Residues Arg568 and Phe592 contribute to an antigenic surface for anti-ADAMTS13 antibodies in the spacer domain

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I. Quantification of anti-ADAMTS13 IgG, IgM and IgA in plasma from patients with acquired thrombotic thrombocytopenic purpura

Most anti-ADAMTS13 antibodies are of immunoglobulin class G (IgG), although in a limited number of patients antibodies of immunoglobulin class M (IgM) and A (IgA) have also been detected.[1,2] While anti-ADAMTS13 antibodies of the IgG4 subclass dominate the immune response to ADAMTS13, IgG1 and IgG2 have been found in approximately 50% of samples analyzed.[3] In this study we examined the Ig class distribution in 48 patients with acquired thrombotic thrombocytopenic purpura (TTP) using human monoclonal antibody II-1 as an internal standard.

Online Supplementary Materials and Methods

Construction and expression of anti-ADAMTS13 IgG1-2-3-4/M/A1-2 antibody II-1

The variable heavy chain of antibody II-1 (IgG1)[4] was subcloned into the pcDNA3.1 vector together with different constant regions of IgG2(Cγ2)-3(Cγ3)-4(Cγ4), IgM (Cμ), IgA1(Cα1) and IgA2(Cα2) with the BamHI/NotI/KpnI restriction sites/enzymes (NEB, Ipswich, UK).[5,6] All variants were transiently expressed in the Freestyle system (Invitrogen) including the light chain of antibody II-1 and quantified using a human IgG or IgM or IgA quantitation kit from Bethyl Laboratories (Montgomery, TX, USA). IgM, IgA1 and IgA2 were co-expressed with the J-chain (present in the pcDNA3.1 vector) in order to let the antibodies multimerize into pentamers/hexamers for IgM or dimers for the IgA1 and IgA2 molecules. All antibody variants were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions. Western blots were stained using an anti-IgG-, anti-IgM- and anti-IgA-horseradish peroxidase (HRP)-labeled antibody (DAKO, Glostrup, Denmark).

Detection of anti-ADAMTS13 IgG/M/A1-2 antibodies in plasma from patients with thrombotic thrombocytopenic purpura

The presence of anti-ADAMTS13 antibodies in plasma from patients with acquired TTP was determined by immobilizing an anti-V5 antibody (1 μg/mL) on Maxisorp microtiter plates (NUNC, Roskilde, Denmark) in 50 mM NaHCO₃ (pH 9.8) overnight at 4°C. Wells were subsequently blocked with phosphate-buffered saline (PBS) 2%, bovine serum albumin (BSA) 0.1%, Tween-20. After washing three times with PBS 0.1% Tween-20, purified recombinant V5-tagged ADAMTS13 (1 μg/mL) was incubated in blocking buffer for 2 h at 37°C. The purification and analysis of ADAMTS13 has been described previously.[7] Subsequently, unbound ADAMTS13 was washed away and plasma samples diluted in PBS 2% BSA 0.1% Tween-20 were added to the wells and incubated for 2 h at 37°C. After washing, immunoglobulins were detected with a mix of HRP-labeled monoclonal anti-human IgG1, IgG2, IgG3 and IgG4 (1:2000; Sanquin Reagents, Amsterdam, the Netherlands) for the detection of total IgG, HRP-labeled monoclonal anti-human IgM (1:750; Sanquin Reagents) and HRP-labeled monoclonal anti-human specific for IgA1 or 2 (1:1000; Southern Biotech, Birmingham, Alabama, USA). A pool of normal human plasma derived from 47 donors and plasma from two patients with congenital TTP were used as negative controls. An equimolar mixture of II-1 IgG1, 2, 3 and 4 was used as an internal standard for determining IgG levels in patients' samples. II-1 IgM was used as an internal standard for determining IgM levels in the patients' samples. II-1 IgA1 and II-1 IgA2 were used as internal standards to determine the level of anti-ADAMTS13 IgA1 and IgA2 in patients' samples. Anti-ADAMTS13 IgG subclass antibodies in plasma from patients with acquired TTP were detected by directly coating ADAMTS13 (1 μg/mL) on Maxisorp microtiter plates (NUNC, Roskilde, Denmark) in 50 mM NaHCO₃ (pH 9.8) overnight at 4°C. After blocking for 1 h at 37°C with PBS 2% BSA 0.1% Tween-20, plasma samples and recombinant anti-ADAMTS13 antibodies (standard; starting dilution of 100 ng/mL for IgG1 and 500 ng/mL for IgG2-3-4) were added for 2 h at 37°C, allowing antibodies to bind ADAMTS13 in solution. Immunoglobulins were detected with HRP-labeled monoclonal anti-human IgG1, IgG3 and IgG4 (1:2000; Sanquin Reagents, Amsterdam, the Netherlands); detection of IgG2 required an additional step of incubation with a primary anti-human IgG2 antibody and then a secondary HRP-labeled antibody (1:2000; Sanquin Reagents, Amsterdam, the Netherlands). A pool of normal human plasma derived from 47 donors was used as a negative control.

