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ADAMTS13-binding IgG are present in patients with thrombotic thrombocytopenic purpura

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Summary

Functional assays are commonly used to measure the antibodies of ADAMTS13 found in patients of thrombotic thrombocytopenic purpura (TTP). In this study we used an enzyme-linked immunoassay to analyze the ADAMTS13-binding IgG levels in six groups of individuals: normal, random hospitalized patients, acute TTP, TTP after receiving plasma therapy, TTP in remission, and other types of thrombotic microangiopathy (TMA). The results showed that ADAMTS13-binding IgG levels were elevated in 100% of the acute TTP group, 75% of the TTP group after receiving plasma therapy, and 40% of the remission group. Overall, the ADAMTS13-binding IgG levels correlated with the inhibitory activity levels against ADAMTS13 ($r = -0.69, P < 0.0001$). The assay also detected elevated IgG binding levels in 5% – 15% of the normal, random, and other TMA control groups. Addition of purified ADAMTS13 protein to the plasma samples suppressed the IgG binding in each of the acute TTP patients, but in none of the non-TTP groups. Serial measurement in a patient that had two exacerbations of TTP within the first three weeks revealed that the ADAMTS13 activity levels remained < 0.1 U/ml during this period, and the ADAMTS13-binding IgG remained elevated, suggesting that ADAMTS13 analysis may provide valuable insight to the disease status during the course of therapy. Analysis of ADAMTS13-binding IgG is helpful for the diagnosis and management of TTP.

Keywords

ADAMTS13; thrombotic thrombocytopenic purpura; antibody

Introduction

Thrombotic thrombocytopenic purpura (TTP), a relatively uncommon but serious disorder, is characterized by widespread platelet- and von Willebrand factor-rich thrombi in the capillaries and kidneys (1). Recent studies have demonstrated that deficiency of ADAMTS13 causes TTP (2). While genetic mutations of ADAMTS13 result in deficiency of the enzyme observed in patients with the hereditary TTP, autoimmune inhibitors of the protease are found in most cases of acquired TTP. Because TTP not infrequently presents with atypical features or overlap in clinical manifestations with other types of thrombotic microangiopathy, analysis of ADAMTS13 status has assumed an essential role in the differential diagnosis of TTP.

Most assays of ADAMTS13 or its inhibitors measure the activity of the enzyme to cleave von Willebrand factor. While such functional assays have been instrumental in identifying and subsequently cloning the enzyme, they are technically demanding, impeding timely investigation of TTP patients. Furthermore, functional assays identify inhibitors in 50%–90% of the TTP patients with severe ADAMTS13 deficiency (3–10). Using IgG isolated from patient plasma samples improves the sensitivity of the mixing assay. However, IgG isolation procedures are not suitable for routine use in clinical laboratories. Recently, less complex assays have been developed to detect ADAMTS13 activity using recombinant VWF fragments in ELISA or FRET format (11,12). Nevertheless, the performance of these newer functional assays has not been rigorously investigated for detection of ADAMTS13 inhibitors. In order to advance our knowledge of how ADAMTS13 is affected in TTP, we used an enzyme-linked immunoassay to investigate the levels of ADAMTS13 antibodies in such patients.

Materials and methods

Plasma samples

The samples used in this study were citrated plasma samples from patients referred for investigation of ADAMTS13. Blood samples were obtained before sessions of plasma exchange, and all the plasma samples were stored at -70°C . The study samples were divided into four groups: acute TTP (TTP: thrombocytopenia, microangiopathic hemolysis, age > 10 years, no plausible causes, and with $\text{Cr} < 3.5 \text{ g/dl}$ throughout the course; 53 cases); TTP after receiving plasma therapy (TTP-Pl: 1–6 units fresh frozen plasma or 1–2 sessions of plasma exchange; TTP-Pl; 16 cases); TTP in remission (Rem: asymptomatic with platelet counts $> 130 \times 10^9/\text{l}$ for at least one week; 22 cases), and other types of thrombotic microangiopathy (TMA; 61 cases). The TMA group included patients with diarrhea-positive hemolytic uremic syndrome (D+HUS, 5 cases), lupus or similar auto-immune diseases (7 cases), metastatic cancers (11 cases), bone marrow or solid organ transplants (9 cases), HELLP syndrome (1 case), prosthetic heart valves (1 case), diarrhea-negative HUS (18 cases), and undetermined cases (9 cases). The undetermined cases were not classified, because their clinical data was not available for evaluation. For comparison, the investigation also included a normal group consisting of blood bank donors (Normal; 40 cases) and a group of randomly selected hospitalized patients (Random; 34 cases). All the samples were investigated in the antibody assay study. Some of the cases were not investigated in subsequent experiments, because the amounts of the samples were insufficient for all the studies. The Institutional Review Board approved the research.

