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ADAMTS13 Autoantibodies Cloned from Patients with Acquired Thrombotic Thrombocytopenic Purpura: 1. Structural and functional characterization *in vitro*

Eric M. Ostertag¹, Stephen Kacir¹, Michelle Thiboutot¹, Gayathri Gulendran¹, X. Long Zheng², Douglas B. Cines¹, and Don L. Siegel¹

¹Departments of Pathology & Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania

²Children's Hospital of Philadelphia, Philadelphia, Pennsylvania

Abstract

BACKGROUND—Acquired thrombotic thrombocytopenia purpura (TTP) is a life-threatening illness caused by autoantibodies that decrease the activity of ADAMTS13, the von Willebrand Factor cleaving protease. Despite efficacy of plasma exchange, mortality remains high and relapse is common. Improved therapies may come from understanding the diversity of pathogenic autoantibodies on a molecular/genetic level. Cloning comprehensive repertoires of patient autoantibodies can provide the necessary tools for studying immunobiology of disease and developing animal models.

STUDY DESIGN AND METHODS—Anti-ADAMTS13 antibodies were cloned from four patients with acquired TTP using phage display and characterized with respect to genetic origin, inhibition of ADAMTS13 proteolytic activity, and epitope specificity. Anti-idiotypic antisera raised to a subset of autoantibodies enabled comparison of their relatedness to each other and to polyclonal IgG in patient plasma.

RESULTS—Fifty-one unique antibodies were isolated comprising epitope specificities resembling the diversity found in circulating patient IgG. Antibodies directed to both the amino terminal domains and those requiring the ADAMTS13 cysteine-rich/spacer region for binding inhibited proteolytic activity, while those solely targeting carboxy-terminal domains were non-inhibitory. Anti-idiotypic antisera raised to a subset of antibody clones crossreacted with and reduced the inhibitory activity of polyclonal IgG from a set of unrelated patients.

CONCLUSIONS—Anti-ADAMTS13 autoantibodies isolated by repertoire cloning display the diversity of epitope specificities found in patient plasma and provide tools for developing animal

Correspondence and reprint requests: Don L. Siegel, Ph.D., M.D., Division of Transfusion Medicine & Therapeutic Pathology, Pathology and Lab Med, Perelman School of Medicine at the University of Pennsylvania, Stellar-Chance Laboratories, Room 509, 422 Curie Blvd., Philadelphia, PA 19104, phone:215-898-9655 fax:215-573-2012, siegeld@mail.med.upenn.edu.

Present affiliations:

Eric M. Ostertag, Poseida Therapeutics, Inc., San Diego, CA.

X. Long Zheng, Division of Laboratory Medicine, Department of Pathology, The University of Alabama at Birmingham, Birmingham, AL.

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models of acquired TTP. Shared idiotypes of inhibitory clones with circulating IgG from multiple patients suggest common features of pathogenic autoantibodies that could be exploited for developing more targeted therapies.

Keywords

Thrombotic thrombocytopenic purpura; ADAMTS13; von Willebrand Factor; microangiopathic hemolytic anemia; therapeutic plasma exchange; phage display

INTRODUCTION

Acquired thrombotic thrombocytopenic purpura (TTP) is a potentially fatal hematologic disease that serves as a paradigm for human autoimmune disorders in which autoantibodies target a single, well-characterized molecule, yet therapies remain non-specific and in some cases are ineffective or of transient benefit. The majority of patients have reduced activity levels of the von Willebrand factor (VWF)-cleaving protease ADAMTS13 due to the development of autoantibodies that inhibit enzyme function.¹⁻⁷ Decreased ADAMTS13 activity can result in the accumulation of ultra-large VWF (UL-VWF) multimers that foster platelet aggregation in the microcirculation leading to thrombocytopenia, microangiopathic hemolytic anemia, organ dysfunction, and death.⁸⁻¹⁰ First-line therapy comprises therapeutic plasma exchange (TPE) that reduces mortality from ~90% to ~15%, presumably by repeated depletion of a fraction of circulating autoantibodies together with replenishment of ADAMTS13 levels until the disease resolves.^{11,12} Notwithstanding earlier recognition and initiation of therapy, and the additional use of systemic immunosuppression,¹³⁻¹⁵ mortality has remained relatively constant since the initial introduction of TPE over 25 years ago.¹²

Improvements in treatment have been stymied because an understanding of the pathogenic humoral immune response -- a prerequisite to the design of specific therapeutic approaches -- has been limited to that which can be gleaned from the study of polyclonal mixtures of immunoglobulin derived from TTP patient sera.¹⁶ Such studies have shown that circulating anti-ADAMTS13 autoantibodies are predominantly IgG₁ and IgG₄¹⁷ and many sera recognize epitopes in the ADAMTS13 spacer domain.¹⁸⁻²² Binding of autoantibodies to the spacer domain is thought to inhibit cleavage of VWF by blocking amino acids involved in formation of a complex between the enzyme and the unfolded A2 domain of VWF.^{18,23-25} However, nearly all patient sera have circulating autoantibodies that recognize at least one additional domain of ADAMTS13.¹⁸⁻²² The functional significance of autoantibodies directed to non-spacer domains has been largely unexplored due to the inherent difficulty in studying complex autoantibody repertoires using heterogeneous mixtures of IgG in patient sera.¹⁶