Results and Discussion

Plasma samples from 48 patients with acquired TTP were analyzed for the presence of IgG, IgM and IgA. In all patients ADAMTS13 activity levels were 10% or less as determined by a FRETS-VWF73 assay[8] (Figure 1). The levels of the immunoglobulins were quantified using human monoclonal anti-ADAMTS13 antibody II-1 as an internal reference. The variable domains of antibody II-1 were fused to the constant regions of human IgG1, IgG2, IgG3, IgG4, IgM, IgA1 and IgA2.

Analysis of supernatants of transfected 293 cells by SDS-PAGE revealed that all variants were expressed (*Online Supplementary Figure S1*). Anti-ADAMTS13 IgG was present in plasma samples from all 48 patients analyzed (Figure 1). Levels of anti-ADAMTS13 IgG ranged primarily from 0.50 – 1.50 µg/mL; higher values of anti-ADAMTS13 IgG were found in 11 plasma samples. The highest level of anti-ADAMTS13 IgG was detected in plasma from patient 7 (19.5 µg/mL). Residual levels of ADAMTS13 activity of 10% were still present in plasma from this patient. Levels of anti-ADAMTS13 IgG did not correlate with residual levels of ADAMTS13 activity (*data not shown*). IgG subclass analysis revealed that IgG1 was present in 35 out of 48 samples and IgG4 in 33 out of 48 samples (Figure 1). Low levels of IgG2 and IgG3 were observed in seven and four samples, respectively. Patients' plasma samples that were negative for the presence of anti-ADAMTS13 IgG1, 2, 3 or 4 were denoted as non-detected (ND). Due to limitations in availability of patients' samples we only assessed the presence of anti-ADAMTS13 IgG2 and IgG3 in plasma diluted 10-fold. We cannot, therefore, exclude that small amounts of IgG2 and IgG3 were present in the patients' samples included in this study. Nevertheless, our findings suggest that anti-ADAMTS13 IgG consists primarily of IgG1 and IgG4. Anti-ADAMTS13 IgM was found in five out of 48 patients. In a previous study the presence of anti-ADAMTS13 IgM was reported in four out of 58 patients.[3] Levels of anti-IgM varied between 0.15 – 1.20 µg/mL. Anti-ADAMTS13 IgA was detected in nine out of 48 patients. This value closely corresponds to results from a previous study in which ten out of 47 patients had anti-ADAMTS13 IgAs.[3] Anti-ADAMTS13 IgA levels were low (0.02 – 0.60 µg/mL) when compared to anti-ADAMTS13 IgG in most patients, with the exception of patient 3 (Figure 1). The percentages of patients positive for the combinations of IgG/IgA, IgG/IgM and IgG/IgM/IgA were similar to those found in a previous study.[2] IgA antibodies can be further subdivided into IgA1 and IgA2. IgA1 is the major component of serum IgA and secretory IgA except in the large intestines and the female genital tract where IgA2 constitutes the majority of IgA found.[9] We found that patients only have subclass anti-ADAMTS13 IgA1 in their plasma; no anti-ADAMTS13 IgA2 was detected. Taken together, our data suggest that the majority of anti-ADAMTS13 antibodies consist of IgG, although significant amounts of IgM and IgA1 antibodies can also be found in plasma of patients with acquired TTP (Figure 1). Our findings are in agreement with those of previous studies on the presence of anti-ADAMTS13 IgA and IgM in patients with acquired TTP.[1,2] IgA consists of two subclasses IgA1 and IgA2; IgA1 has a unique hinge region, lacking in IgA2, containing O-linked glycans that can mediate binding to various lectins (galectin-1, Fcα1/δR on T cells[9]). Serum IgA is exclusively monomeric, and is mostly composed of IgA1 (~80%). In contrast, IgA produced at the mucosa is mostly dimeric or polymeric and associated with the J-chain, allowing for interaction with the polymeric Ig-receptor (pIgR) expressed on mucosal epithelial cells.[10] The pIgR transports the IgA to mucosal surfaces of the gastro-intestinal, respiratory and urinogenital tracts where it prevents pathogen penetration, interacts with dietary antigens and controls commensal microbes through immune exclusion.[11,12] Here, IgA1 is also more abundant than IgA2 (except in the large intestine and female genital tract), and both subclasses remain covalently attached to the extracellular part of the pIgR (secretory component). The monomeric IgA in serum, on the other hand, has been suggested to be a second line of defense against incoming pathogens, which are eliminated by FcαRI expressed on monocytes and neutrophils and Küpffer cells in the liver.[13] FcαRI interacts exclusively with serum IgA, but not secretory IgA due to steric hindrance by the secretory component, and can mediate a strong degranulation of neutrophils and release of inflammatory mediators upon receptor crosslinking.[6,12,13] Deposits of systemic IgA immune complexes can give rise to purpura in the skin and kidney failure in patients with Henoch-Schönlein purpura and patients with IgA nephropathy, respectively.[14] High titers of anti-ADAMTS13 IgA and IgG1 have previously been linked to clinical outcome following a first TTP event.[2,3] Here we show that anti-ADAMTS13 IgA present in TTP patients is exclusively composed of subclass IgA1. It is not currently known whether immune complexes consisting of anti-ADAMTS13 IgA1 contribute to the pathogenesis of acquired TTP.