ADAMTS13 antibody ELISA

The measurement of ADAMTS13-binding antibody levels was performed using IMUBIND® ADAMTS13 autoantibody kit (American Diagnostica, Stamford, CT, USA), according to the manufacturer's inserts. Briefly, for measurements of ADAMTS13-binding antibody, 100 μl aliquots of plasma samples diluted 1:20 with the assay buffer were added to microwells coated with a full-length recombinant ADAMTS13 fusion protein, which was expressed in HEK293 cells and had a FLAG peptide sequence (DYKDDDDK) at the carboxyl terminus for affinity purification. The excessive binding sites in the microwells were blocked with SuperBlock (Pierce, Rockford, IL, USA). Following a washing step, a goat anti-human IgG antibody labeled with horseradish peroxidase was added to the microwells. Following another washing step, a perborate-3,3'-5,5'-tetramethylbenzidine (TMB) substrate was added to generate a blue colored reaction. Sulfuric acid was added to stop the color reaction. The optic absorbance was measured at 450nm. A reference curve based on five serial dilutions of a random TTP plasma sample was constructed for each assay. The levels of ADAMTS13-binding IgG were expressed in arbitrary units per ml (AU/ml). One AU/ml equals 1 $\mu\text{l}/\text{ml}$ of affinity-purified anti-ADAMTS13

IgG isolated from the same random TTP plasma sample. When analyzed against the affinity purified IgG, the random plasma sample contained 60 AU/ml ADAMTS13-binding IgG.

ADAMTS13 activity and inhibitor assay

ADAMTS13 activity levels were determined by using the SDS PAGE based assay previously developed in our laboratory (4,5). The assay yielded a range of 0.79–1.27 U/ml (mean $\pm$ standard deviation, 1.03 ± 0.12 U/ml) in normal subjects. To detect inhibitors of ADAMTS13, a test sample was heated at 56°C for one hour before being added at a 3:1 ratio to normal control plasma. After incubation at room temperature for 30 minutes, the residual ADAMTS13 activity levels in the mixtures were analyzed. A patient was considered to have inhibitors of ADAMTS13 if the residual ADAMTS13 activity level in the plasma mixture was less than 70% of the control mixture (5).

ADAMTS13 proteins

ADAMTS13 protein was isolated from fresh plasma of normal blood donors using previously described procedures (13). The activity of isolated protease was 14 U/mg proteins.

ADAMTS13 protein with a tag of V5 sequence and 6xHis sequence at its carboxyl terminus was expressed in COS-7 cells as described elsewhere (14).

Statistics

Normally distributed data were expressed in mean $\pm$ standard deviation. Non-normally distributed data were represented by the ranges and medians. Paired data sets were compared by paired t-test or Wilcoxon signed rank test. Unpaired data sets were analysed with unpaired t-test, ANOVA with post-test multiple comparisons, Mann-Whitney test, or Kruskal-Wallis test with post-test. Contingency tables were analyzed with Fisher's exact test. All comparison and correlation analyses were performed using GraphPad InStat (GraphPad Softwares, Inc.).

Results

ADAMTS13-binding IgG assay

A representative standard curve of the antibody assay is shown in Figure 1. Overall, the assay provided good resolutions for 2 – 60 AU/ml antibody levels. Plasma samples with higher IgG binding levels were diluted and re-assayed. The intra and inter-assay coefficients of variation were 6.7% and 7.8% respectively.

