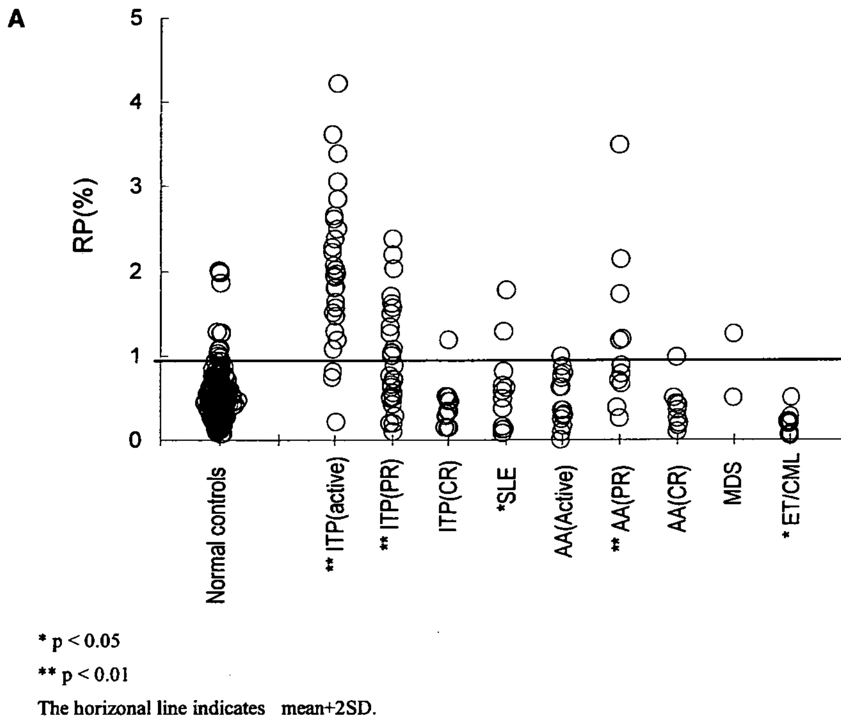


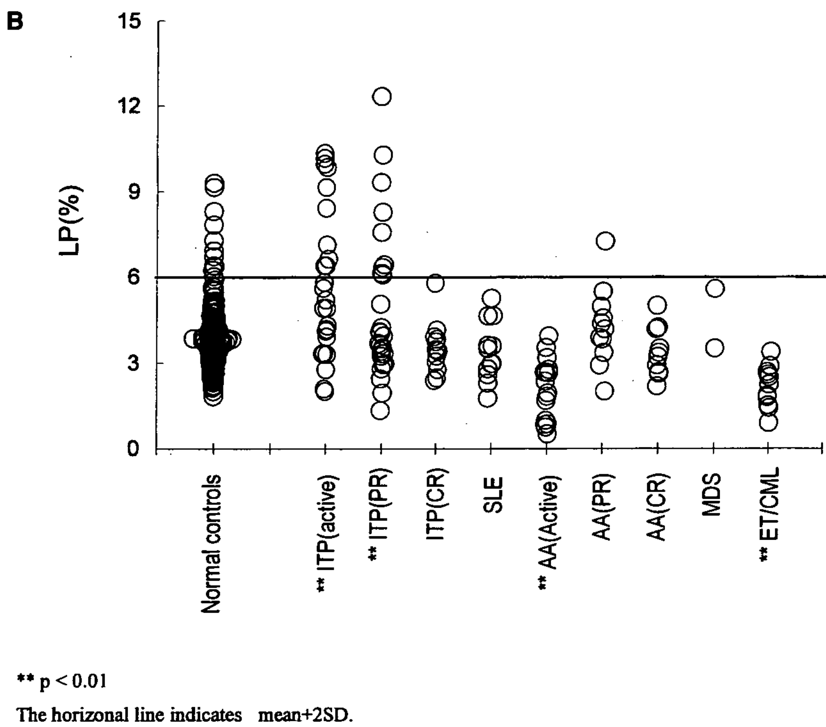
FIG. 1. Dot plot showing fluorescence intensity versus forward scatter intensity. Each line automatically discriminates RP, mature red blood cells, reticulocytes, mature platelets, and white blood cell fragments.

TABLE 1. RP, LP, and Platelet Counts in Healthy Volunteers and Patients with Thrombocytopenia

Subjects	N	RP (%) (mean ± 1SD)	Platelet Count ($\times 10^9$/L) (mean ± 1SD)	LP (%) (mean ± 1SD)
Normal controls	287	0.48 ± 0.29	247.5 ± 48.6	3.78 ± 1.14
ITP (all)	76	1.21 ± 0.94	119.0 ± 89.1	5.17 ± 3.44
Active phase	28	2.03 ± 0.88	45.5 ± 20.6	6.80 ± 4.42
PR phase	28	0.98 ± 0.61	116.8 ± 57.6	4.85 ± 2.60
CR phase	20	0.39 ± 0.25	225.1 ± 78.1	3.33 ± 0.89
AA (all)	37	0.66 ± 0.65	91.7 ± 60.0	3.03 ± 1.44
Active phase	17	0.46 ± 0.29	34.5 ± 13.6	2.04 ± 1.00
PR phase	11	1.21 ± 0.89	122.2 ± 35.2	4.24 ± 1.32
CR phase	9	0.38 ± 0.25	162.6 ± 24.4	3.42 ± 0.85
SLE	5	0.92 ± 0.56	298.4 ± 69.5	3.87 ± 0.92
MDS	2	0.88 ± 0.38	119.0 ± 8.0	4.54 ± 1.05
ET/CML	11	0.20 ± 0.12	740.8 ± 603.2	2.15 ± 0.70



* p < 0.05
** p < 0.01
The horizonal line indicates mean+2SD.



** p < 0.01
The horizonal line indicates mean+2SD.

FIG. 2. RP and LP in healthy volunteers and patients with thrombocytopenia or underlying diseases. ITP, idiopathic thrombocytopenic purpura; AA, aplastic anemia; SLE, systemic lupus erythematosus; **/*, significantly different (**$p < 0.01$, *$p < 0.05$). **A:** RP, **B:** LP.

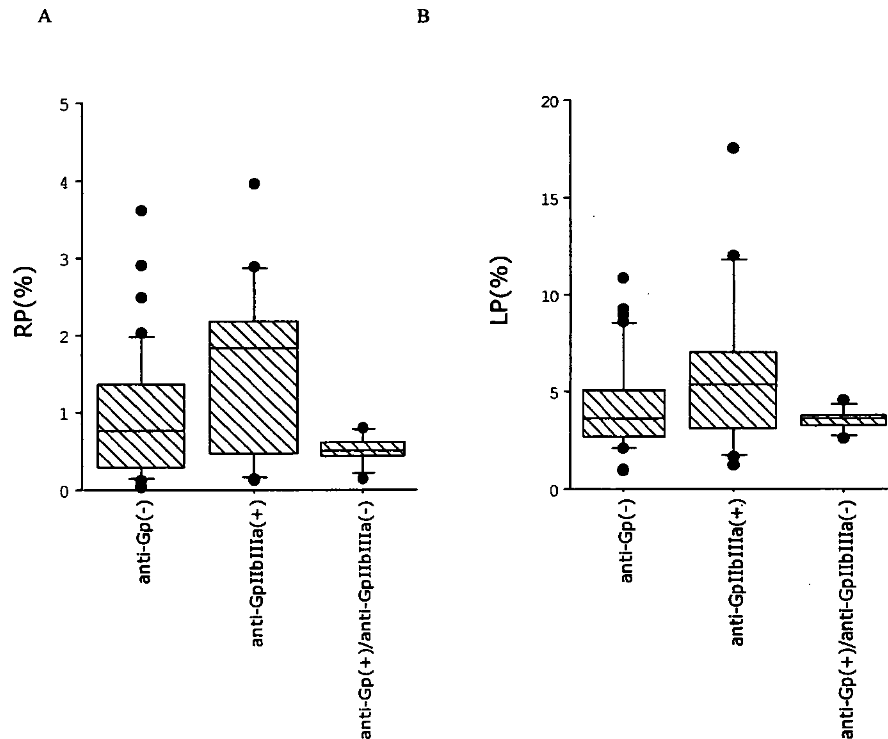


FIG. 3. RP and LP in patients with antibodies. anti-Gp(-): patients negative for any GP antibody (N=37); anti-GpIIbIIIa(+): patients positive for anti-GP IIb/IIIa antibody (N=17); anti-Gp(+)/GpIIbIIIa(-): patients positive for some anti-GP antibodies; exception with anti-GP IIb/IIIa antibody (N=7). **A:** RP, **B:** LP.

RESULTS

RP was significantly higher in patients with ITP (1.21 ± 0.94%, $p < 0.01$), especially in active phase (2.03 ± 0.88%, $p < 0.01$) and PR phase of AA (1.21 ± 0.89%, $p < 0.01$), than in healthy volunteers (0.48 ± 0.29%), the patients with ITP and AA in CR phase showed low RP rate; however, it was not significant (Table 1). RP was markedly lower in patients with ET or CML (0.20 ± 0.12%, $p < 0.05$) (Fig. 2A). LP was significantly higher in patients with ITP (5.17 ± 3.44%, $p < 0.01$) than in healthy volunteers (3.78 ± 1.14%), especially in active phase (6.80 ± 4.42%, $p < 0.01$), while LP was markedly lower in patients in the active phase of AA (2.04 ± 1.00%, $p < 0.01$) and with ET or CML (2.15 ± 0.70%, $p < 0.01$). On the other hand, RP was not significantly different among healthy volunteers and patients with ITP or AA in CR phase. There were no difference in LP rate between healthy volunteers and patients with SLE (Fig. 2B). As shown in Fig. 3, RP was significantly higher in the patients positive for anti-GpIIb/IIIa antibody (1.53 ± 1.10%) than in those negative for it ($p < 0.05$), and LP tended to be higher in the patients positive for it (5.89 ± 4.13%) (Fig. 3). RP and LP were poorly correlated with PAIgG ($R = 0.14$ and 0.12) (Fig. 4), and poorly correlated with plasma TPO level (Fig. 5). We next investigated the association with number of platelet, RP, and LP on the patients with ITP in active phase. RP and LP were negatively correlated with platelet count (R= 0.302 and 0.197) (Fig. 6).