Cloning individual antibodies could help address these issues but examples of human monoclonal anti-ADAMTS13 autoantibodies cloned from patient B-cells have been limited to those directed to the spacer domain of ADAMTS13.²⁶⁻²⁸ Molecular characterization of anti-ADAMTS13 autoantibodies specific for amino- and carboxy-terminal ADAMTS13 domains is lacking, and it is not clear whether non-spacer domain binding autoantibodies

also affect ADAMTS13 function.^{12,29} A few of the cloned human spacer domain-specific inhibitory antibodies were found to be encoded by the human heavy chain variable region gene V_H1-69^{29,30} suggesting a potential characteristic of ADAMTS13 inhibitory antibodies to exploit therapeutically.²⁷ However, the structural features conferred by V_H1-69 that impart inhibition of ADAMTS13 proteolytic activity are not clear, nor is the extent to which V_H1-69-encoded antibodies are representative of circulating autoantibodies expressed in patient plasma.

To address these issues, we used repertoire cloning by antibody phage display to capture a diverse set of anti-ADAMTS13 binding specificities in TTP patients and then characterized the antibodies with respect to their genetic origins, clonality, and effects on ADAMTS13 proteolytic activity. Anti-idiotypic antibodies were raised to a subset of these monoclonal ADAMTS13 autoantibodies to explore relatedness to one another and to those in patient sera. In an accompanying manuscript⁵⁷, we test the pathogenicity of these antibodies in a novel murine model of human autoantibody-mediated TTP.

MATERIALS AND METHODS

Cloning human anti-ADAMTS13 autoantibodies

Antibody phage display libraries expressing IgG₁₋₄(κ/λ)-derived scFv's were created from the splenic or peripheral blood B-cells of 4 unrelated patients (TTP1-TTP4) with autoantibody-mediated TTP (Table 1) using methods previously published by our laboratory.³¹⁻³³ Briefly, cDNAs encoding immunoglobulin heavy/light chain variable regions were amplified by PCR and cloned into the pComb3X vector (Scripps Research Institute, La Jolla, CA). After electroporation into *E. coli* and co-infection with helper phage, DNA encoding each single chain variable region fragment (scFv) was packaged into a phage particle expressing the encoded antibody fragment on its surface. Citrated peripheral blood for library construction was collected from patients TTP2, TTP3, and TTP4, just prior to their first plasma exchange at the Hospital of the University of Pennsylvania. Mononuclear cells were isolated by density sedimentation (Ficoll-Paque, GE Healthcare, Pittsburgh, PA). Splenic tissue (15 g) from TTP1 was obtained following splenectomy after a 42-week relapsing/remitting course.

Antibody libraries were selected against full-length human recombinant ADAMTS13 coated to wells of immunoassay plates (Technoclone GmbH, Vienna, Austria) using solid-phase panning.³³ For TTP1, the library was also selected against the TSP1 5-8/CUB fragment of ADAMTS13 prepared as described.³⁴ Positive binding clones (>10-fold absorbance above background with irrelevant phage-displayed scFv) were identified from each patient's library. Nucleotide sequences of the heavy/light chains of each scFv were determined using pComb3X-specific sequencing primers³³ to identify unique antibodies. Sequences were analyzed for homology to human immunoglobulin germline gene segments using IMGT/V-QUEST.³⁵

Production of soluble scFv antibody fragments

Soluble scFv preparations (i.e., antibodies unlinked to phage) of each positive clone identified through phage ELISA were used to confirm ADAMTS13 binding and to perform ADAMTS13 inhibition assays, epitope mapping, and to generate rabbit anti-idiotypic antibodies. The TOP10F' non-suppressor strain of *E. coli* (Invitrogen, Life Technologies, Grand Island, NY) was infected with individual phage clones³⁶ and scFv molecules with carboxy terminus 6xHis sequence (for purification) and a hemagglutinin (HA) peptide tag (for detection) were then purified from bacterial extracts by nickel-chelation affinity chromatography.³³ ScFv binding to ADAMTS13 (150 ng scFv per well) was assessed by ELISA using HRP-conjugated anti-HA secondary antibody (Roche Diagnostics, Indianapolis, IN).³⁶ Negative controls included E1M2, an RBC Rh(D)-specific scFv³⁷ and PX4-3, a keratinocyte-specific scFv.³³ For experiments requiring a V5 vs. HA tag on the carboxyl termini of scFv's, antibodies were expressed using a modified pMT/BiP/V5-His A plasmid (Invitrogen) in *Drosophila* S2 cells as described in Supplementary Methods.

Epitope mapping anti-ADAMTS13 antibodies

Epitope mapping of scFv was performed by immunoprecipitation (IP) of recombinant full-length and truncated forms of ADAMTS13.²¹ Incubation mixtures comprising 500 μ L PBS containing 25–100 ng of an ADAMTS13 construct, 600 ng of scFv, 0.1% protease inhibitor cocktail (Sigma, St. Louis, MO), 0.05% Tween 20 (Pierce, Rockford, IL), and 1% BSA were rotated overnight at 4°C followed by addition of 30 μ L anti-HA agarose beads (Roche). Beads were incubated for 2 hours at room temperature, washed 5 times with 1 mL of 0.05% Tween 20/PBS, and the final bead pellet was resuspended in 50 μ L gel electrophoresis sample buffer containing DTT (Novex) and heated at 85°C for 5 min. Electrophoresis and Western blotting were performed per manufacturer's instructions using NuPAGE 4–12% Bis-Tris gels. Immunoprecipitated ADAMTS13 constructs were visualized with a chemiluminescent substrate (ECL, GE Healthcare Life Sciences) on PVDF membranes developed with HRP-conjugated mouse anti-V5 antibody (Invitrogen) at 1:5000 dilution in blocking buffer (2.5% non-fat dry milk, 0.5% Tween, TBS).

