Acquired von Willebrand Disease Caused by an Autoantibody Selectively Inhibiting the Binding of von Willebrand Factor to Collagen

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An 82-year-old man with a low-grade malignant non-Hodgkin lymphoma and an IgG, A monoclonal gammapathy presented a recently acquired bleeding tendency, characterized by recurrent epistaxis, easy bruising, and episodes of melaena, requiring packed red blood cell transfusions. Coagulation studies showed a von Willebrand factor (vWF) defect [Ivy bleeding time, >15 minutes; vWF antigen (vWF:Ag), 0.08 U/mL; ristocetin cofactor activity (vWF:RCoF), <0.05 U/mL; collagen binding activity (vWF:CBa), 0.01 U/mL; absence of the high molecular weight multimers of vWF on multimeric analysis]. Mixing experiments suggested the presence of an inhibitor directed against the vWF:CBa activity of vWF without significantly inhibiting the FVIII:C, vWF:Ag, and vWF:RCoF activities. The inhibitor was identified as an antibody of the IgM class by immunoabsorption of vWF and inhibitor-vWF complexes from the plasma of the patient. Subsequent immunoprecipitation experiments using recombinant fragments of vWF showed that the inhibitor reacted with both the glycoprotein Ib binding domain (amino acids [aa] 422-826) and the A3 (aa 909-1112) domain of vWF, but not with the A2 (aa 716-908) or D4 (aa 1183-1535) domains. We conclude that the IgM autoantibody inhibits the vWF:CBa activity by reacting with an epitope present on both the glycoprotein Ib and A3 domains of vWF.

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CASE REPORT

A 82-year-old man with an IgG, A paraprotein (5 g/L) was referred to our department in 1989 for further analysis of a prolonged partial thromboplastin time discovered before a liver biopsy in the staging procedure of a non-Hodgkin lymphoma. Physical examination showed a slight hepatosplenomegaly, without enlarged lymph nodes. Hemoglobin levels and leukocyte, lymphocyte, and platelet counts were normal, as were the serum values for total IgG, IgA, and IgM. The bone marrow aspirate showed a normocellular hematopoiesis with 8% monoclonal plasma cells (cIgM+G A). Additionally, a monoclonal B-cell population (slgM A) represented 5% and 21% of the total B-cell population in blood and bone marrow, respectively. Skeletal x-rays showed no osteolytic lesions. The diagnosis was made as low-grade malignant non-Hodgkin lymphoma stage IV with paraproteinemia IgG, A.

Before 1989, the patient had no personal or family history of a bleeding tendency. Previous hemostatic challenges (appendectomy, dental extractions, and cholecystectomy) were uneventful. From 1989 on, the patient developed several episodes of easy bruising, recurrent epistaxis, and severe gastrointestinal blood loss, necessitating extensive replacement therapy with packed red blood cells and factor VIII (FVIII)/vWF concentrates. Gastrointestinal radiographic and endoscopic studies did not show a source of blood loss, except for a mild gastritis at one occasion. A transurethral prostatectomy was successfully performed with the administration of DDAVP and frequent cryoprecipitate infusions. However, trial of cyclophosphamide (2 mg/kg body weight) for 6 weeks did not improve the coagulation defect.

MATERIALS AND METHODS

Coagulation assays. Bleeding times were measured according to Ivy et al. Platelets were counted in EDTA blood samples using the Platelet Analyzer 810 (Baker Instruments, Allentown, PA). Platelet aggregation induced by 0.63 and 1.75 mg/mL ristocetin (H. Lundbeck Co, Copenhagen, Denmark) was recorded with the use of a serial aggregometer (Payton model 300B; Scarborough, Canada) in platelet-rich plasma at a standard count of 200 x 10^7/L. FVIII coagulant activity (FVIII:C) was assayed by means of the Automatic Coagulation Laboratory (ACL; Instrumental Laboratory, Ijsselstein, The Netherlands) using FVIII-deficient plasma (Ortho Diagnostic Systems, Beersse, Belgium).