The case report patient was 35 years old, female. She was diagnosed with ITP at 24 years of age. She has been treated with low-dose corticosteroid. She has medicated by intravenous immunogloblin for her delivery. The progression of platelet count and RP and LP before and after medication is shown in Fig. 7. She had low platelet counts (38×10^9/L), high RP (4.3%)

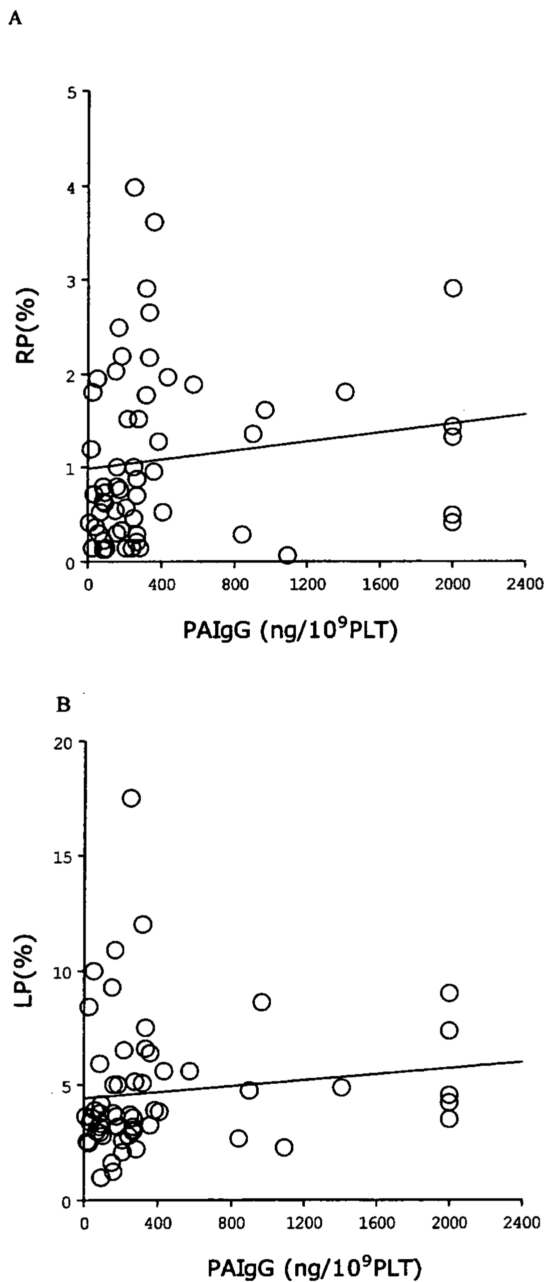


FIG. 4. Relationship between PaIgG and RP or LP in patients with ITP. **A:** RP, **B:** LP.

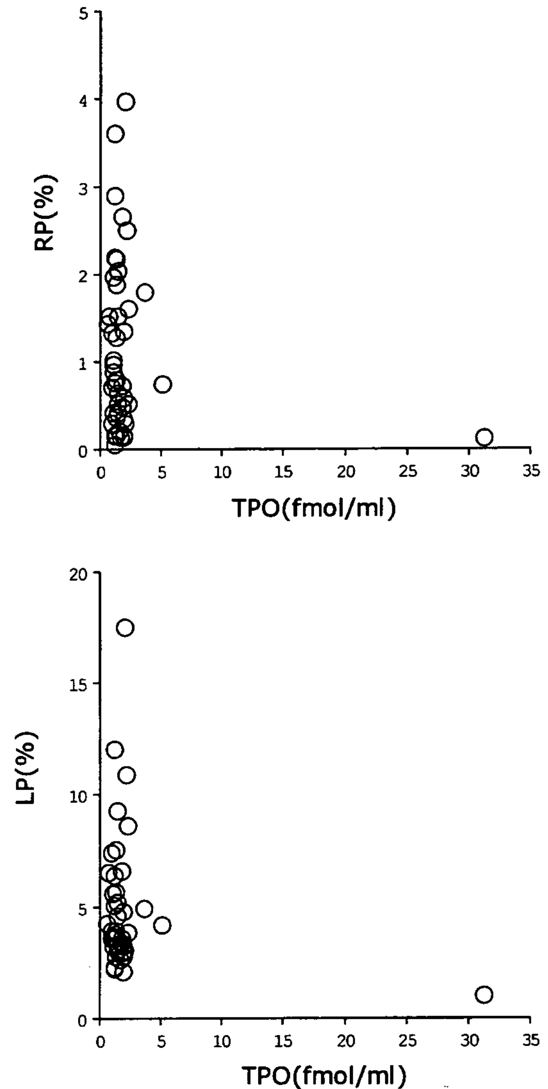


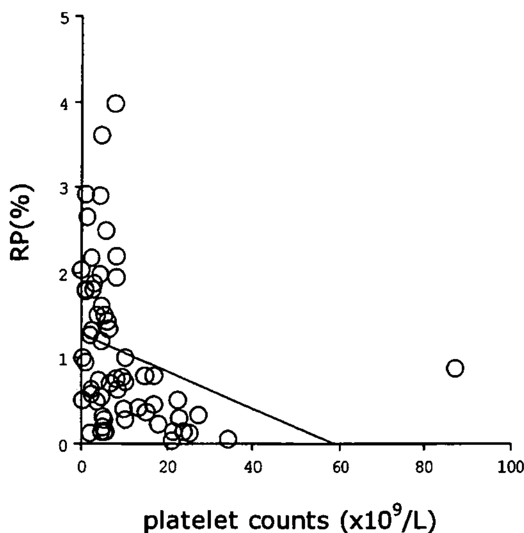
FIG. 5. Relationship between TPO and RP or LP in patients with ITP. **A:** RP, **B:** LP.

and LP (16.7%) before treatment with high-dose gamma globulin. She was given gamma-globulin 20 g per day for 5 days intravenously (day 0–day 5). After treatment, platelet count (134×10^9/L) increased, while RP (1.15%) and LP (5.56%) decreased. Platelet count (26×10^9/L) decreased again 40 days after treatment, while RP (2.36%) and LP (9.42%) increased again (Fig. 7).

DISCUSSION

Recently, RP measurement (7,16,17) has been developed to evaluate or diagnose the pathophysiology of thrombocytopenia, but is still not established. We found that RP in healthy volunteers was approximately 0.48%, with no significant differences between females and males or

A



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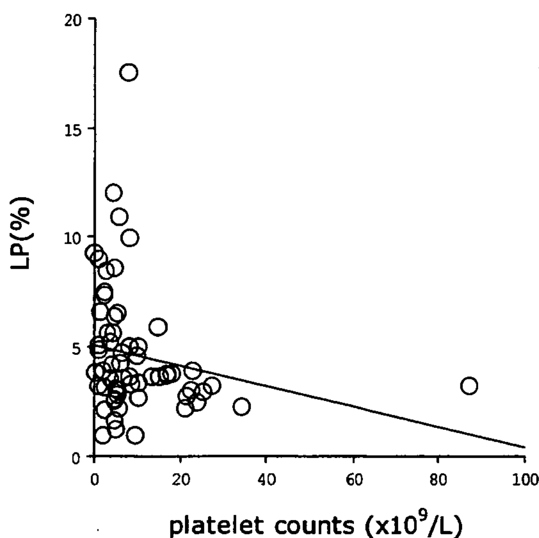

FIG. 6. Relationship between platelet counts and RP or LP in patients with ITP. **A:** RP, **B:** LP.

among various age groups. Platelet count and RP were negatively correlated in healthy volunteers and patients with thrombocytopenia, suggesting that RP may reflect platelet generation in normal bone marrow (16,17).

In our study, RP was significantly higher in ITP, especially in the active phase, and PR phase of AA, while RP was markedly lower in patients with ET or CML. These results suggest that RP may reflect clinical phase of ITP and that clearance of platelet is not enhanced in CML and ET. Although RP in AA was within normal range in active phase and CR, it was high in PR phase of AA. Increase in RP may reflect recovery of platelet production in bone marrow in patients in PR phase of AA. RP are reported to be younger

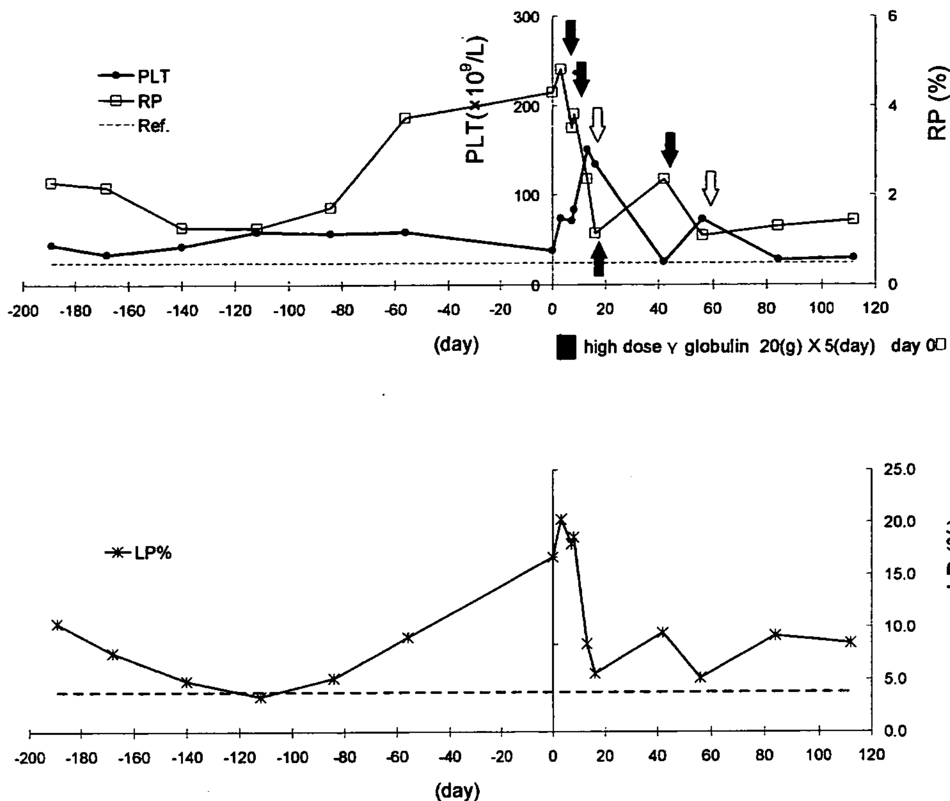

FIG. 7. ITP case.

platelets that have been newly released into the circulation (19). High levels of RP are more valuable but low levels of RP are less valuable. LP measurement (11,16) has also been developed to evaluate thrombocytopenia. In this study, LP was significantly higher in ITP, especially in active phase, while LP was markedly lower in active phase of AA and ET or CML. LP measurement is useful for the diagnosis of thrombocytopenia, especially AA. Both measurements of RP and LP may be more sensitive for diagnosis for thrombocytopenia.