Generation of rabbit anti-ADAMTS13 anti-idiotypic antibodies

New Zealand White rabbits were immunized with bacterially-produced HA-tagged scFv by Pocono Rabbit Farm & Laboratory (Canadensis, PA) following their standard protocol. Rabbit IgG was purified with Protein G as described,³⁸ and scFv immunity was verified by comparing rabbit pre-immune with post-immune IgG by ELISA using microplates pre-coated with 10 μ g/mL mouse anti-V5 tag antibody (Invitrogen) to capture S2 cell-produced V5-tagged scFv's and developed with HRP-conjugated donkey anti-rabbit IgG (Amersham, Pittsburgh, PA). V5-tagged scFv's were used in all assays with rabbit anti-idiotypic IgG to avoid detecting rabbit anti-HA antibodies produced from bacterially-produced HA-tagged scFv immunogens.

ADAMTS13 activity assays

ADAMTS13 activity was measured in the presence or absence of scFv, TTP patient plasma, and/or rabbit anti-idiotypic IgG using FRETS-VWF73 peptide (Peptide International,

Louisville, KY). Analytes were mixed in volumes of 8 μ L and added to 42 μ L of substrate buffer and 50 μ L of diluted FRETs-VWF73 reagent.³⁹ Fluorescence emission of cleaved FRETs-VWF73 was measured using a Synergy 2 Multi-Mode Reader (BioTek, Winooski, VT) equipped with 340 nm excitation and 440 nm emission filters. Additional details are in Supplementary Methods.

Study approval

Human studies research was approved by the University of Pennsylvania's Institutional Review Board (protocol #802720). Written informed consent was received from participants prior to inclusion.

RESULTS

Cloning TTP patient anti-ADAMTS13 autoantibodies

Antibody phage display libraries expressing IgG₁ through IgG₄ κ/λ isotypes of single chain variable region fragments (scFv) (i.e., heavy and light chain variable regions tethered together by a short peptide linker) were created from the splenic B-cells ("TTP1") or peripheral blood B-cells ("TTP2", "TTP3", "TTP4") of four unrelated patients with autoantibody-mediated TTP. Patients were diagnosed with acquired TTP on the basis of thrombocytopenia, microangiopathic hemolytic anemia, and <10% plasma ADAMTS13 activity in the setting of inhibitory immunoglobulin. Table 1 shows relevant demographic and clinical data. Peripheral blood for library construction was collected from TTP2-TTP4 just prior to their first plasma exchange and comprised $\sim 1 \times 10^6$ IgG-positive B-cells. Splenic tissue from TTP1 was obtained following splenectomy after a 42-week relapsing/remitting course and comprised $\sim 6 \times 10^9$ IgG-positive B-cells. Antibody libraries contained 4.6×10^8 , 3.6×10^8 , 7.4×10^8 , and 6.6×10^7 independent transformants, respectively, which represent complexities within (or higher) than the range considered ideal for libraries constructed from immune vs. non-immune sources.⁴¹

Antibody libraries for each patient were each selected ("panned") against full-length human recombinant ADAMTS13. Library TTP1 was also panned against recombinant TSP1 5-8/CUB fragment of ADAMTS13.³⁴ After four rounds of panning for each library, antigen-positive clones were identified by ADAMTS13 ELISA. Nucleotide sequences for the heavy and light chains of each were determined to identify the number of unique antibodies obtained from each patient. The last column of Table 1 tabulates the number of antibody clones sampled from each patient library, the number of positives, and, of these, the number of unique antibodies. In sum, a cohort of 51 unique human monoclonal anti-ADAMTS13 antibodies was assembled for further study. Nomenclature for antibody clones are in the form "X-Y" where "X" is TTP patient number and "Y" is an arbitrary number.

Structural analysis of anti-ADAMTS13 autoantibodies shows evidence of clonal expansion and somatic mutation

The nucleotide sequences of the 51 antibodies showed use of the human heavy chain variable region gene V_H1-69 for 75% of the anti-ADAMTS13 antibodies (Table 2), a bias reported previously.²⁷ However, 13 of our 51 antibodies were derived from diverse V_H3- and

V_H6-family genes as well. That splenic tissue derived from TTP1 vs. peripheral blood lymphocytes from TTP2-TTP4 yielded the largest number of unique antibodies with the greatest genetic diversity was not surprising given that the TTP1 library was constructed from >1000-fold more B-cells. Furthermore, the spleen may be a reservoir for long-lived memory B-cells producing ADAMTS13 autoantibodies given that splenectomy is associated with long-term remission in TTP patients.⁴²