II. Quantification of binding of spacer domain variants to patient's IgG

In order to quantitatively determine differences in reactivity of the spacer domain variants for anti-ADAMTS13 IgG present in the patient samples we performed densitometric scanning of the western blots shown in Figure 2.

## III. VWF processing of ADAMTS13 spacer domain variants

The activity of the MDTCS variants included in this study was determined using the fluorogenic FRETS-VWF73 assay (Peptides International, Liousville, KY, USA).[8] Unexpectedly, VWF processing activity of MDTCS-F568A and MDCTS-F592A was increased when compared to wild type MDCTS. Introduction of both amino acid substitutions did not further enhance the VWF processing activity of MDTCS (see results obtained for MDTCS-R568A/F592A). In accordance with previous findings the VWF processing activity of MDTCS-RYY was strongly reduced when compared to wild type MDTCS.[7] Introduction of the F592A, R568A or both the F592A and R568A substitutions resulted in an increased activity when compared to MDTCS-RYY. Overall, our findings suggest that introduction of the R568A and/or F592A substitutions does not induce major structural alterations. The strongly reduced binding of patient IgG to MDTCS-RYY-R568A/F592A and the relatively modest effect of these amino acid substitutions on ADAMTS13 activity may provide a basis for the design of spacer domain variants with reduced antigenicity which at least partially retain their ability to process VWF.