In ITP, RP and LP were significantly higher in patients positive for anti-GpIIb/IIIa antibody, suggesting that this antibody may be essential for the onset of ITP. As shown in several reports (20,21) that immunodominant epitopes on GpIIb-IIIa are recognized by autoreactive T cells in ITP, anti-GpIIb/IIIa antibodies would be most important in ITP. RP and LP were poorly correlated with PAIgG in our ITP cases. Although PAIgG (15,22) was as an anti-platelet antibody developed to diagnose ITP, its sensitivity and specificity were low. RP and LP were in this study poorly correlated with plasma TPO level, although it was reported that TPO level was negatively correlated with platelet counts and megakaryocytes in bone marrow (12–14,23). Comparison between TPO and RP or LP in our study was performed for only patient with ITP. It is reported as another reason that autoantibodies against either platelet GPIb or GPIIb/IIIa in ITP plasma are not only involved in platelet destruction, but may also contribute to inhibition of platelet production (24). In the clinical course, RP in our cases reflected platelet count in ITP. Recombinant TPO has been reported to increase RP before increase in platelet count in patients with ITP (25).

ACKNOWLEDGMENTS

This study was supported in part by research grants from the Japanese Ministry of Health, Labor and Welfare and from the Japanese Ministry of Education, Science, Sports and Culture.

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Usefulness of Fully Automated Measurement of Reticulated Platelets Using Whole Blood

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Summary: Reticulated platelets (RP) were measured with an automated hematology analyzer (modified R-2000) in 287 healthy volunteers and in 212 patients with thrombocytopenia. In healthy volunteers, the RP was 0.48 ± 0.26% in men and 0.48 ± 0.32% in women. No significant difference in the RP values due to gender or age (21–60 years) was observed. Furthermore, the reverse correlation was observed between platelet counts and RP. The RP was high in patients with idiopathic thrombocytopenic purpura (ITP), those with high fibrinogen and fibrin degradation products (FDP), and those with high C-reactive protein (CRP), but low in patients after chemotherapy. The RP was highest in active phase of ITP, and relatively high in the partial remission phase of aplastic anemia. In patients after chemotherapy, the patients had a minimum phase of RP and then a maximum phase of RP before platelet counts increased. RP was significantly high in the maximum phase and significantly low in the minimum phase. The relationships between platelet count and RP were negatively correlated in patients with ITP, high FDP, or high CRP, but were not correlated in patients with aplastic anemia, liver disease, or after chemotherapy. These results show that RP reflects the pathology of thrombocytopenic disorders and the measurement of RP is useful for the differential diagnoses and analysis of platelet kinetics.
Key Words: Reticulated platelet—Chemotherapy—ITP—Thrombocytopenia—Aplastic anemia.

Platelets were identified to have a coarse, punctuated reticulum (reticulated platelets; RP) by using a new methylene blue dye in 1969 (1). It was suggested that those cells might contain increased amounts of cytoplasmic RNA, reflecting thrombopoiesis in bone marrow. Subsequently, it was reported that platelet nucleic acid could be detected with flow cytometry utilizing thiazole orange as dye (2). RNA-rich platelets were reported to be readily measured by flow cytometry and to provide information on thrombopoietic activity in thrombocytopenic patients (3). Analysis of RP was indicated to provide a good indication of the rate of platelet production in bone marrow (4). In the diagnosis of immune thrombocytopenia, flow cytometric analysis of RP was reported to be better than platelet-associated IgG (5). Furthermore, a significant difference in reticulated platelet count between normal men and women had been shown using erythrocyte thiazole orange fluorescence in whole blood as an internal standard (6).

Idiopathic thrombocytopenic purpura (ITP) is caused by an immunologic mechanism of antiplatelet antibodies (7). In this state, platelet production is normal or increased but platelets are hyper-destroyed in spleen. On the other hand, patients with aplastic anemia have low platelet, leukocyte, and erythrocyte counts in peripheral blood and hypoplastic bone marrow. The flow cytometric analysis of RP is suggested to be a useful method for estimating thrombocytopenia. Recently, the instruments for fully automated measurement of RP (modified R2000 fully automated reticulocyte counter), capable of measuring the RP in the peripheral whole blood immediately, was developed.

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In this study, we measured the RP in healthy volunteers, thrombocytopenic patients, and in patients with underlying diseases due to thrombocytopenia to examine the usefulness of the RP count for different diagnosis of thrombocytopenia.

MATERIALS AND METHODS

RPs were examined in 287 healthy volunteers (144 female and 143 male; median age, 32 years old; range, 21–60 years old). RP was also measured in 212 patients with thrombocytopenia and underlying diseases of thrombocytopenia: 58 ITP, 23 aplastic anemia (AA), 7 systemic lupus erythematosus (SLE), 49 liver diseases, 8 leukemic patients after chemotherapy, 22 patients with high FDP (> 10 μg/mL), and 45 patients with high CRP (more than 4 mg/dL). The study protocol was approved by the Human Ethics Review Committees of Mie University School of Medicine. ITP was diagnosed by thrombocytopenia, normal bone marrow with more than normal count of megakaryocytes and no other disease due to thrombocytopenia; active phase, less than 50,000/μL of platelet counts; partial remission (PR), 50,000–120,000/μL of platelet counts with or without treatment; complete remission (CR), more than 120,000/μL of platelet counts without treatment. Aplastic anemia was diagnosed by thrombocytopenia, anemia, and leukocytopenia without other disease due to pancytopenia; active phase, less than 50,000/μL of platelet counts; partial remission (PR), 50,000–100,000/μL of platelet counts with or without treatment; complete remission (CR), more than 100,000/μL of platelet counts without treatment.

RP was measured by a previously described method (8). Briefly, approximately 2 mL of whole blood was collected from healthy volunteers or patients and was anticoagulated with ethylenediamine tetraacetic acid (EDTA)-2K. All blood samples were kept at room temperature until analysis. Counts of RP are reported to be stable during 2–6 hours after collection (9). Total platelet counts and frequency of RP were determined by the modified R2000 (Sysmex Inc., Kobe, Japan) with special software (9–11). For measurements of RP, 10 μL of blood was aspirated by the modified R2000 and automatically mixed with 40 μL of of 2.6% auramine O in 95.9% ethylene glycol solution (Ret-Search dye, Sysmex Inc.) to stain the RNA present in reticulocytes and RP. The samples were then irradiated by an argon laser beam at the wavelength of 488 nm. The resulting fluorescence intensity and forward light scatter were measured with flow cytometry (Fig. 1) (9). The frequency of RP was expressed as the percentages of total platelet

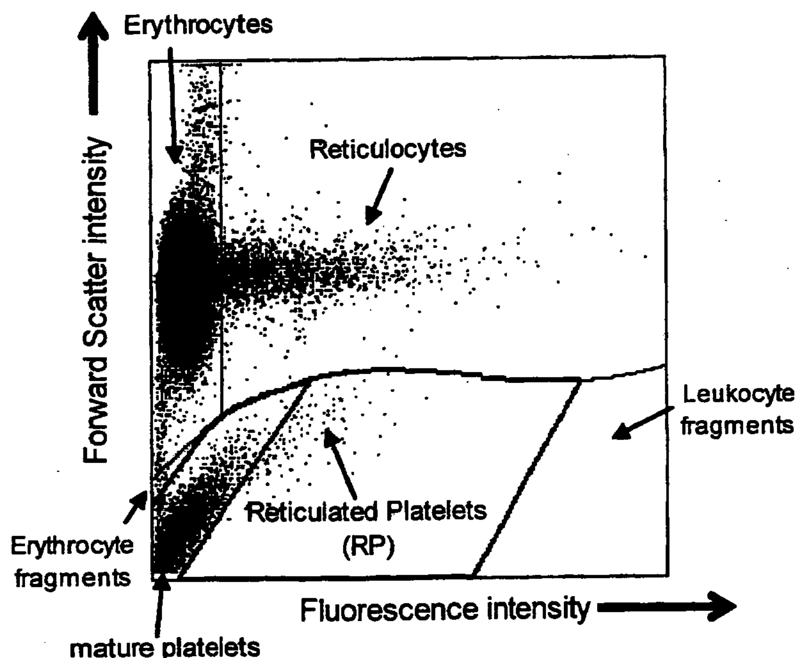

FIG. 1. Dot plot showing fluorescence intensity versus forward scatter intensity. Each line discriminates automatically. RP, mature red blood cell, reticulocyte, mature platelets, white blood cell fragments.

count. The time required to complete all these measurements was approximately 90 seconds. C-reactive protein (CRP) was measured using N-assay TIA CRP-S (Nittobo Company, Tokyo, Japan). Fibrin and fibrinogen degradation products (FDP) were measured using LPIA-FDP (Dia-Iatron Company, Tokyo, Japan). Prothrombin time was measured using a Thromborel S (Dade Behring, Tokyo, Japan).

Statistical Analysis

Results are expressed as mean ± SD. Differences between groups were evaluated by one-way ANOVA and the Bonferroni test. For assessment of correlations, coefficients of correlation were determined by Pearson's correlation coefficient and linear regression analysis. In both cases, a p value less than< 0.05 was considered significant.

RESULTS

Mean value of platelet count was 251.5 ± 50.0 × 10^9/L in female and 243.6 ± 46.8 × 10^9/L in male. Mean value of RP was 0.48 ± 0.32% in female and 0.48 ± 0.26% in male, and median of RP was 0.40 (range, 0.07%–2.01%) in female and 0.44 (range, 0.09%–2.00%) in male, respectively; there was no difference of RP between female and male (Fig. 2). There was no significant difference in the RP values due to age differences (Fig. 3). The relationship between platelet count and RP was negatively correlated in healthy volunteers ($r= 0.348$, $p < 0.01$) (Fig. 4). RP was significantly higher in patients with ITP ($p < 0.01$), those with high CRP ($p < 0.01$), those with high FDP ($p < 0.01$), those with SLE ($p < 0.05$) and those with liver disease ($p < 0.05$) than in healthy volunteers (Fig. 5). In ITP, RP was highest in active phase and that in CR was similar to normal controls (Table 1). In aplastic anemia, RP was within normal range at CR or active phase, but was significantly increased at PR phase ($p < 0.01$). RP was high in PR phase of aplastic anemia and in those with SLE. In patients after chemotherapy, there were two phases: minimum and maximum of RP. After chemotherapy, the patients had firstly minimum phase of RP and then had maximum phase of RP before increase of platelet counts. RP was significantly higher in the maximum phase ($p < 0.01$) and significantly lower in minimum phase ($p < 0.05$) than in healthy volunteers. The relationship between platelet count and RP was negatively correlated in patients with thrombocytopenia and healthy volunteers ($r = 0.404$, $p < 0.01$) (Fig. 6). The relationship between platelet count and RP was negatively correlated in patients with ITP ($r = 0.489$, $p < 0.01$) (Fig. 7A) but were