Table 2 also shows the D and J_H gene segments that had rearranged with V_H genes to form the entire heavy chain variable regions (i.e., V_HDJ_H) and their complementarity determining region-3 (HC-CDR3), the region of greatest diversity in an antibody's heavy chain. By exploiting the fact that there is only a remote probability that two B-cells will not only randomly select an identical combination of V_H, D, and J_H, but will also splice the genes together to create identical HC-CDR3 regions (theoretical probably <1 in 10¹¹),⁴³ one can use an HC-CDR3 to identify a B-cell clonotype. This is indicated in Table 2 by each separate line. For example, the heavy chains of antibodies 1-416, 1-428 and 1-304 would be predicted to have each been derived from the same original parental B-cell within patient TTP1 because they each share the identical HC-CDR3 ("AMDSVYGNFDF") though those 3 heavy chains are otherwise unique due to somatic mutation elsewhere in the heavy chain (Supplementary Fig. 1). Counting the number of different HC-CDR3 regions suggests that the 51 scFv heavy chains were derived from clonal expansion of 30 individual B-cells. Antibody light chain analysis showed use of both kappa and lambda, but light chains lack D segments which make it difficult to confidently assign discrete B-cell origins to every light chain that share similar CDR3's (Supplementary Table 1). Supplementary Fig. 1 provides sequence alignments of all 51 antibody heavy and light chains and indicates positions of replacement and silent mutations with respect to their most likely immunoglobulin germline genes.

Together, these data demonstrate that within each of the four TTP patients, the autoimmune response to ADAMTS13 was oligoclonal with multiple B-cells expanding to produce groups of related antibodies that underwent further somatic mutation. The relatively high ratios of replacement-to-silent mutations in HC-CDR1 and HC-CDR2 (>4.7, Supplementary Fig. 1A) are characteristic of antigen-driven clonal expansion⁴⁴ as we have previously found in the autoimmune repertoires in idiopathic thrombocytopenic purpura³² and pemphigus,³³ and alloimmune Rh(D) repertoire.³⁷

Relationship of ADAMTS13 inhibitory autoantibodies to genetic background and epitope specificity

Inhibitory activities of anti-ADAMTS13 antibodies varied from 0% to ~100% residual ADAMTS13 activity (Fig. 1A). For reference, the germline V_H gene from which the particular recombinant scFv was derived is shown above each bar. With only two exceptions (1-437 and 1-404), antibodies that significantly inhibited ADAMTS13 were encoded by V_H1-69 ($P = 1.4 \times 10^{-6}$). In contrast, anti-keratinocyte PX4-3 scFv³³ is also encoded by V_H1-69 (Fig. 1B) and had no effect on ADAMTS13 activity even when incubated with ADAMTS13 at a 5-fold greater concentration, indicating inhibitory activity is not conferred simply by the use of this heavy chain gene.

Epitope mapping was performed with a subset of 23 scFv's with different genetic backgrounds and inhibitory activities in order to ask whether the ability of an anti-ADAMTS13 antibody to inhibit ADAMTS13 proteolytic activity *in vitro* is related to where it binds the enzyme. Figure 2 summarizes the results (raw data in Supplementary Fig. 2 and Supplementary Table 2) and illustrates a diversity in epitope specificities similar to that found in patient plasma.^{18–22}

With only one clear exception (1-437), antibodies that inhibit ADAMTS13 proteolytic activity *in vitro* require the cysteine-rich/spacer region for binding. This is consistent with previous studies suggesting that antibodies that bind to the cysteine-rich/spacer region interfere with engagement of ADAMTS13 with VWF substrate.^{18,23–25} The fact that all 13 V_H1-69-encoded scFv's in this group require this region for binding is consistent with other reports^{26–28} and suggests that there is a feature expressed by V_H1-69 (independent of HC-CDR3 that is encoded primarily by the D gene, not V_H) that is either permissive or required (but not sufficient vis-a-vis anti-keratinocyte PX4-3 above) for an antibody to recognize features presented by immunodominant residues in cysteine-rich/spacer region-containing domains of ADAMTS13. For scFv's 1-420, 1-416, and 3-301, these results are also consistent with those of a separate study using hydrogen-deuterium exchange mass spectrometry in which we showed their specificity for the ADAMTS13 spacer region at near amino acid resolution.⁴⁵

Of the non-V_H1-69 inhibitory antibodies, 1-437 maps to a fragment containing the metalloprotease domain (potentially explaining its inhibitory activity), and the idiotope of 1-404 appears to make contact independently with both cysteine-rich/spacer-containing and CUB domains, perhaps stabilizing ADAMTS13 in a “closed” inactive conformation.^{46,47} ScFv 1-410 also binds to both cysteine-rich/spacer-containing domains and TSP 5-8/CUB domains but is not inhibitory. The 7 remaining scFv's target the C-terminal domains and do not inhibit enzymatic activity *in vitro*. It should be recognized, however, that ADAMTS13 activity assayed by measuring the cleavage of VWF peptides vs. VWF multimers may miss pathogenic effects of certain antibodies including those that target the C-terminal domains of ADAMTS13.

ADAMTS13 autoantibodies share crossreactive idiotypes

The observation that 13 of the 15 inhibitory scFv's in our subset of antibodies bind to identical ADAMTS13 regions and were encoded by the same V_H gene suggests that their idiotypes share features. If inhibitory anti-ADAMTS13 antibodies share idiotypes within and across patients, there would be rationale for developing therapies that recognize these common features to block antibody binding or attenuate their production. However, in general, the most important contributing factors to the structure of an antibody's idiotype are its heavy and light chain CDR3 loops which, for these antibodies, appear to be quite varied in length and amino acid sequence (Table 2, Supplementary Table 1, Supplementary Fig. 1). This would suggest that their idiotypes are quite different.