vWF assays. vWF antigen (vWF:Ag) was assayed by an enzyme-linked immunosorbent assay (ELISA), as described by Cerjak. Ristocetin cofactor activity (vWF:RCoF) was assayed with formalin-fixed platelets using a PAP 4 Model Aggregometer (Bio/Data Corp, Hatboro, PA).
**vWF binding to collagen.** The collagen binding activity of vWF (vWF:CBA) was measured according to the ELISA-based method of Brown and Bosak with slight modifications. Microtiter plate wells (AS S Nunc, Roskilde, Denmark) were coated overnight at 4°C with 100 μL of a 0.2 mg/mL suspension of collagen (bovine achilles tendon, type I; Sigma Chemicals, St Louis, MO) in 20 mMol/L acetic acid. After washing with phosphate-buffered saline (PBS)-0.05% Tween 20 (PBS-Tween), 100 μL of 1/40 dilutions of plasmas in PBS-Tween 20-1% albumin (PBS-Tween-Albumin) were added to the wells and incubated for 2 hours at room temperature. After rinsing, the wells were incubated with 100 μL of a 1/200 dilution in PBS-Tween-Albumin of horseradish peroxidase-conjugated rabbit polyclonal Igs to human vWF (Dakopatts A/S, Glostrup, Denmark) for another 2 hours at room temperature. After washing, 100 μL ABTS substrate solution (27.4 mg ABTS (2,2’-azino-di-[3-ethylbenzthiazolsulphonate)]) dissolved in 10 mL buffer, containing 10 mMol/L citric acid, 100 mMol/L NaH2PO4.H2O, and 10 μL 30% H2O2) was added. After 15 minutes of incubation, the color development was stopped by the addition of 10 μL concentrated acetic acid and the extinction was measured at 414 nm.

**vWF multimeric analysis.** The multimeric composition of vWF was analyzed by sodium dodecyl sulphate (SDS) 0.9% agarose gel electrophoresis according to Brostad et al.14

**Inhibitor screening.** Assay of an inhibitor against FVIII-C was performed as described by Kasper et al.15 Inhibitors of vWF:Ag, vWF:RCoF, and vWF:CBA were sought by incubating 1 vol of patient plasma (PP) with increasing volumes of pooled normal plasma (NP) for 1 hour at 37°C. Equal volumes of normal pooled plasma and PBS served as control samples.

**Infusion studies.** The commercial vWF concentrate Haemate P (Behring, Marburg, Germany) was infused (3,000 IU) at the time of the construction and expression of recombinant vWF (NWF) in 20 mmol/L acetic acid. After washing with phosphate-buffered saline (PBS)-0.05% Tween 20-1% albumin (PBS-Tween-Albumin) were added to the wells and incubated for 2 hours at room temperature. After rinsing, the wells were incubated with 100 μL of a 1/200 dilution in PBS-Tween-Albumin of horseradish peroxidase-conjugated rabbit polyclonal Igs to human vWF (Dakopatts A/S, Glostrup, Denmark) for another 2 hours at room temperature. After washing, 100 μL ABTS substrate solution (27.4 mg ABTS (2,2’-azino-di-[3-ethylbenzthiazolsulphonate()]) dissolved in 10 mL buffer, containing 10 mMol/L citric acid, 100 mMol/L NaH2PO4.H2O, and 10 μL 30% H2O2) was added. After 15 minutes of incubation, the color development was stopped by the addition of 10 μL concentrated acetic acid and the extinction was measured at 414 nm.