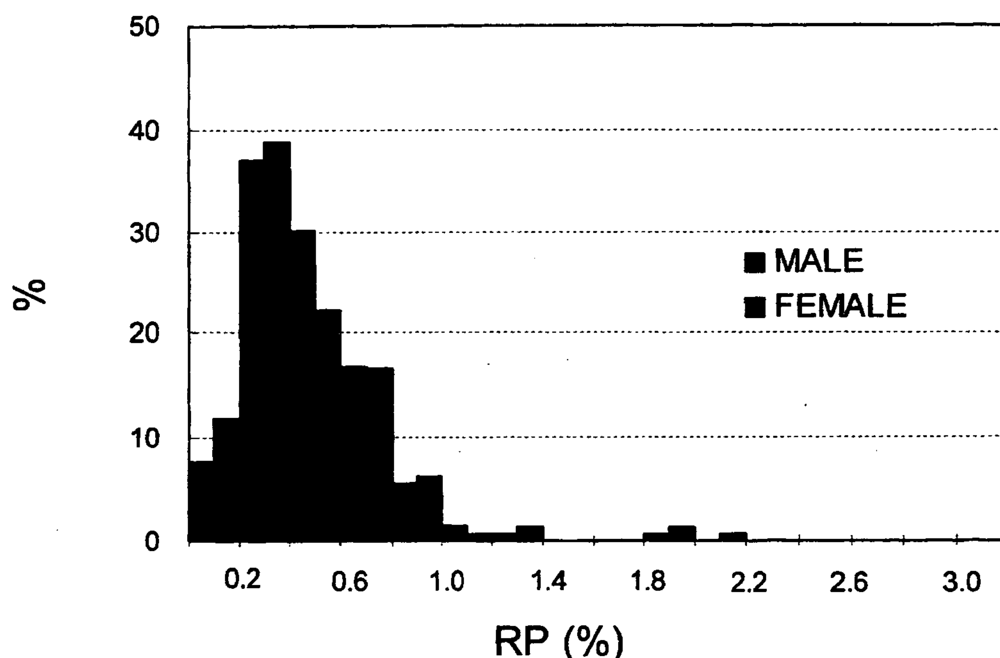

FIG. 2. Histogram of RP in healthy volunteers. Gray bar, female; black bar, male.

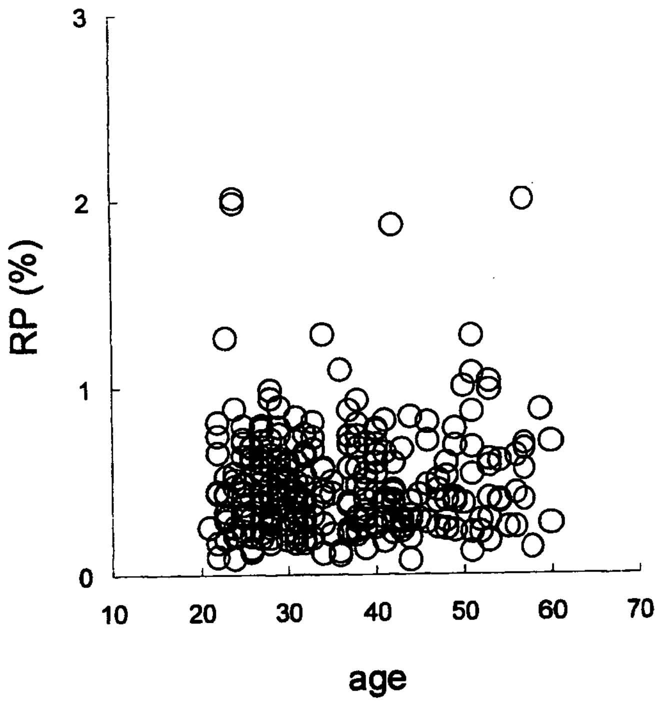

FIG. 3. Relationship between RP and age. There was no significant difference of RP among ages (from 21 to 60 years old).

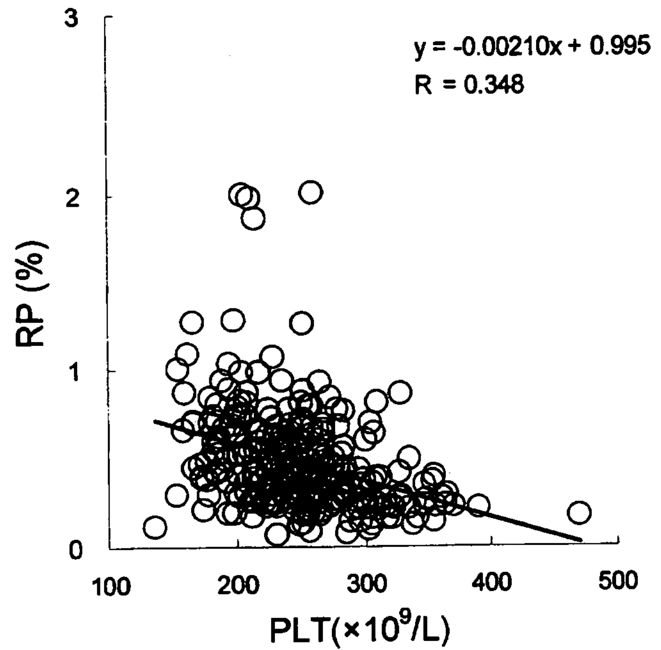

FIG. 4. Relationship between platelet count and RP in healthy volunteers.

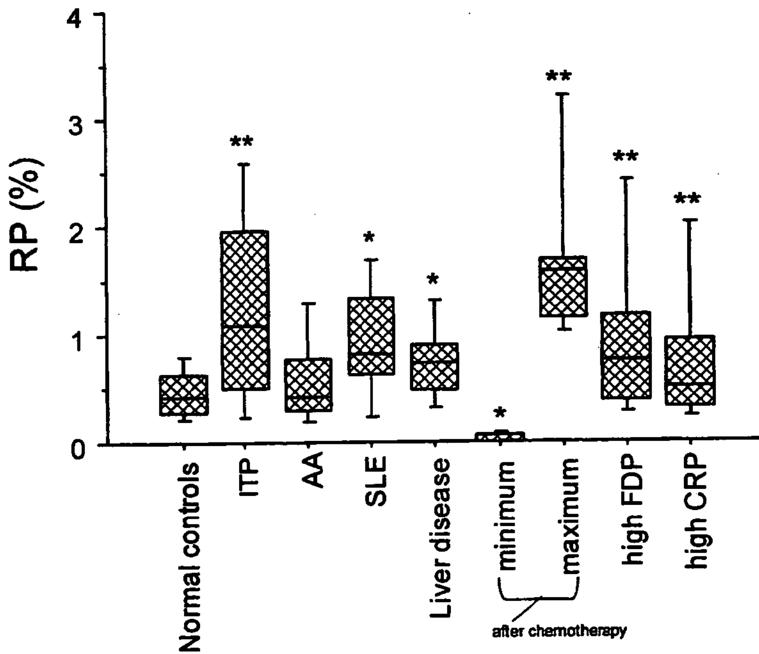

FIG. 5. RP in healthy volunteers and the patients with thrombocytopenia or the underlying diseases. ITP, idiopathic thrombocytopenic purpura; AA, aplastic anemia; SLE, systemic lupus erythematosus; **/*, significantly different (**$p < 0.01$, *$p < 0.05$). The box plots show the standard elements. The lowest boundary of the box indicate the 25th percentiles and the highest boundary of the box indicate the 75th percentiles. The upper whiskers indicate the 90th percentiles and the lowest whiskers indicate 10th percentiles. The solid line in the box represents the median value.

TABLE 1. Reticulated Platelet in Healthy Volunteers and Patients with Thrombocytopenia or the Underlying Diseases

Subjects	Number	RP (%)	Platelet Count (×10⁹/L)
Healthy volunteers	287	0.48 ± 0.29	247.5±48.6
ITP (all)	58	1.28 ±0.94*	113.3±91.5
Active phase	26	1.99±0.85*	48.7±25.3
PR phase	19	0.89±0.56†	111.7±42.6
CR phase	13	0.41±0.26	245.0±87.8
Aplastic anemia (all)	23	0.69±0.71	102.8±53.8
Active phase	9	0.51±0.20	42.1±16.9
PR phase	7	1.36±0.94*	135.4±32.0
CR phase	7	0.25±0.10	148.1±15.7
SLE	7	0.94±0.51†	250.7±96.6
Liver disease	49	0.77±0.37†	76.2±24.6
After chemotherapy	8		
Minimum‡	8	0.02±0.04†	38.1±11.4
Maximum§	8	1.60±0.80*	61.4±34.1
High FDP**	22	1.18±1.63*	202.3±143.1
High CRP††	45	0.84±0.92*	232.6±122.4

‡ Minimum phase of RP after chemotherapy.
§ Maximum phase of RP after chemotherapy.
** A group of the patients with high FDP (> 10 μg/mL).
†† A group of the patients with high CRP (> 4 mg/dL).
*$p < 0.01$; †$p < 0.05$.

tend to be positively correlated in patients with aplastic anemia (Fig. 7B). The relationship between platelet count and RP was negatively correlated in patients with high FDP ($r = 0.425$, $p < 0.05$) and those with high CRP ($r = 0.402$, $p < 0.01$) but not in patients with liver diseases ($r = 0.196$) and in patients after chemotherapy (Fig. 8). However, RP was not significantly correlated with CRP, FDP, or PT (Table 2).

DISCUSSION

Thrombocytopenia is a common hematologic abnormality that is caused by hypoproduction, hyperdestruction, and sequestration of platelets. It is difficult to distinguish between hypoplastic and hyperdestructive forms of thrombocytopenia. The presence of normal or increased numbers of megakaryocytes in the bone marrow essentially excludes the diagnosis of hypoplastic thrombocytopenia due to aplastic anemia or amegakaryocytic thrombocytopenia. To date, no simple diagnostic test exists to diagnose hyperdestructive thrombocytopenia such as ITP and thrombotic thrombocytopenic purpura (TTP).