ADAMTS13-binding IgG levels

The platelet counts, the ADAMTS13 activity levels, and the percentage with positive inhibitors for the study groups are depicted in Table 1. The individual IgG binding values are depicted for each group in Figure 2. Because the IgG levels detected by the immunoassay did not exhibit a normal distribution in some of the study groups, the values were compared by using non-parametric multiple comparisons, which revealed that the ADAMTS13-binding IgG values of the TTP group were significantly higher than those of the normal, random, and TMA groups ($P < 0.001$). The values of the TTP group were also higher than those of the TTP-remission group ($p < 0.001$). The overlapping ranges of the groups raised questions of whether the assay had sufficient power to reliably identify individual cases of TTP.

Further analysis of the data in Figure 2 revealed that two (5%) of the 40 normal subjects, four (12%) of the 34 random samples, and seven (11%) of the 61 other TMA samples had IgG values in the range observed in the acute TTP group. The seven cases with elevated binding values in the other TMA group included two cases with lupus, two cases with metastatic cancers, two cases with bone marrow or renal grafts, and one case with causes undetermined.

When the two seemingly outliers were excluded from the normal group, the data of the remainders exhibited a normal distribution, with the mean $\pm$ standard deviation = 5.3 ± 1.7 AU/ml. Using 10.3 AU/ml (mean + 3SD of the normal group, excluding the two outliers) as the threshold, 100%, 75%, and 45%, respectively, of the acute TTP, TTP-plasma, and TTP-remission groups were found to have abnormally high IgG levels. Fourteen samples in the three non-TTP groups (normal, 2/40 = 5%; random, 5/34 = 15%; and TMA, 7/61 = 11%) were above this threshold, yielding a specificity of 89.6% for the test. A lower threshold (e.g. normal mean + 2SD) would slightly decrease the specificity of the assay without improving its sensitivity for TTP.

To determine whether the ADAMTS13-binding IgG levels measured by ELISA correlated with the ADAMTS13 inhibitory activity levels in the plasma samples, we plotted the IgG value of each TTP sample against the residual ADAMTS13 activity level of normal plasma after it was mixed with a three-volume aliquot of heated patient plasma sample (Fig. 3). In the mixing assay, a patient sample with higher inhibitor activity suppressed the ADAMTS13 activity in normal plasma to lower residual level. Figure 3 shows that the ADAMTS13-binding IgG levels correlated inversely with the residual normal plasma ADAMTS13 activity levels for the acute TTP samples ($\blacklozenge$; $r = -0.69$; $P < 0.0001$) or the acute TTP samples plus the samples of TTP after receiving plasma therapy or during remission (Δ ; $r = -0.78$; $p < 0.0001$).

Competitive suppression of IgG binding with ADAMTS13

To determine whether immobilized ADAMTS13 competed with ADAMTS13 in solution for binding with IgG, we performed competitive binding experiments in which the ADAMTS13 protein purified from normal plasma was added at a maximum concentration of 7.0 U/ml to the study plasma samples before the IgG binding assay was performed. Addition of ADAMTS13 decreased the IgG binding in each of the 33 acute TTP samples examined, but did not decrease the IgG binding in any of the seven normal/random or six TMA cases with high IgG-binding values (Fig. 4A). More specifically, after incubation with ADAMTS13, the IgG binding value was $\leq 81\%$ of its own buffer control in each of the acute TTP samples. In contrast, the corresponding values were $\geq 91\%$ the non-TTP groups (Fig. 4B, $P < 0.0001$). Three samples from the acute TTP Group and six samples from the non-TTP groups were also tested after incubation with recombinant ADAMTS13 at 10 U/ml. Recombinant ADAMTS13 suppressed the IgG binding by each of the acute TTP samples but none of the non-TTP samples (data not shown).

To further determine whether ADAMTS13 protein isolated from normal plasma completely suppressed the IgG binding in the TTP samples, we plotted the IgG binding values after incubation with ADAMTS13 against the corresponding values after incubation with control buffer (Fig. 4C). The graph revealed that ADAMTS13 obviously caused less suppression of IgG binding in seven of the TTP samples contained non-suppressible IgG.