To explore idiotypic diversity within a set of ADAMTS13 autoantibodies, rabbit antisera were raised to V_H1-69-encoded 1-416, 1-420, 1-428, and 1-431. Antibodies 1-416 and 1-428 share the same heavy chain CDR3 while the heavy chain CDR3's of antibodies 1-420 and

1-431 are each distinct (Table 2) as are the light chain CDR3's in each of the 4 antibodies (Supplementary Table 1).

Binding of post-immune rabbit IgG to its scFv immunogen was ~100-fold greater than pre-immune IgG by ELISA (data not shown). Post-immune, but not pre-immune, IgG blocked its respective scFv's ability to inhibit ADAMTS13 as illustrated for 1-416 (Figure 3A). Similar patterns of reactions were found for rabbit IgG raised against 1-420, 1-428, and 1-431 (data not shown). The ability to block scFv-mediated inhibition of ADAMTS13 is not simply due to rabbit IgG molecules directed to human isotype (e.g., Fc γ) because scFv have no isotype (they comprise only V_H and V_L chains). The ability to block scFv-mediated inhibition cannot be due to rabbit IgG being directed to conserved human V_H/V_L framework regions or even to conserved V_H1-69-specific structural elements because the ability of rabbit IgG raised against 1-428 to block 1-428's inhibition of ADAMTS13 is unaffected by the presence of 8-fold excess human V_H3-33-encoded E1M2 (an anti-Rh(D) scFv)³⁷ or human V_H1-69-encoded PX4-3 (an anti-keratinocyte scFv)³³ (Fig. 3B). In this experiment, the amount of rabbit IgG was titrated down to the point of just being able to block 1-428 in order to increase its sensitivity to any effects of E1M2 or PX4-3.

To explore whether the idiotypes among the four scFv's share common features, anti-idiotypic IgG raised against a given scFv was tested for its ability to block the inhibition of ADAMTS13 by the other 3 scFv's. To increase the sensitivity of these assays, the amounts of scFv used were 2.5-fold less than the amounts used in Fig. 1. As shown in Table 3, there was evidence of broad crossreactivity among 3 of the 4 scFv. Marked inhibition of ADAMTS13 by 1-420 could not be blocked by anti-idiotypic IgG to any of the other 3 scFv's nor could anti-idiotypic IgG raised against 1-420 block the inhibition of ADAMTS13 by the other 3 scFv's. This finding serves as a convenient internal control showing that the rabbit IgG are not acting by binding to common human or V_H1-69 structures. This finding also suggests that for inhibitory antibodies directed to cysteine-rich/spacer region-containing domains, V_H1-69 features alone do not define the antibody's idiootype.

These data suggest that with one exception, a small set of ADAMTS13 inhibitory monoclonal antibodies derived from a single TTP patient share idiotypic determinants that can be targeted to prevent inhibition of ADAMTS13. The larger question is how representative these idiotypes are of the repertoire of idiotypes of polyclonal inhibitory immunoglobulin in the plasma of patients other than TTP1 from which the scFv's were derived.

To address this question, inhibition of ADAMTS13 by plasma from TTP4 and three additional TTP patients (TTP5-TTP7) was measured in the presence of anti-idiotypic IgG. As shown in Fig. 3C, inhibition of ADAMTS13 by polyclonal patient plasma-derived immunoglobulin was blocked to varying extents by anti-idiotypic IgG generated to a single monoclonal scFv from a completely unrelated TTP patient. The ability to block ADAMTS13 inhibition was striking in some cases (e.g., TTP7) with each of the anti-idiotypic IgG. These results cannot be attributed to rabbit antibodies to patient IgG Fc domains because the rabbits were immunized with scFv. That rabbit antisera might have pre-existing reactivity to human IgG was ruled out through the use of preimmune sera.

Therefore, these data support the clinical relevance of these cloned scFv's and suggest their use as targets for the design of small molecules that could block enough ADAMTS13 inhibitory IgG to raise ADAMTS13 activity above a clinical threshold.

DISCUSSION

Described in this report are the first examples of human antibodies specific for ADAMTS13 amino-terminal (MDT1) and carboxy-terminal (T5-8/CUB) domains, and their apparent diversity in V_H gene usage (Table 2) contrasts significantly to the marked V_H1-69 restriction of antibodies targeting ADAMTS13 domains containing the cysteine-rich/spacer region. Antibodies directed toward these amino- and carboxy-terminal domains are known to be present in TTP patient plasma^{19,21,48} and correlate with platelet count at disease onset,²¹ but their ability to inhibit ADAMTS13 proteolytic activity has not been demonstrated. We found that MDT1-binding 1-437 is as potent an inhibitor as any cysteine-rich/spacer region-directed antibody, perhaps by interfering with catalysis mediated by the metalloprotease domain. Our antibody cohort includes 6 CUB-specific antibodies and one TSP 2-8/CUB-specific antibody, none of which inhibit ADAMTS13 activity as assessed by cleavage of VWF peptide. However, 1-404 is an inhibitory antibody and independently binds to cysteine-rich/spacer-containing fragments and CUB regions, suggesting that its epitope comprises amino acid residues located in both regions. In light of recent reports proposing that ADAMTS13 normally circulates in a “closed” inactive form comprising an intramolecular CUB-to-spacer binding interaction subject to allosteric activation by VWF,^{46,47} we speculate that 1-404 may be exemplary of a class of autoantibodies that exert their pathogenic effect by stabilizing the enzyme's closed conformation. Though CUB-binding antibodies could reduce ADAMTS13 activity by enhanced clearance^{49–51} or by inhibiting other functions of the protease,^{34,52–55} they might function synergistically to stabilize the enzyme in an open conformation allowing spacer domain-specific antibodies to bind and block VWF binding to ADAMTS13.