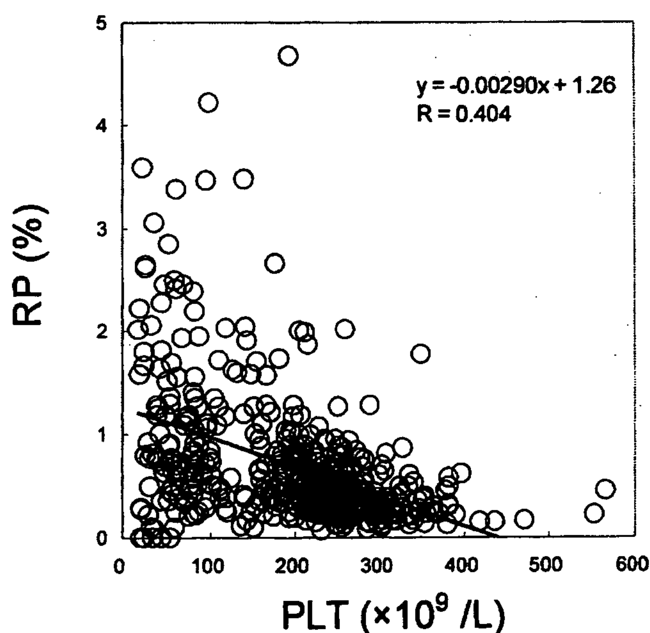

FIG. 6. Relationship between RP and platelet counts in healthy volunteers and patients with thrombocytopenia.

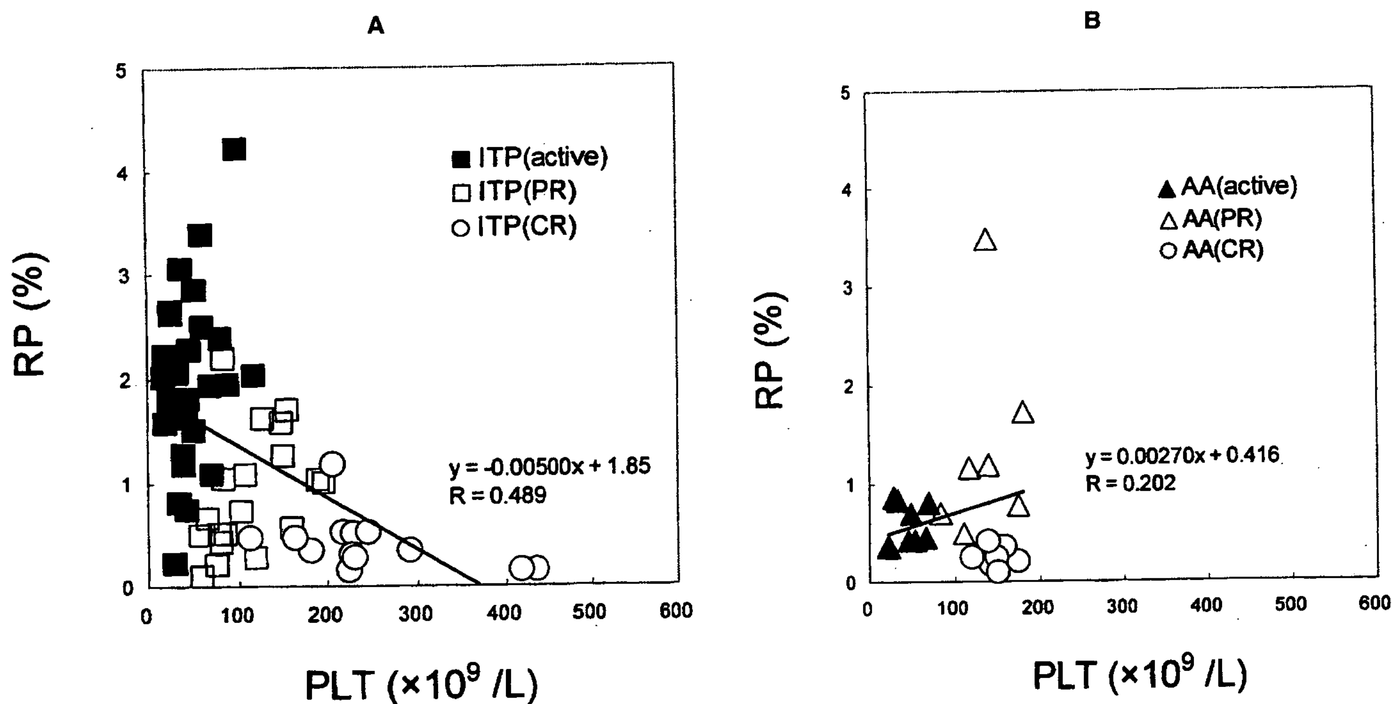

FIG. 7. Relationship between RP and platelet count in patients with ITP (A) and aplastic anemia (B). (A) active phase; ■, PR phase; □ □ CR; ○ (B) active phase; ▲, PR phase; □ □ CR; ○.

TABLE 2. Relationship Between RP and CRP, FDP or PT

	CRP	FDP	PT
RP	R = 0.294	R = 0.0447	R = 0.200

Although platelet-associated immunoglobulin G (PAIgG) (5,12) as anti-platelet antibodies was developed to diagnose ITP, the sensitivity or specificity was low. Recently RP (1,8,9) and thrombopoietin (TPO) (13,14) have been developed to evaluate for thrombocytopenia. RP are reported to be younger platelets that have been newly released into circulation (15).

We found that, in healthy volunteers, RP was approximately 0.48%, with no significant difference between female and male, and among various ages, which was different from the previous report (6). The relationships between platelet count and RP were negatively correlated in healthy volunteers, suggesting that RP may reflect the platelet generation in normal bone marrow.

In various diseases, RP was significantly high in patients with ITP, in those with high CRP, and in those with high FDP. ITP is an autoimmune disease characterized by increased platelet clearance caused by anti-platelet auto-antibodies (16). The patients with ITP usually have increased megakaryocytes in the bone marrow, and are considered to have high RP (10,17) and normal TPO levels (14,18). In ITP, RP was highest in active phase and reduced similar to normal controls in CR, suggesting that RP may reflect to clinical phase of ITP. Although RP was within normal range in patients with active phase or CR of aplastic anemia, RP was high in PR phase. The increase in RP may reflect to the recovery of platelet production in bone marrow in patients with aplastic anemia. RP was also increased in patients with SLE, those with high FDP, and those with high CRP. SLE also has an autoimmune mechanism for thrombocytopenia such as ITP. In those with high FDP and high CRP, aggregation of platelets may be caused by thrombin or an inflammatory reaction and platelet production may be enhanced. In patients after chemotherapy, the patients had a minimum phase of RP and then had a maximum phase of RP before platelet counts increased. RP was sig-

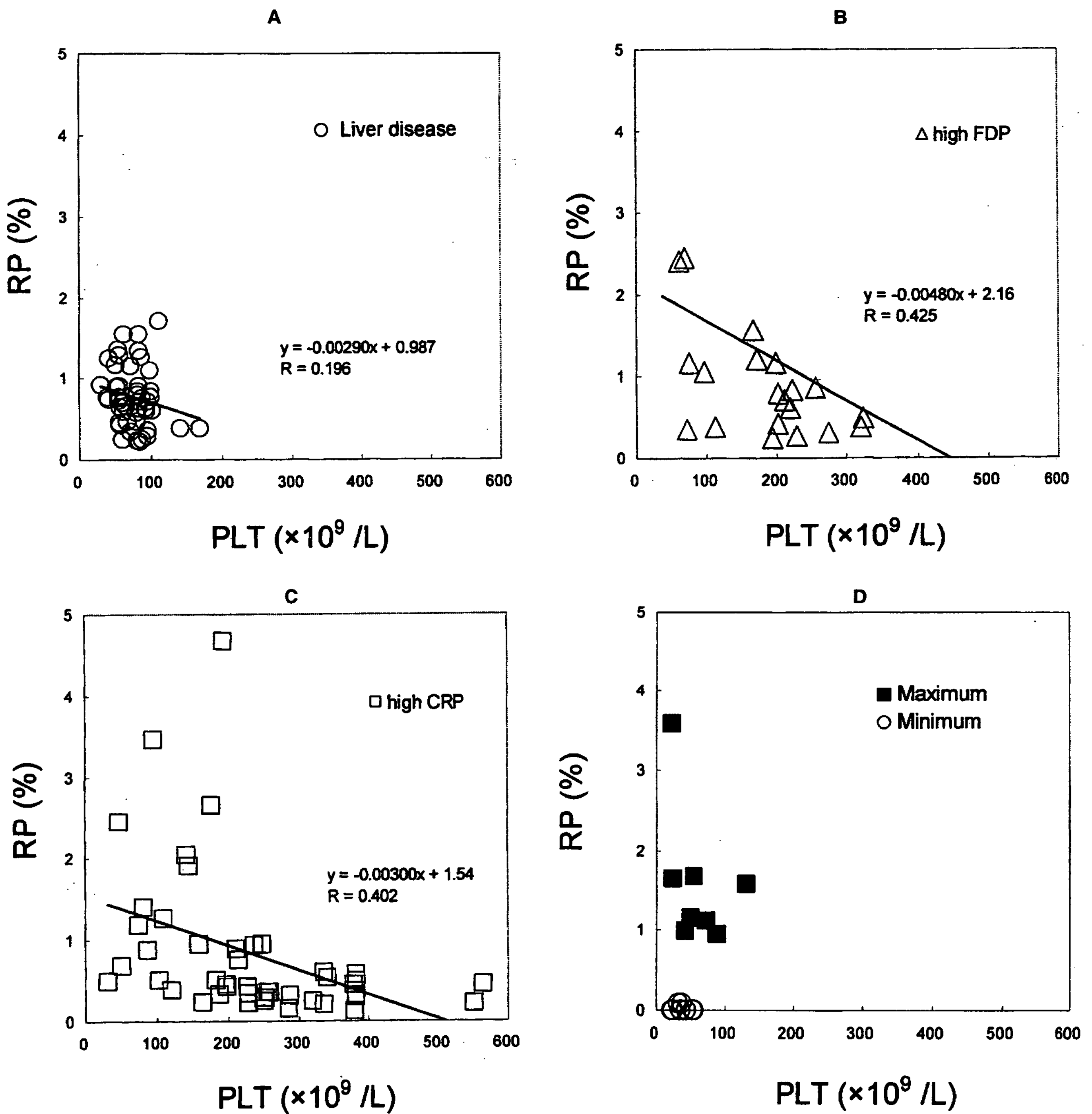

FIG. 8. Relationship between RP and platelet count in patients with liver diseases (**A**) and patients with high FDP (**B**) and patients with high CRP (**C**) and patients after chemotherapy (**D**).

nificantly low in minimum phase and significantly high in maximum phase, suggesting that RP may reflect platelet production in bone marrow after chemotherapy. Measurement of RP may be useful for determining the necessity and/or timing of platelet transfusion in patients with thrombocytopenia after chemotherapy. RPs were negatively correlated in patients with ITP,

in patients with high FDP, and in those with high CRP, but were not correlated in patients with aplastic anemia and those after chemotherapy. These results suggest that RP is negatively correlated with platelet count in hyperdestructive thrombocytopenia but not correlated with that in amegakaryocytic thrombocytopenia.