To determine whether the IgG reacted with the FLAG sequence in the recombinant ADAMTS13 on the microwells, we performed blocking experiments on six non-TTP and nine acute TTP cases by adding a synthetic FLAG peptide at a final concentration of 5.0 mM to the study samples. In none of the samples did the FLAG peptide decrease the binding of IgG (Fig. 5).

Evolution of ADAMTS13 activity and antibody levels during the course of TTP

To explore how ADAMTS13 activity and antibody levels might be used to evaluate the severity of TTP, we serially investigated a patient that had two recurrence of thrombocytopenia within the first three weeks, despite having initial brisk response to plasma exchange (Fig. 6A). The platelet counts rose quickly in response to plasma exchange, but declined precipitously on day

7 and again on day 18, one and four days after plasma exchange was discontinued, respectively. Eventually the patient achieved sustained remission by day 40. Analysis of the ADAMTS13 activity revealed that the protease activity was less than 0.1 U/ml at presentation and remained below this level prior to day 21 (Fig. 6B). The protease activity became detectable by day 23, reaching a maximum of 1.13 U/ml on day 24, before it declined again to 0.2 U/ml by day 48, when plasma exchange had discontinued for seven days. The initial ADAMTS13-binding IgG level was 34 AU/ml in his patient (Fig. 6B). This IgG binding level decreased to 11.8 AU/ml after the plasma sample was incubated with plasma derived ADAMTS13, indicating that most of the observed IgG binding was specific for ADAMTS13. The ADAMTS13-binding IgG level increased to the highest of 60.2 AU/ml on day 10, but steadily decreased thereafter to 8.6 and 8.8 AU/ml on days 27 and 48.

Discussion

In this study we have used an enzyme-linked immunoassay to investigate the presence of ADAMTS13-binding antibodies in patients with TTP. The results reveal that the assay is very sensitive in detecting antibodies of ADAMTS13. Nevertheless, the specificity of its results requires validation with a competitive binding assay.

The presence of ADAMTS13-binding IgG is consistent with the current view that autoantibodies of ADAMTS13 cause deficiency of the enzyme in patients with TTP. Among the patients with severe deficiency of the enzyme, defined as < 5%–10% of normal control depending on the assays used, ADAMTS13 inhibitors have been detected in 50%–90% of the cases (Table 2) (3–10). Variation in assay methods is believed to account for most of the observed difference. Using an immunoassay similar in design to the present one, Rieger et al. detected elevated IgG binding in 97% (35/36) of acute TTP cases (16). Taken together, the results of these two ELISA studies suggest that ADAMTS13-binding IgG in TTP.

The Percentage of patients with elevated IgG binding was substantially lowered from 100% to 75% after the patients received plasma infusion or exchange, presumably because the ADAMTS3 given by transfusion decreased the free ADAMTS13-binding antibody levels. This difference before and after plasma therapy has practical implications, because, in clinical practice, a blood sample may be obtained for ADAMTS13 studies after the patient has received blood transfusion or plasma exchange. In such samples a borderline or low ADAMTS13-binding IgG level does not exclude the diagnosis of TTP. The decrease of ADAMTS13-binding IgG observed after plasma therapy is consistent with previous observations that many patients of TTP have low levels of the inhibitors and readily improve upon plasma infusion therapy (5,16,17).

The course of the ADAMTS13 activity and antibody levels described in Figure 6 suggested that serial analysis of ADAMTS13 activity and antibody levels may be quite informative in following the response of the patients to plasma therapy. Intense immune reaction during the first three weeks of the course may explain why ADAMTS13 remained undetectable in this patient despite daily plasma exchange, and why TTP exacerbated immediately after plasma exchange was performed less frequently. On the other hand, each plasma exchange probably did raise the ADAMTS13 activity to levels sufficient for inducing a response in the platelet count.