Observations identifying the use of heavy chain gene V_H1-69 for spacer domain-directed inhibitory antibodies^{26,27} led investigators to hypothesize a “shape complementarity” between V_H1-69-encoded variable domain residues and exposed exosites in the spacer domain.³⁰ A unique hydrophobic “Ile-Ile-Pro-Ile-Phe” motif was noted in V_H1-69 CDR2 that might serve to facilitate interaction with hydrophobic residues present in the ADAMTS13 spacer domain, including Tyr661 and Tyr665.^{23,25} Recently, 4 additional V_H1-69-encoded spacer domain-directed patient antibodies were reported that also have an “Ile-Ile-Pro-Ile-Phe” in their CDR2.²⁸ Alignment of these 7 previously-reported V_H1-69-encoded antibodies bearing this CDR2 motif with the 38 V_H1-69-encoded antibodies reported here reveals some variability in amino acid residues occupying these CDR2 positions, though much of the variability is conservative (Table 4). The variability is, in part, because only certain alleles of the V_H1-69 gene (e.g., *I-69*01*) encode the “Ile-Ile-Pro-Ile-Phe” motif that was described initially. Of note, 37 of our 38 V_H1-69-encoded antibodies are derived from *I-69*09* or *I-69*10* alleles that have a leucine at position 62. Overall, all antibodies retain a proline at position 58, and two of the previously-reported antibodies, II-1²⁷ and 3b,²⁸ substitute a tyrosine for the phenylalanine at position 62.

Also shown in Table 4 is the CDR2 region of PX4-3, a V_H1-69-encoded anti-keratinocyte autoantibody that does not bind to or inhibit ADAMTS13 (Fig. 1) or prevent anti-idiotypic IgG from neutralizing ADAMTS13 inhibitory scFv (Fig. 3B). Unless amino acid residues isoleucine, valine, or methionine at position 59 cannot be replaced by threonine as in PX4-3, our results suggest that the hydrophobic CDR2 motifs of V_H1-69-encoded heavy chains cannot be solely responsible for ADAMTS13 binding. Our findings that anti-idiotypic IgG crossreact and block ADAMTS13 inhibition mediated by 1-416, 1-428, and 1-431, but not 1-420 (Table 3), when considered in the context of their CDR2 residues (Table 4), also suggest that the idiotopes of spacer domain-directed V_H1-69-encoded inhibitory antibodies comprise more than just their V_H CDR2 regions.

The collection of anti-ADAMTS13 autoantibody clones described in this report closely mimics the diversity of ADAMTS13 binding domains found in the plasma of patients with acquired TTP. It should be appreciated that the repertoires of anti-ADAMTS13 autoantibodies cloned from these four patients represent “snapshots” with respect to the full spectrums of pathogenic antibodies that may evolve over time, particularly during relapse as we recently demonstrated in patients with pemphigus vulgaris, an autoantibody-mediated blistering skin disease.⁵⁶ The idiotypic relatedness of our set of inhibitory antibodies to patient IgG supports their clinical relevance and suggests that they might serve as useful targets for the design of therapeutic agents that block IgG binding. In the second manuscript in this series⁵⁷, we explore antibody pathogenicity *in vivo* by transfecting mice with cDNA encoding these inhibitory antibodies and visualizing the effects of sustained antibody-mediated ADAMTS13 inhibition.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Inhibitory activity of human anti-ADAMTS13 monoclonal antibodies. ADAMTS13 activities of normal human plasma were measured using FRETs-VWF73 in the presence of (A) recombinant anti-ADAMTS13 scFv clones or PBS control (defined as 100% activity) or (B) >5-fold more of an irrelevant keratinocyte-binding human V_H1-69-encoded scFv PX4-3. Values for each scFv are the averages of 3 independent measurements (+/- SD). For reference, germline V_H genes from Table 2 are indicated above each bar.

Fig. 2.
Epitope specificities of anti-ADAMTS13 scFv. Using overlapping fragments of ADAMTS13 and immunoprecipitation with selected scFv, binding regions for antibodies were derived and indicated in cartoon map of ADAMTS13. For reference, heavy chain germline V_H genes from Table 2 and ADAMTS13 inhibitory activities (percent residual activity from Fig. 1) are indicated in parentheses next to the name of each clone. Raw data for this experiment are in Supplementary Table 2 and Supplementary Figure 2. Domain abbreviations: M, metalloprotease; D, disintegrin; 1 through 8, thrombospondin type 1 motifs 1 through 8; C, cysteine-rich domain; S, spacer domain; CUB, pair of CUB domains (complement C1r/C1s, Uegf, bone morphogenic protein 1).

Fig. 3.