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Severe secondary deficiency of von Willebrand factor–cleaving protease (ADAMTS13) in patients with sepsis-induced disseminated intravascular coagulation: its correlation with development of renal failure

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Deficiency of ADAMTS13 is found in patients with thrombotic thrombocytopenic purpura (TTP), and the genetic defects in the *ADAMTS13* gene or the autoantibody against ADAMTS13 is thought to be responsible for the development of TTP. The clinical correlation and mechanisms of secondary ADAMTS13 deficiency in other disease states were investigated. In addition to TTP, ADAMTS13 levels were severely decreased in patients with sepsis-induced disseminated intravascular coagulation (DIC). The incidence of acute renal failure and serum creatinine levels in patients with ADAMTS13 activity levels lower than 20% (incidence, 41.2%; creatinine, 160 ± 150 μM [1.81 ± 1.70 mg/dL]) ($P < .05$) were significantly higher than they were in patients with ADAMTS13 activity levels higher than 20% (incidence, 15.4%; creatinine, 84 ± 67 μM [0.95 ± 0.76 mg/dL]) ($P < .01$). Additionally, unusually large von Willebrand factor multimers were detected in 26 (51.0%) of 51 patients with ADAMTS13 activity levels lower than 20%. Lower molecular weight forms of ADAMTS13 were found in the plasma of patients with sepsis-induced DIC, suggesting that the deficiency of ADAMTS13 was partially caused by its cleavage by proteases in addition to decreased synthesis in the liver. These data suggested that severe secondary ADAMTS13 deficiency can be associated with sepsis-induced DIC and may contribute to the development of renal failure. (Blood. 2006;107:528-534)

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Introduction

Deficiency of the von Willebrand factor (VWF)–cleaving protease,[1-5] ADAMTS13 (a disintegrin-like metalloprotease with thrombospondin type 1 repeats) is found in most patients with thrombotic thrombocytopenic purpura (TTP), and this deficiency is thought to be responsible for platelet aggregation and microthrombi formation in the circulation, which in turn cause typical thrombotic microangiopathies (TMAs) to develop.[6-9] Deficiency of ADAMTS13 in patients with TTP is caused by genetic defects in the *ADAMTS13* gene (familial TTP, Upshaw-Schulman syndrome) or by autoantibodies against ADAMTS13. Although hemolytic uremic syndrome (HUS) is clinically similar to TTP, the role of ADAMTS13 deficiency in the development of HUS is controversial because reports conflict about whether ADAMTS13 activity remains unchanged[6-8] or decreases.[10-13] It also is possible that secondary deficiency of ADAMTS13 may account for the development of microthrombi formation in disease states other than TTP. To search for the clinical correlation of secondary ADAMTS13 deficiency in disease states, we measured ADAMTS13 activity levels by the standard method[14] and determined antigen levels by our newly developed monoclonal antibody–based enzyme-linked immunosorbent assay (ELISA) for ADAMTS13 in patients with TTP and in patients with sepsis-induced disseminated intravascular coagulation (DIC). We found that severe secondary ADAMTS13 deficiency could occur in patients with sepsis-induced DIC and that it had a clinical correlation with the development of renal failure.

Patients, materials, and methods

Blood samples

All samples were obtained with informed consent from patients according to the Declaration of Helsinki. Blood was drawn from 113 patients (65 men, aged 17-83; 44 women, aged 21-81; idiopathic TTP, 3 patients; Upshaw-Schulman syndrome, 1 patient; sepsis-induced DIC, 109 patients). The diagnosis of TTP was made with note of the presence of typical clinical features (fever, bleeding tendency, neurologic symptoms) laboratory examination results (thrombocytopenia, hemolytic anemia with red blood cell fragmentation, increased levels of LDH, increased levels of serum creatinine), and effectiveness of plasma exchange treatment. Patients with definite infection, such as bacteremia, pneumonia, urinary tract infection, biliary tract infection, or pathogenic *Escherichia coli* O-157 infection, were excluded from the TTP group. The patient with Upshaw-Schulman syndrome had TTP, and plasma transfusion was effective in preventing recurrence.

The diagnosis of DIC was made according to the criteria established in 1988 by the Japanese Ministry of Health and Welfare. Criteria for DIC were

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reported previously.[15] Briefly, the presence of underlying disease—such as infection and malignancies—specific clinical conditions (bleeding symptoms, organ dysfunction), and results of laboratory examinations (platelet counts, prothrombin time, fibrinogen, fibrin degradation products) were quantified based on score. If the score was 7 or more, the diagnosis of DIC was made. In patients with hematologic malignancy, scores on the bleeding symptom and platelet counts were excluded, and the diagnosis of DIC was made if the total score was 4 or more.

The diagnosis of sepsis was made according to the guidelines of the Society of Critical Care Medicine Consensus Conference Committee.[16] Briefly, patients had to meet at least 3 of the 4 criteria for systemic inflammatory response and had to have a known infection or a suspected infection, as evidenced by one or more of the following: bacteremia, pathologic microorganisms or white blood cells in a normally sterile body fluid such as urine or joint fluid; purulent sputum; radiographic evidence of pneumonia; clinical signs associated with high risk for infection (eg, cholangitis, peritonitis) or increased levels of endotoxin, β-D-glucan, or *Candida* antigen.

Thirty-nine patients with DIC were shown to have bacteremia, as evidenced by their blood cultures. Twelve patients, whose bacteremia was not evidenced by blood culture, had increased levels of endotoxin, β-D-glucan, or *Candida* antigen. Twenty-eight patients who were negative for bacteria in blood culture or who did not have increased levels of endotoxin, β-D-glucan, or *Candida* antigen, had pneumonia as evidenced by radiography, 11 patients had urinary tract infection, 4 patients had wound infection during postoperative periods, 1 patient had biliary tract infection, 1 patient had bacterial arthritis, 1 patient had bacterial osteomyelitis, and 12 patients had suspected respiratory infection with the presence of pathogenic microorganisms, such as methicillin-resistant *Staphylococcus aureus* in sputum cultures.

Citrated platelet-poor plasma samples were prepared and stored at −80°C until use. Blood was also drawn from 12 healthy volunteers (7 men, aged 25-53; 5 women, aged 25-48) for the preparation of normal pooled plasma. Laboratory analyses of patients' blood were performed by the standard methods using automated analyzers. Complete blood cell counts, serum creatinine (normal range, 35-97 μM [0.4-1.1 mg/dL]), serum bilirubin (normal range, 3-21 μM [0.2-1.2 mg/dL]), aspartate aminotransferase (AST; normal range, 8-35 IU/L), alanine aminotransferase (ALT; normal range, 5-40 IU/L), serum albumin (normal range, 39-51 g/L [3.9-5.1 g/dL]), and C-reactive protein (CRP; normal range, less than 5 mg/L [0.5 mg/dL]) were measured in this study.

Determination of ADAMTS13 antigen and activity levels

The human *ADAMTS13* cDNA used in this study was described previously.[5] Human ADAMTS13 was expressed in human embryo kidney 293 cells stably transfected with pCAG-ADAMTS13 Neo and was purified. Murine monoclonal antibodies (mAbs) to human ADAMTS13 were generated by the standard method[17] after immunization of BALB/c mice with recombinant human ADAMTS13. Two mAbs, WH10 and WH2-22-1A, were selected for ELISA, which was shown to bind to the third TSP-1 motif and to the disintegrin domain of ADAMTS13 by the binding study to recombinant ADAMTS13 mutants, respectively.[5,14,18] WH10 (2 μg/mL) was used for microtiter plate coating (Maxi Sorp plate; Nalge Nunc International, Rochester, NY). After blocking with 1% casein, plasma samples from healthy subjects and patients were diluted in phosphate-buffered saline, pH 7.2/0.1% casein, and then incubated in WH10-coated plates. ADAMTS13 bound to the microtiter plates was detected by peroxidase-conjugated WH2-22-1A. Purified recombinant ADAMTS13 was used as the standard to determine ADAMTS13 antigen levels in normal plasma. The ADAMTS13 level in each patient's plasma was expressed as the percentage of that in normal pooled plasma. ADAMTS13 activity levels in plasma were measured according to the previously described method.[14] Briefly, 10 μL plasma was mixed with purified VWF (1 μg) in 100 μL reaction buffer (5 mM Tris [pH 8.0]/1.5 M urea/10 mM $BaCl_2$/0.4 mM Pefabloc SC [Roche Diagnostics, Mannheim, Germany]) at 37 °C for 24 hours. Reaction was terminated by the addition of 10 μL of 500 mM EDTA, pH 8.0.[14] Portions of samples were subjected to 1.4% sodium dodecyl sulfate–agarose gel electrophoresis to determine the extent of VWF degradation. After electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes, and VWF multimers were detected by peroxidase-labeled rabbit anti–human VWF antibodies (Dako, Glostrup, Denmark).[12-14]

Quantification of molecular markers of DIC

Plasma levels of fibrin degradation products (FDPs) were quantified with commercial kits (Roche Diagnostics, Tokyo, Japan) used for laboratory examinations. Given that the quantification of free thrombin concentration in plasma is technically difficult, we used ELISA (Sysmex, Kobe, Japan) to quantify plasma levels of thrombin/antithrombin III (TAT) complexes. Similarly, plasma levels of plasmin/α2 plasmin inhibitor complexes (PICs) were measured using ELISA with commercial kits (Sysmex) used for laboratory examinations. Plasma plasminogen activator inhibitor 1 (PAI-1) levels were quantified by the latex photometric immunoassay by using a commercial kit (Mitsubishi Kagaku Iatron, Tokyo, Japan), as described previously.[19] The granulocyte elastase digests of cross-linked fibrin (granulocyte elastase–dependent fibrin degradation products [E-XDPs]) were measured by the automated latex photometric immunoassay using IF-123 monoclonal antibody, which is specific for the fibrin fragment D species generated by granulocyte-elastase digestion.[20] Monoclonal antibody IF-123–bound latex particles (Mitsubishi Kagaku Iatron) were used for the assay. A 2.4-μL aliquot of sample plasma was mixed with 32 μL latex reagents in 250 μL Tris-buffered saline, and then absorbance changes were analyzed with an automated analyzer for latex photometric immunoassay (model LPIA-NV7; Mitsubishi Kagaku Iatron). The standard E-XDP was purified according to the method of Kohno et al.[20] The normal range of plasma E-XDP levels is less than 3 U/mL.

Effect of granulocyte elastase on ADAMTS13

Recombinant ADAMTS13 (250 nM) was incubated in 20 μL Tris-buffered saline, pH 7.4, in the absence or presence of granulocyte elastase (Elastin Products, Owensville, MO) at 5 nM and 50 nM. Aliquots (5 μL each) were harvested after incubation at 37°C for 5, 15, and 30 minutes. The reaction of each aliquot was terminated by addition of the sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer containing 2% SDS. Samples were then analyzed by SDS-PAGE followed by Western blotting with anti-ADAMTS13 monoclonal antibody WH2-22-1A and peroxidase-labeled anti–mouse IgG.