The low ADAMTS13 activity level observed in TTP patients during remission suggested that the immune response to ADAMTS13 did not completely disappear in many TTP Patients. Institution of ticlopidine therapy has been associated with the development of ADAMTS13 antibodies (18–21) Therefore, it is not unreasonable to conjecture that exposure to certain compounds or infectious microorganisms may induce in susceptible individuals the production

of antibodies that cross-react with ADAMTS13. This scheme is analogous to the hemolysis caused by anti-I antibodies following mycoplasma infection, and may explain why anti-ADAMTS13 activity was more intense in the first few weeks of presentation, before it declined to lower levels thereafter.

A main concern with the current assay is that it yielded high IgG values in 5%–12% of the subjects without TTP. The prevalence rates of elevated IgG did not appear to differ among the various non-TTP groups. This finding is analogous to that reported by Rieger et al. who detected elevated IgG binding results 14%–13% of individuals that did not have TTP (15). Although it is tempting to suggest that some individuals without TTP have ADAMTS13-binding antibodies, there is no direct evidence to substantiate such an interpretation.

On the contrary, the competitive binding experiments demonstrated that both recombinant ADAMTS13 and plasma-derived ADAMTS13 failed to suppress the IgG binding in any of the non-TTP subjects, while the proteins decreased IgG binding in each of the TTP cases investigated. By eliminating the non-suppressible samples, the competitive assay increased the specificity of the antibody immunoassay to 100% for acute TTP. The competitive assay also suggests that the ELISA detected ADAMTS13-binding IgG only when binding capacity of the antibody exceeds the ADAMTS13 level in the circulation. Nevertheless, prospective studies are needed to confirm the sensitivity and specificity of the current assays.

The causes of non-suppressible IgG binding detectable in some individuals without TTP remain unclear. Preliminary investigations suggest that multiple causes might have contributed to the observed phenomenon. Analysis of the individual data showed that ADAMTS13 suppressed some of the acute TTP samples less effectively than others, suggesting that these TTP samples might also contain non-suppressible IgG.

In this study, the antibody assay detected elevated IgG binding in 41% of the remission group. Based on the limited competitive binding data available for the remission group, the percentage of samples with specific ADAMTS13-binding IgG may be even lower than this level of ADAMTS13 antibodies tend to decline to below the circulating ADAMTS13 levels in TTP patients as they achieve remission.

Overall, the ADAMTS13-binding IgG levels showed a clear correlation with the inhibitory activity levels in TTP plasma samples. However, the data in Figure 3 exhibits a wide range of deviation from the regression line. At least two reasons may account for the scattering of data: the difference in the types of samples used in the assays and the detection of non-suppressible binding in association with the ELISA. Firstly, for the mixing experiments it was necessary to heat the samples at 56°C to inactivate the endogenous ADAMTS13 activity in the plasma samples. This heating step denatured ADAMTS13 and might have dissociated the antibodies from the immune complexes of ADAMTS13, rendering the antibodies detectable in the mixing experiments. For the IgG binding assay, plasma samples were not heated because preliminary experiments revealed that, after heating, the normal plasma samples exhibited a broader range of distribution with substantial overlapping of the TTP and non-TTP samples. Secondly, as mentioned above, the IgG values detected by ELISA might contain a non-suppressible component in some of the TTP samples, giving rise to falsely high IgG values. Additionally, we could not exclude the possibility that some of the TTP cases might contain non-inhibitory anti-ADAMTS13 antibodies (22). These considerations suggest that the inhibitor assay and the ADAMTS13-binding IgG assay may complement each other for evaluation of TTP patients. Further studies are needed to delineate which assay better correlates with the clinical course of TTP.

In conclusion, this study demonstrates that essentially all patients with the acquired form of TTP and ADAMTS13 deficiency have ADAMTS13-binding IgG that is detectable by using a simple

enzyme-linked immunoassay. The level of ADAMTS13-binding IgG may become undetectable after the patients receive plasma infusion or exchange. The assay may yield false positive results in a subset of the general population in which the IgG binding is not suppressible by ADAMTS13 protein in solution. Serial analysis of ADAMTS13 activity and antibody levels may be instrumental in the diagnosis and management of autoimmune TTP.

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Figure 1. A representative reference curve for determination of ADAMTS13-binding IgG levels
The regression equation for this curve: $\text{concentration} = 28.95 \times \text{OD}^2 + 17.93 \times \text{OD} - 1.37$; $r^2 = 0.9998$.