Blocking ADAMTS13 inhibition with rabbit anti-idiotypic IgG. (A) ScFv 1-416 inhibited ADAMTS13 in the absence of rabbit IgG and in the presence of pre-immune rabbit IgG, but not in the presence of post-immune anti-scFv 1-416 rabbit IgG (right-hand set of bars; *, $P=0.006$; **, $P=0.008$; N.S., not significant). No scFv or irrelevant human anti-Rh(D) scFv E1M2 had any effect on ADAMTS13 activity under any rabbit IgG conditions (left-hand and middle sets of bars). Residual ADAMTS13 activity with scFv 1-416 and no rabbit IgG or pre-immune IgG (white and gray bars in right-hand set of bars) is higher than in Fig. 1 because the amount of scFv was 2.5-fold lower here to increase the sensitivity of rabbit post-immune IgG blocking. (B) In the presence of scFv 1-428, ADAMTS13 activity was inhibited when mixed with no rabbit or pre-immune rabbit IgG, but was “rescued” in presence of post-immune rabbit IgG (4th set of bars) as for scFv 1-416 in (A) (*, $P=0.002$; **, $P=0.0006$). Results were unchanged if performed in the presence of an 8-fold excess of human V_H3-33 -encoded anti-Rh(D) scFv E1M2 (5th set of bars) or human anti-keratinocyte V_H1-69 -encoded PX4-3 (6th set of bars). First 3 sets of bars are controls showing that scFv’s E1M2 and PX4-3 had no effect on ADAMTS13 activity themselves. In this experiment, the amount of rabbit IgG was titrated down to the point of just being able to block 1-428 in order to increase its sensitivity to any effects of E1M2 or PX4-3. (C) Gray bars show reduction of ADAMTS13 activity in normal plasma when mixed with heat-treated (56°C for 30 min to destroy any residual patient plasma ADAMTS13) TTP4-TTP7 plasmas and pre-immune rabbit IgG. Black bars show various degrees of “rescue” of ADAMTS13 activity if TTP4-TTP7 plasmas were pre-incubated with post-immune rabbit anti-idiotypic

IgG raised against one of 4 inhibitory scFv's as indicated. All differences significant (P range, 0.005 – 0.041) except for those marked "N.S." (P range, 0.059 – 0.098). ADAMTS13 activities are averages of 2 measurements except for TTP7/1-431 which were measured once (*).

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TABLE 1

Patient demographics, clinical data, and number of anti-ADAMTS13 antibodies isolated

Patient	Age/Sex/Race	Hgb (g/dL)	Plt ($\times 10^9/L$)	LDH (U/L)	Cr (mg/dL)	No. TPE	Source of B cells	No. scFv sampled/positive/unique
TTP1 [*]	10y/M/W	8.7	9	8845	0.9	109	spleen [†]	75/67/31 [‡]
TTP2 [§]	28y/F/W	9.6	7	2763	0.5	64	PB//	32/22/14
TTP3 [¶]	47y/F/B	11.5	35	761	1.1	48	PB//	16/12/4
TTP4 [#]	59y/M/W	9	14	3427	1.5	13	PB//	17/10/2

Patients were diagnosed with acquired TTP on the basis of thrombocytopenia, microangiopathic hemolytic anemia, and <10% plasma ADAMTS13 activity in the setting of inhibitory IgG.

M indicates male; F, female; W, White; B, Black; Hgb, hemoglobin concentration; Plt, platelet count; LDH, lactate dehydrogenase; Cr, creatinine; TPE, therapeutic plasma exchange procedure; PB, peripheral blood

^{*} Patient's course of TTP relapsed/remitted over period of 42 weeks until splenectomy. No recurrence in 12 years of follow-up.

[†] Antibody library constructed from 15 g spleen comprising $\sim 6 \times 10^9$ IgG-positive B cells.

[‡] Of 31 unique positive clones, 28 were obtained from selection against full-length ADAMTS13 and 3 were obtained from selection against the TSP1 5-8/CUB fragment of ADAMTS13.

[§] Patient had history of well-controlled lupus since 18 years of age. Experienced miscarriage a few months before diagnosis of TTP. Clinical course complicated by multiple subarachnoid hemorrhages and grand mal seizures. Diagnosed with lymphoma 12 years post-TTP.

[¶] Antibody library constructed from $\sim 1 \times 10^6$ IgG-positive B cells isolated from 50 ml peripheral blood collected prior to first plasma exchange.

^{¶¶} This episode of TTP was a relapse from initial diagnosis of TTP made 2 years earlier. Had splenectomy after this episode and no recurrence in over 10 years.

[#] Patient responded rapidly to TPE and has not had relapse in the past 8 years.