**Identification of the Ig class of the inhibitor with the use of anti-vWF immunobeads.** To identify the Ig class of the inhibitor, rabbit polyclonal Igs to human vWF (Dakopatts) were coupled to Immunobeads (Biorad, Hercules, CA) according to the instructions of the manufacturer. Two hundred microliters of PP (vWF:Ag as estimated by ELISA 0.25 ± 0.03 U/mL), 200 μL of NP (vWF:Ag 1.00 ± 0.04 U/mL), or, to allow complex formation between eventually free inhibitor and vWF, 200 μL of a mixture of PP and NP (ratio, 76:1) was diluted in 1,000 μL PBS-Tween-Albumin. After incubation overnight at 4°C, 40 μL of anti-vWF immunobeads was added and incubated for 3 hours at room temperature. It was previously established that the binding capacity of 40 μL of anti-vWF immunobeads was in excess for 200 μL NP (data not shown). The immunobeads were then pelleted (for 10 minutes at 1,500g at room temperature) and washed three times with 10 mL PBS-Tween and resuspended in 500 μL of a 1/1,000 dilution in PBS-Tween-Albumin of horseradish peroxidase-conjugated rabbit polyclonal Igs to either human IgM, IgG, or IgA (Dakopatts). After 3 hours of incubation at room temperature, the immunobeads were again pelleted and washed three times with PBS-Tween and finally resuspended in 1,000 μL of ABTS substrate solution. After 10 minutes, the color development was stopped with the addition of 100 μL concentrated acetic acid. After pelleting the immunobeads (for 10 minutes at 1,500g at room temperature), the extinction of the supernatant was measured at 414 nm. The background extinction was estimated by performing this experiment with 1,000 μL PBS-Tween-Albumin instead of plasma. All experiments were performed in duplicate.

**Expression of recombinant vWF fragments.** A detailed description of the construction and expression of recombinant vWF (rWF) and rWF fragments in baby hamster kidney (BHK) cells will be published elsewhere (Vink et al, submitted). Briefly, the coding sequence for the GPIbα binding domain of vWF (amino acids [aa] 422-826), the A2 domain (aa 716-908), the A3 domain (aa 909-1112), and the D4 domain (aa 1183-1535), were spliced to the coding sequence of the signal peptide of vWF (Fig 1). This construct was introduced in the mammalian expression vector pNUT15 and stable cell lines were obtained after transfection of BHK cells and selection with methotrexate. The transient expression of rWF and a mutant lacking the A1 domain (ΔA1-rWF) in Cos cells, has been described.17 Both these constructs were introduced in the pNUT vector and stable cell lines were established in BHK cells.

**Metabolic labeling.** Cell lines were washed twice with Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Paisley, UK) medium and starved for 1 hour in DMEM without methionine and cysteine. Trans-S-label (ICN Biomedicals, Costa Mesa, CA) was added at 500 μCi and the cells were incubated for 4 hours at 37°C. Unlabeled methionine and cysteine (150 μg/mL) medium was then added. The conditioned medium was harvested after 20 hours. Dead cells were spun down and protease inhibitors were added (1 mMol/L phenylmethylsulfonyl fluoride, 10 mMol/L EDTA, 10 mMol/L benzamidine, and 5 mMol/L N-ethylmaleimide [N-EM]).

**Immunoprecipitation.** Conditioned medium (100 μL) was mixed with 400 μL immunoprecipitation buffer (10 mMol/L Tris, 150 mMol/L NaCl, 1% Nonidet P-40, and 0.5% bovine serum albumin, pH 7.4), mixed with 5 μL serum of the patient, and rotated for 2 hours at 4°C. Twenty microliters of rabbit polyclonal Igs to human IgM (Dakopatts) was added and rotated for 1 hour at 4°C, after which 40 μL protein A-sepharose (Pharmacia, Uppsala, Sweden) was added and rotated for 1 hour at 4°C. The Sepharose beads were pelleted (for 30 seconds at 14,000g at room temperature) and washed four times with 1 mL of immunoprecipitation buffer. Bound protein was eluted (for 5 minutes at 10°C) with reducing sample buffer and analyzed by polyacrylamide gel electrophoresis (PAGE) on a 5% 25% gel in the presence of SDS, performed according to Laemmli.26 Competition experiments with unlabeled fragments were performed by incubating 100 μL conditioned medium, with 100 μL 10× immunoprecipitation buffer and 800 μL of BHK
medium or a mixture of the GPIb and A3 domain followed by immunoprecipitation, as described above. Immunoprecipitation of $^{125}$I-vWF was performed by incubating 1 $\mu$g $^{125}$I-vWF (10 $\mu$L) with 100 $\mu$L 10$\times$ immunoprecipitation buffer, 10 $\mu$L of the patient's serum, and 900 $\mu$L of the culture media, followed by an immunoprecipitation, as described. The $^{125}$I-vWF precipitations were scanned with a Phospholmager (Molecular Dynamics, Sunnyvale, CA) and analyzed using ImageQuant software (Molecular Dynamics).