Figure 2. Depiction of the individual ADAMTS13-binding IgG values

The dashed line indicates the level of mean - 3 SD for the normal group, excluding the two outliers. Norm: normal group. Rand: randomly selected hospitalized patients. TTP: patients with acute TTP before receiving plasma therapy. T-PI: Acute TTP after receiving plasma infusion or exchange, but before achieving remission. Rem: TTP in remission. TMA: other types of thrombotic microangiopathy.

Figure 3. A plot of the ADAMTS13-binding IgG levels against the residual ADAMTS13 activity levels in mixing studies

A lower residual activity value indicates a higher inhibitor level in the plasma sample. The coefficient of correlation (r) was -0.69 ($p < 0.0001$) for the acute TTP samples (◆) and was -0.78 ($P < 0.0001$) when the TTP samples obtained after initiation of plasma therapy or during remission (△) were also included in the analysis.

Figure 4. ADAMTS13 protein suppresses the IgG binding of TTP

A) ADAMTS13 protein decreased the IgG binding of acute TTP samples. Each sample is represented by two data points: the left-hand point indicates the control IgG binding value after the sample was incubated with buffer, and the right-hand point represents the value after the sample was incubated with plasma derived ADAMTS13. B) Deviation of the individual IgG binding values after incubation with ADAMTS13, expressed as a percentage of its own buffer control. Each horizontal bar indicates the median level of the group. The dashed line indicates the mean + 2SD of the TTP group. C) A plot of IgG binding values in the presence of ADAMTS13. "◆" represents acute TTP samples; "◇" represents non-TTP samples. A data

point below the line of unity (solid line) indicates the sample contains ADAMTS13-suppressible IgG binding. The dashed line indicates the upper limit (mean + 2SD) of normal samples for IgG binding in the presence of ADAMTS13. Norm/Rand: normal and randomly selected hospitalized patients, TTP: patients with acute TTP before receiving plasma therapy. PI-Rem: TTP after receiving plasma therapy or in remission. TMA: other types of thrombotic microangiopathy.

Figure 5. FLAG peptide does not suppress the IgG binding

For each pair of data connected by a straight line, the left-hand point represents the control IgG binding level, while the right-hand data represents the IgG binding level, while the right-hand data represents the IgG binding level after the sample was incubated with the FLAG peptide at 5 mM. The non-TTP data included samples from the normal, random, and TMA groups.

Figure 6. The courses of the platelet counts (A) and of the ADAMTS13-binding IgG and ADAMTS13 activity levels (B) in a patient presenting with acute TTP
In panel A, each ◇ represents one session of plasma exchange.

Table 1

Platelet counts and ADAMTSI3 levels of the study groups.

Group	Number of cases	Platelet count median (range) $\times 10^9/l$	ADAMTSI3 activity median (range) U/ml	ADAMTSI3 inhibitor positive %
Acute TTP	53	11 (5 - 140)	<0.1 (<0.1)	93
TTP-plasma (T-Pl)	16	34 (9 - 83)	<0.1 (<0.1 - 0.39)	73
Remission (Rem)	22	210 (138 - 581)	0.13 (<0.1 - 0.54)	86
TMA	61	26 (3 - 128)	0.84 (0.27 - 1.58)	0

Table 2
Prevalence of ADAMTS13 antibodies in TTP.

Authors, years of publication	Numbers positive/investigated [*]	% positive
<i>Inhibitors of ADAMTS13 (functional assay)</i>		
Furlan, et al., 1998	20/24	83
Tsai, et al., 1998	31/39	79
Tsai, et al., 2001	31/41	76
Veyradier, et al., 2001	30/59	51
Rick, et al., 2002	26/29	90
Studt, et al., 2003	31/50	62
Coppo, et al., 2004	17/31	55
Peyvandi, et al., 2004	42/48	87
<i>ADAMTS13-binding IgG (ELISA)</i>		
Rieger, et al., 2005	35/36	97
This study	53/53	100

^{*} Only patients with severe ADAMTS13 deficiency (<5% - 10%) were included.