Detection of ADAMTS13 molecular forms in plasma

Western blot analysis of ADAMTS13 in plasma by mAb WH2-22-1A was performed after immunoprecipitation with anti-ADAMTS13 polyclonal antibody immobilized to protein G-Sepharose.

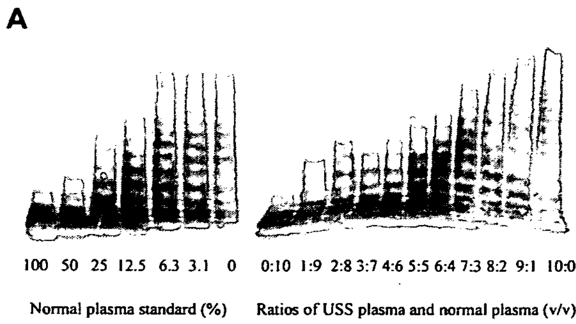
Analysis of VWF multimers in patient plasma

VWF multimers in patient plasma were analyzed by SDS-agarose gel electrophoresis according to the method described previously.[12-14]

Results

ELISA for ADAMTS13

We generated mAbs against recombinant human ADAMTS13 and used them to develop an mAb-based ADAMTS13 ELISA. To determine the specificity of this assay, plasma obtained from a patient with Upshaw-Schulman syndrome was mixed with normal plasma at various ratios, and the ADAMTS13 activity and antigen levels were measured. As shown in Figure 1, ADAMTS13 activity and ADAMTS13 antigen levels in the plasma of the patient with Upshaw-Schulman syndrome were less than 1%, and the ADAMTS13 antigen level in the patient plasma increased linearly in parallel with the ADAMTS13 activity in the presence of increasing amounts of normal plasma. The correlation coefficient



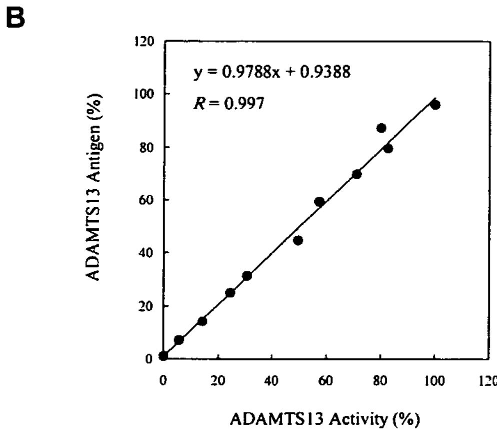


Figure 1. Analysis of ADAMTS13 activity and antigen levels in plasma of patients with Upshaw-Schulman syndrome. ADAMTS13 activity and antigen levels were determined in the plasma of a patient with Upshaw-Schulman syndrome (USS) mixed with normal pooled plasma at various ratios. (A) Result of ADAMTS13 activities in the plasma of the USS patient mixed with normal plasma at various ratios (0:10-10:0). (B) Correlation of ADAMTS13 activity and antigen levels in these samples.

between ADAMTS13 antigen and ADAMTS13 activity was 0.997. The ADAMTS13 level in normal pooled plasma was 1.57 μg/mL when recombinant human ADAMTS13 was used as the standard. The calibration curve was linear ($r = 0.999$), and the ELISA could distinguish absorbance changes of ADAMTS13 at 0.3% of the normal plasma level from ADAMTS13-depleted plasma. Interassay variability in samples containing 50% and 100% of ADAMTS13 were 7.9% and 5.2%, respectively.

ADAMTS13 levels in disease states

ADAMTS13 antigen and activity levels in the plasma of patients with sepsis-induced DIC or TTP were studied (Figure 2). The

Table 1. Correlation between the ADAMTS13 levels and molecular markers of DIC in patients with sepsis-induced DIC

	ADAMTS13*		
	Activity	Antigen	Activity-antigen ratio
Fibrinogen	−0.347	−0.244	−0.219
FDP	0.354	0.242	0.274
TAT	0.246	0.379	0.367
PIC	0.370	0.357	0.327
PAI-1	−0.230	−0.300	−0.006
Platelet	0.260	0.245	0.239
E-XDP	−0.399†	−0.404†	−0.229

n = 109 patients.
*Values are *rs* determined by Spearman rank correlation test.
†Statistically significant ($P < .01$).

correlation coefficient of ADAMTS13 antigen and ADAMTS13 activity was 0.80. As shown in Figure 2A, discrepancies between ADAMTS13 antigen levels and activity levels were observed in many samples. These discrepancies mainly were caused by the decreased level of specific ADAMTS13 activity compared with the ADAMTS13 antigen level. Some samples had higher specific activity of ADAMTS13. To explore the possibility that decreased levels of the ADAMTS13-specific activity correlated with disease states, Western blot analysis of ADAMTS13 molecular forms in patient plasma was performed. Low molecular–weight ADAMTS13 species were observed in DIC patient plasma by Western blotting (Figure 2B), indicating that proteolytic cleavage of ADAMTS13 could occur in this disease state. The recent report showed that ADAMTS13 could be digested in vitro by proteases such as thrombin and plasmin.[21] Because thrombin and plasmin can be generated in DICs, we tested the correlation between ADAMTS13 levels and molecular markers of coagulation and fibrinolysis. There was no correlation of ADAMTS13 activity, antigen, or specific activity level with levels of fibrinogen, FDP, TAT, PIC, PAI-1, or platelet counts (Table 1). We could only find a negative correlation between activity levels and antigen levels of ADAMTS13 and plasma levels of granulocyte elastase digests of fibrin (E-XDP) (Table 1; Figure 3A-B). Based on these results, we studied the effects of granulocyte elastase on ADAMTS13 in vitro. In accordance with previous reports, recombinant ADAMTS13 was determined to migrate at approximately 190 kDa by SDS-PAGE, followed by Western blotting.[14,21] As shown in Figure 3C, recombinant ADAMTS13 migrating at approximately 190 kDa was converted to the 120-kDa and 100-kDA fragments and finally to the approximately 40-kDa fragment on incubation with granulocyte

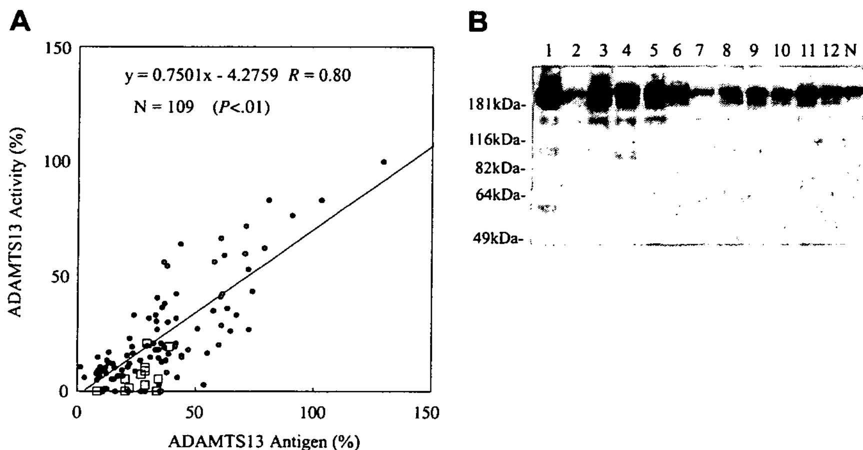


Figure 2. Analysis of ADAMTS13 activity, antigen, and molecular forms in plasma of patients with sepsis-induced DIC. (A) ADAMTS13 activity and antigen levels in the plasma of patients with sepsis-induced DIC were determined as described in "Patients, materials and methods." Samples (□) were subjected to immunoprecipitation followed by Western blotting to investigate the cleavage of ADAMTS13, as described in "Patients, materials, and methods." (B) Typical Western blot of degraded ADAMTS13 found in the patients' plasma indicated in panel A (□) is shown. Western blotting of ADAMTS13 antigen in normal pooled plasma (N) is shown as the control. ADAMTS13 molecules in normal plasma migrated at approximately 190 kDa.

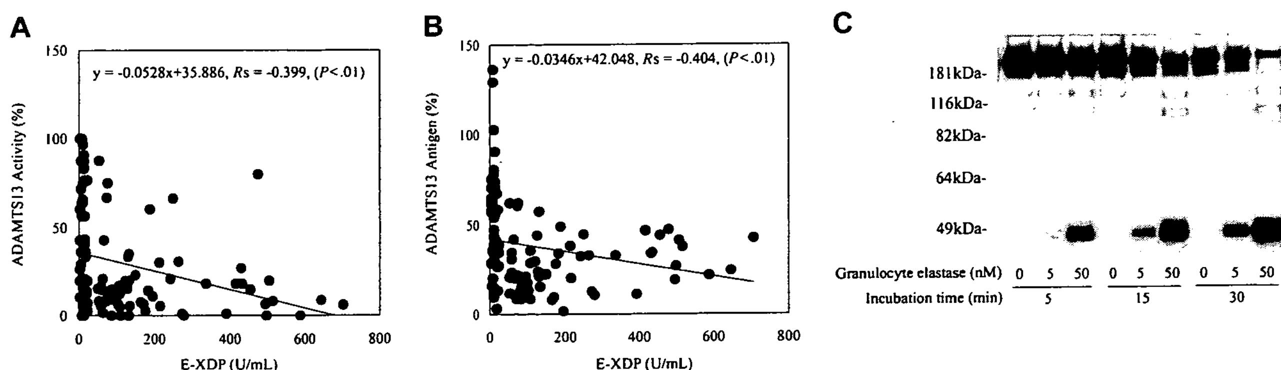


Figure 3. Correlation between the ADAMTS13 levels and the granulocyte elastase digests of cross-linked fibrin (E-XDP) levels in plasma of patients with sepsis-induced DIC and the effect of granulocyte elastase on ADAMTS13 in vitro. Correlations between the activity levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (A) and between the antigen levels of ADAMTS13 and the plasma levels of granulocyte-elastase digests of fibrin (E-XDP) (B) in patients with sepsis-induced DIC are shown. Values were analyzed by Spearman correlation coefficient by rank test. Recombinant ADAMTS13 was incubated with granulocyte elastase at 5 nM or 50 nM, and degradation of ADAMTS13 by granulocyte elastase was studied after the indicated time and analyzed as described in "Patients, materials, and methods" (C).

elastase in a dose-dependent and a time-dependent manner in vitro. A variety of lower molecular–weight ADAMTS13 fragments were detected in DIC patient plasma by Western blot (Figure 2B). According to the previous report, the ADAMTS13 fragments migrating approximately 150 to 170 kDa could be generated by thrombin.[21] ADAMTS13 fragments migrating approximately 120 kDa and 100 kDa in patient plasma might correspond to the granulocyte elastase digests of ADAMTS13. However, the 120-kDa ADAMTS13 fragment and the 100-kDa ADAMTS13 fragment could be generated by thrombin and plasmin, respectively.[21] It also is possible that thrombin-cleaved ADAMTS13 or plasmin-cleaved ADAMTS13 could be digested by granulocyte elastase or vice versa. These data may suggest that granulocyte elastase, together with other proteases (thrombin and plasmin plays a role in ADAMTS13 cleavage under certain pathologic conditions), may partially account for the decrease of the ADAMTS13-specific activity observed in DIC patients.