TABLE 2

Genetic features and clonality of anti-ADAMTS13 heavy chains

Patient	Antibodies grouped by heavy chain clonotype	V _H family	V _H gene	D gene	J _H gene	HC-CDR3
TTP1	1-416, 1-428, 1-304	1	1-69*09	D5-12*01	J4*02	AMDSVYGNFDF
	1-431, 1-417, 1-303	1	1-69*09	D221*02	J4*02	ARDLGDFGDS
	1-408	1	1-69*09	D1-20*01	J4*02	ARDSVIGTSD
	1-406	1	1-69*09	D4-23*01	J4*02	ARDVGDFGDS
	1-458 [†] , 1-401 [†] , 1-420	1	1-69*09	D1-26*01	J4*02	AREFSGGNYFDF
	1-438, 1-434	1	1-69*09	D2-8*01	J6*02	ARFLWGLDV
	1-432	1	1-69*09	D6-13*01	J3*01	ARGVAAGWNAFDV
	1-405	1	1-69*09	D3-22*01	J4*02	ARSSYSTFDY
	1-450	1	1-69*09	D1-26*01	J6*02	ASGDYYYDMAV
	1-423	1	1-69*09	D3-16*01	J4*02	SIGRYTYGHFDT
	1-418, 1-413	1	1-69*09	D6-19*01	J4*02	(T/V)SNGWSNFDV
	1-437	3	3-21*01	D3-3*01	J6*02	AAAYDFWSGYYF
	1-404, 1-441	3	3-30*04	D2-21*01	J4*02	ARDLRGGEDY
	1-403 [†] , 1-415 [†]	3	3-30*04	D3-3*01	J4*02	ARDTFSYYDFWRAFDY
	z1-402	3	3-30*04	D2-2*01	J4*02	AASSYFFPDF
TTP2	1-410, 1-407	3	3-43*01	D3-9*01	J4*02	AKDNGYDILTLYLD(S/Y)
	1-440, 1-451, z1-201	3	3-9*01	D3-22*01	J4*02	AKDPNSLYRSGSFDY
	z1-303	6	6-1*01	D6-19*01	J5*02	AREGQWLPNYFDP
	2-204, 2-102	1	1-69*09	D2-8*01	J4*02	ARDKGYANNYGAY
	2-207 [†] , 2-301 [†] , 2-304	1	1-69*09	D2-15*01	J4*02	ARDQGYANDYGAY
	2-103, 2-106, 2-305 [†] , 2-406 [†]	1	1-69*09	D2-8*01	J4*02	ARDQGYANNYGAY
	2-302	1	1-69*09	D6-6*01	J4*02	ARDQVFGAY
	2-203	1	1-69*09	D3-16*01	J4*02	ARDRGYANTYGAY
	2-206	1	1-69*09	D3-16*01	J4*02	ARDRGYDNKYGAY
	2-408	1	1-69*09	D2-8*01	J4*02	ARDRGYSNNYGAY
	2-108	3	3-7*01	D1-14*01	J4*02	ARSPGYFEDY
	3-305 [†] , 3-405 [†] , 3-302 [†]	1	1-69*01	D1-26*01	J3*01	AREARDSFDF

Patient	Antibodies grouped by heavy chain clonotype	V _H family	V _H gene	D gene	J _H gene	HC-CDR3
TTP4	3-301	1	1-69*10	D2-8*02	J4*02	ARDDTGRDDYFEY
	4-307	1	1-69*01	D5-12*01	J4*02	ARSGYSDAFDI
	4-303	1	1-69*09	D1-26*01	J4*02	ARGGGSYDFFDY

All antibodies were obtained by selecting phage display antibody libraries against full-length ADAMTS13 except those with names beginning with “z” which were isolated by selecting the TTP1 library against the TSPI 5-8/CUB fragment of ADAMTS13.

HC-CDR3 indicates heavy chain complementarity determining region 3.

[‡]Members of a clonotype identical at the heavy chain amino acid level but comprise unique antibodies due to mutations in the associated light chains (see Supplementary Table 2 and Supplementary Figure 1B).

TABLE 3

Blocking of scFv-induced ADAMTS13 inhibition by rabbit anti-idiotypic IgG

Rabbit IgG added	% ADAMTS13 activity in presence of scFv inhibitor			
	scFv 1-416	scFv 1-420	scFv 1-428	scFv 1-431
none	20 *	0	7 *	19 *
anti-scFv 1-416	93	0	84	13
anti-scFv 1-420	0	99	0	0
anti-scFv 1-428	75	8	100	72
anti-scFv 1-431	70	3	94	92

* Residual ADAMTS13 activities with scFv's alone are higher than in Fig. 1 because a 2.5-fold lower amount of scFv was used in order to increase sensitivity of blocking scFv by rabbit IgG

TABLE 4

Comparison of CDR2 amino acid residues for V_H1-69 encoded antibody heavy chains

Antibody	Heavy chain amino acid							Reference
	56	57	58	59	60	61	62	
<i>I-69*01 germline gene</i>	I	I	P	I	--	--	F	IMGT ³⁵
<i>I-69*09 & *10 germline genes</i>	.	.	.	.	--	--	L	IMGT ³⁵
I-9, I-10, I-27	.	.	.	.	--	--	.	Lukens ²⁶ , Pos ²⁷
B-2, B-6, B-5, 3j	.	.	.	.	--	--	.	Schaller ²⁸
II-1	.	.	.	.	--	--	Y	Pos ²⁷
3b	.	.	.	.	--	--	Y	Schaller ²⁸
I-416	V	.	.	V	--	--	L	current study
I-420, I-401, I-458, I-450, I-418, I-413, 2-103, 2-106, 2-302, 4-303, 3-302, 3-305, 3-405, 3-301	.	.	.	.	--	--	L	
I-428, I-304, I-408, I-405, I-423	.	.	.	V	--	--	L	
I-431	F	V	.	.	--	--	L	
I-417, I-303, I-406	F	.	.	.	--	--	L	
I-438	V	.	.	.	--	--	L	
I-434, I-432, 2-204, 2-305, 2-406, 2-203, 2-206, 2-408	.	V	.	.	--	--	L	
2-102	V	V	.	.	--	--	L	
2-207, 2-301, 2-304	.	T	.	.	--	--	L	
4-307	.	.	.	M	--	--	.	
PX4-3	.	.	.	T	--	--	L	Payne ³³

Antibodies are grouped by identical CDR2 amino acid residues in positions 56-62. Replacement mutations are indicated with letters, identities with ".", and gaps with "--". CDR2 numbering designations per Brochet et al.³⁵