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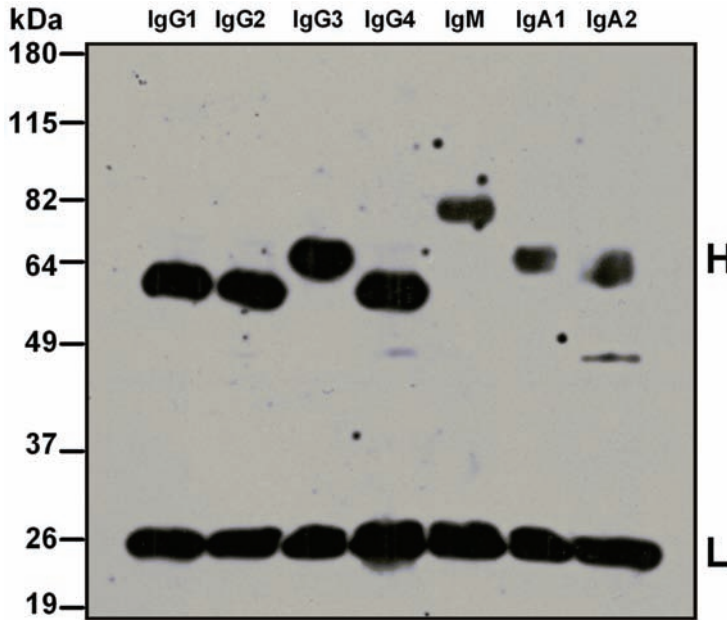
Online Supplementary Figure S1. Western blot analysis of II-1 antibodies of different isotype formats (IgG1-2-3-4, IgM, IgA1 and IgA2). Antibodies were subjected to SDS-PAGE analysis and detected with an anti-IgG/M/A antibody by western blotting. Although the same amount was administered to the gel under reducing conditions, the detecting antibody was not able to recognize the IgM, IgA1 and IgA2 antibody as well as the IgG. Both heavy chains (H) and light chains (L) are indicated. The heavy chains of IgG1, IgG2 and IgG4 (~58kDa) migrate at the same apparent molecular weight whereas IgG3 (~65kDa), IgM (~80kDa) and both IgA (~65kDa) migrate at a higher apparent molecular weight.
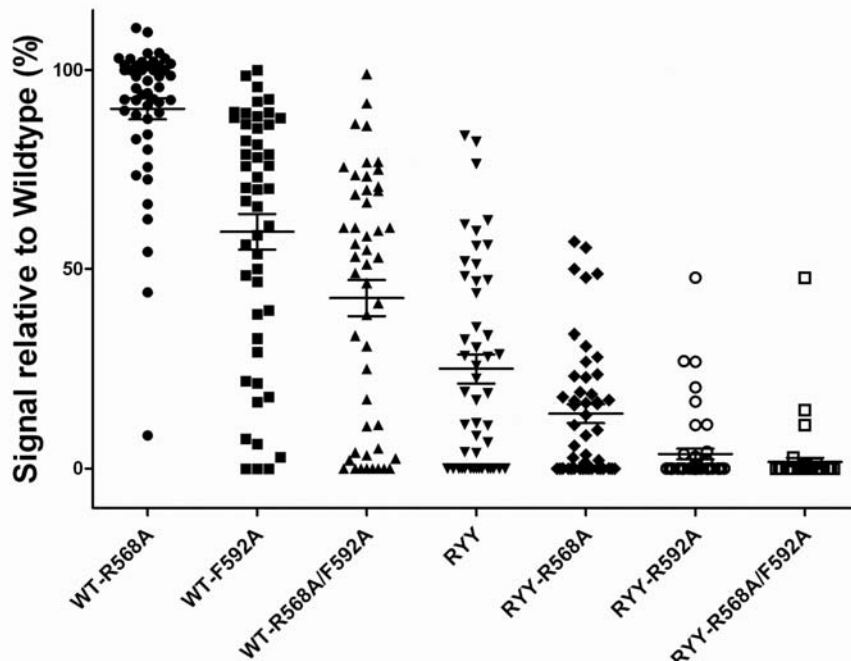
Online Supplementary Figure S2. Quantitification of binding of spacer domain variants to patients' IgG. Signals obtained for binding of patient-derived IgG to the different spacer domain variants were plotted as percentage of binding to wild type MDTCS. The spacer domain variants used in this study are shown on the x-axis. Each data point indicates the reactivity of IgG present in a single patient's sample to the variant listed on the x-axis. Means and standard errors of the means of the reactivity of the 48 samples with the different variants are indicated by horizontal bars. There was a progressive decline in reactivity of patients' IgG in the order WT, WT-R568A, WT-F592A, WT-R568A/F592A, RYY, RYY-R568A, RYY-F592A and RYY-R568A/F592A.

Online Supplementary Figure S3. VWF processing activity of spacer domain variants. Spacer domain variants (5 nM) were incubated with 2 μM of FRETS-VWF73 substrate for 60 min at 30°C. The fluorescence signal was measured using a Wallac 1420 ARVO multilabel counter. The VWF processing activity of the different spacer domain variants is expressed as percentage of that observed for wild-type MDTCS.