ADAMTS13 deficiency in disease states

ADAMTS13 antigen and activity levels in patient groups and in healthy subjects are shown in Figure 4. The plasma ADAMTS13 antigen and activity levels in untreated patients with TTP (no plasma exchange treatment, no fresh frozen plasma transfusion) were 13.5% ± 7.1% (range, 5.1%-19.6%) and 6.3% ± 5.7% (range, 0%-12.5%), respectively (idiopathic TTP 3, Upshaw-Schulman syndrome 1). Decreased levels of ADAMTS13 antigen and activity were observed in patients with sepsis-induced DIC compared with healthy subjects ($P < .01$) in this study, and severe decreases of ADAMTS13 activity and antigen levels were observed in patients with sepsis-induced DIC. Of the 109 patients with sepsis-induced DIC, decreases in ADAMTS13 activity levels (less than 5%) were found in 17 (15.6%) patients; clinical features and laboratory data of these patients are summarized in Table 2. Consciousness disturbance, thrombocytopenia, decreased hemoglobin levels, and increased LDH levels were commonly found in these patients. Clinical features were indistinguishable from those of patients with TTP, though patients with sepsis-induced DIC had evidence of the infection. Given that the highest ADAMTS13 activity level in patients with TTP without plasma exchange or blood transfusion was 12.5%, patients with sepsis-induced DIC were divided into 2 groups. One included patients with decreased ADAMTS13 activity levels (less than 20%; n = 51), and the other included patients with ADAMTS13 activity levels greater than 20% (n = 52). Patients with chronic renal failure before infection were excluded from this analysis. Patients were in severe condition; 25 (49.0%) of 51 patients in the former group and 35 (67.3%) of 52 patients of the latter group received transfusions of fresh frozen plasma, platelet concentrates, or both within 5 days of the determination of ADAMTS13 levels. This might have affected the activity and antigen levels of ADAMTS13.

Correlation between secondary ADAMTS13 deficiency and organ failure

Analyses of clinical and laboratory data showed that the patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) had elevated serum creatinine levels (Figure 5) that were

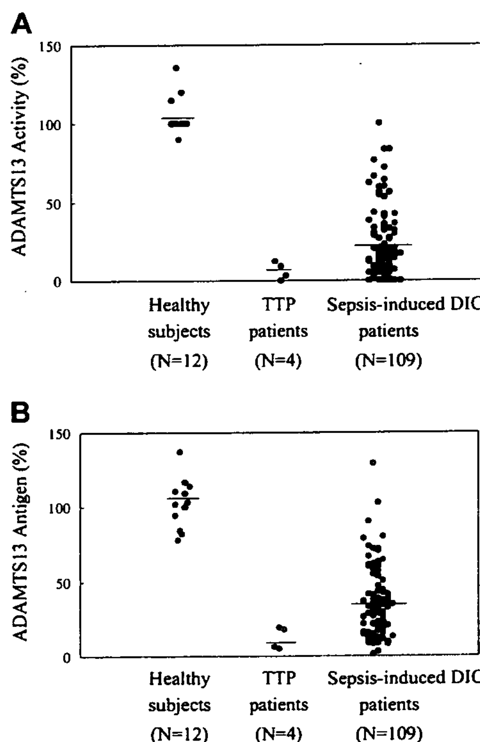


Figure 4. Plasma ADAMTS13 levels in patients and healthy subjects. ADAMTS13 activity levels (A) and antigen levels (B) of healthy subjects, patients with TTP (idiopathic TTP, 3; Upshaw-Schulman syndrome, 1) before plasma exchange treatment, and patients with sepsis-induced DIC (n = 109) are shown. Differences in the mean values (horizontal lines) between the healthy subject group and patient groups were statistically significant (nonrepeated measures ANOVA and Dunnett test; $P < .01$).

Table 2. Clinical profiles of patients with sepsis-induced DIC whose ADAMTS13 activity levels were lower than 5%

Characteristic	Value
Age, y	56.9 ± 21.3
Consciousness disturbance, no. (%)	8 (47.1)
Blood transfusion, no. (%)	11 (64.7)
ADAMTS13 antigen, %	25.5 ± 13.6
Creatinine, mg/dL	1.88 ± 2.06
Albumin, g/dL	2.2 ± 0.5
WBC count, cells/μL	11 200 ± 7 500
RBC count, × 10⁴/μL	260 ± 86
Hemoglobin, g/dL	8.3 ± 2.0
Platelet count, × 10⁴/μL	6.7 ± 5.3
LDH, IU/L	2481.3 ± 4107.8
CRP, mg/dL	18.11 ± 13.41

n = 17 patients.

Values for all categories except consciousness disturbance and blood transfusion are mean ± SD.

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert WBC count from cells per microliter to $\times 10^9$ cells per liter, divide cells per microliter by 1000.

To convert RBC count from $\times 10^4$ cells per microliter to $\times 10^{12}$ cells per liter, divide $\times 10^4$ cells per microliter by 100.

To convert hemoglobin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert platelet count from $\times 10^4$ platelets per microliter to $\times 10^9$ per liter, multiply $\times 10^4$ platelets per microliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

significantly higher than those in patients with ADAMTS13 levels higher than 20% (Table 3). The incidence of renal injuries in patients with severe ADAMTS13 deficiency (ADAMTS13 activity less than 20%) was significantly higher than in patients with ADAMTS13 activity levels higher than 20% (Table 3). However, there were no differences in the incidence of liver dysfunction or serum levels of bilirubin, AST, and ALT among these groups (Table 3), suggesting that severe ADAMTS13 deficiency in these patients may be linked to the development of renal injuries. There was a significant difference in serum albumin levels between both groups, suggesting that the decrease of ADAMTS13 activity and antigen levels in patients was at least partially caused by reduced synthesis in the liver.

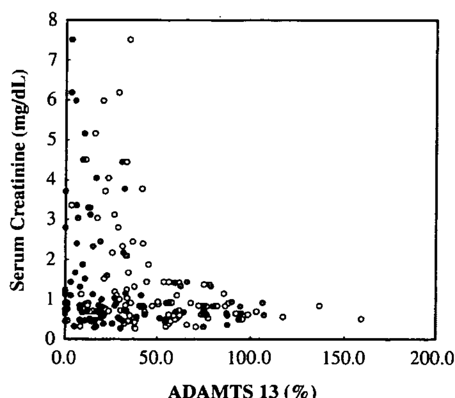


Figure 5. Correlation between the plasma ADAMTS13 levels and the serum creatinine levels. Correlation between serum creatinine levels and ADAMTS13 activity (●) levels or antigen (○) levels in patients with sepsis-induced DIC is shown (n = 103). Patients with a history of chronic renal failure were excluded from the study.

Table 3. Correlation between ADAMTS13 levels and organ injury in patients with sepsis-induced DIC

	ADAMTS13 activity less than 20%, n = 51	ADAMTS13 activity greater than 20%, n = 52	P
Creatinine, mg/dL	1.81 ± 1.70	0.95 ± 0.76	< .01*
AST, IU/L	106 ± 128	182 ± 290	NS
ALT, IU/L	72 ± 109	122 ± 160	NS
Bilirubin, mg/dL	2.70 ± 3.13	2.20 ± 2.53	NS
Albumin, g/dL	2.3 ± 0.4	2.9 ± 0.7	< .05*
CRP, mg/dL	13.50 ± 10.51	6.90 ± 8.61	< .01*
Organ injury, no. (%)			
Renal injury	21 (41.2)	8 (15.4)	< .05†
Liver injury	40 (78.4)	38 (73.1)	NS

Values for all categories except organ injury are mean ± SD. Renal injury: serum creatinine greater than 1.2 mg/dL.

Liver injury: elevation of bilirubin (> 2.0 mg/dL), AST (> 40 IU/L), or ALT (> 40 IU/L).

To convert creatinine from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 88.4.

To convert bilirubin from milligrams per deciliter to micromoles per liter, multiply milligrams per deciliter by 17.1.

To convert albumin from grams per deciliter to grams per liter, multiply grams per deciliter by 10.

To convert CRP from milligrams per deciliter to milligrams per liter, multiply milligrams per deciliter by 10.

NS indicates not significant.

*Statistically significant (Welch *t* test).

†Statistically significant (Fisher exact probability test).

Analysis of VWF multimers in patients with severe secondary ADAMTS13 deficiency

Additionally, unusually large VWF multimers were detected in the plasma of patients with severe secondary ADAMTS13 deficiency (ADAMTS13 activity less than 20%), as shown in Figure 6. Serum creatinine levels in patients in whom unusually large VWF multimers and severe ADAMTS13 deficiency were detected were significantly higher than in patients in whom the unusually large VWF multimers were absent (Table 4). There was no significant difference in ADAMTS13 activity (Table 4) and ADAMTS13-specific activity (activity-antigen ratio) between these patient groups (not shown).

There was a significant difference in CRP levels between the ADAMTS13 activity less than 20% group and the ADMATS13 activity greater than 20% group, but their platelet counts were not significantly different (not shown), indicating that the decrease in ADAMTS13 may be related to inflammatory responses. These results are consistent with the data showing a negative correlation between the activity and antigen levels of ADAMTS13 and the plasma levels of granulocyte elastase digests of fibrin (E-XDP).

Discussion

ADAMTS13 has been shown to play an important role in VWF processing.[1-14,22,23] As shown previously, ADAMTS13 may cleave the unusually large multimers of VWF on the endothelial cell surface, preventing entrance of such unusually large multimers into the circulation.[8,24] Without this processing of VWF multimers, the unusually large multimers of VWF secreted from endothelial cells would enter the circulation and initiate platelet thrombus formation, which in turn would cause the development of TMA.[8,24] Patients with primary ADAMTS13 deficiency caused by defects in the *ADAMTS13* gene or with autoantibodies against ADAMTS13 have been shown to develop TTP, suggesting the important physiologic role of ADAMTS13-catalyzed cleavage of these