**RESULTS**

**Platelet function and vWF-related activities in plasma.** The initial hemostatic data of our patient were characterized by a prolonged Ivy bleeding time (>15 minutes; normal, ≤4 minutes), absent ristocetin-induced platelet aggregation, low levels of FVIII:C (0.10 U/mL; normal, 0.60 to 1.40 U/mL) and vWF:Ag (0.08 U/mL; normal, 0.60 to 1.40 U/mL), and very low to undetectable levels of vWF:RCoF (<0.05 U/mL; normal, 0.60 to 1.40 U/mL) and vWF:CBA (0.01 U/mL; normal, 0.60 to 1.40 U/mL), confirming the diagnosis of vWD. A selective absence of the high molecular weight multimers of vWF, as seen in patients with type IIa congenital vWD, was noted (Fig 2, preinfusion value). The absence of the high molecular weight multimers of vWF was not caused by increased proteolysis, as shown by subunit analysis of vWF, or caused by the binding of vWF to malignant lymphoma cells (results not shown).

**Infusion studies.** Infusion of 3,000 U of the vWF concentrate Haemate P (Fig 2) resulted in a partial correction of the bleeding time. In addition, a rapid clearance to preinfusion levels of the FVIII/vWF-related activities and the high molecular weight multimers of vWF was observed. Interestingly, the vWF:CBA activity remained decreased immediately after Haemate P infusion, despite normal levels of ristocetin cofactor activity and vWF antigen.

**Inhibitor of FVIII/vWF.** The patient plasma contained no inhibitory activity against FVIII:C (data not shown). Incubation of different volumes of normal pooled plasma with 1 vol of PP did not induce significant inactivation of the vWF:Ag and vWF:RCoF activities (results not shown). However, a significant reduction of the vWF:CBA activity was observed in the mixture of 1 vol of PP with 1 vol of normal pooled plasma (0.29 ± 0.02 U/mL) as compared with controls (0.49 ± 0.02, P < .001). Incubation of 1 vol of PP with increasing volumes of normal pooled plasma showed that the inhibitory effect of the PP on the vWF:CBA activity was lost after incubating 1 vol of PP with 9 vol of normal pooled plasma (1.02 ± 0.03 U/mL v controls 1.04 ± 0.06; P > .05). No significant changes of the vWF:Ag, vWF:RCoF, and vWF:CBA activities were measured after prolonged incubation (data not shown). The mixture of normal and patient plasma was found to retain the high molecular weight multimers of vWF, indicating no specific in vitro proteolysis of vWF (data not shown).

**Identification of the Ig class of the inhibitor with the use of anti-vWF immunobeads.** The results of a representative experiment of immunoabsorption of vWF or vWF-Ig complexes are shown in Fig 3. Two times more IgM, bound to vWF isolated by immunoabsorption of plasma from the patient, was observed in comparison to the results found when using 200 $\mu$L of normal pooled plasma. This indicates the presence of IgM-vWF complexes in the plasma of the patient. Preincubation of normal pooled plasma with an excess of PP (ratio 1:76) resulted in a sevenfold increase in the binding of IgM to vWF isolated from the mixture of normal pooled and patient plasma as compared with normal pooled plasma. The binding of IgG and IgA was comparable. Furthermore, after the plasma of the patient was depleted of IgM, no inhibition of the vWF:CBA activity was observed in mixing experiments with normal pooled plasma (data not shown).

**Immunoprecipitation of rvWF fragments by the inhibitor.** To determine which part of vWF interacted with the inhibitor, recombinant fragments of vWF expressed in mammalian cells (Fig 1) were immunoprecipitated. As shown in Fig 4A, the inhibitor reacted both with the GPIb binding domain and the A3 domain of vWF, but not with the A2 or the D4 domains of vWF. This indicates that either the inhibitor has an epitope present on aa 422-715 and aa 909-1112 or that two anti-vWF antibodies are involved. To further show that the inhibitor reacted exclusively with the vWF GPIb and A3 domains, we performed additional inhibition experiments. Immunoprecipitation studies (Fig 4B) showed that an excess of mixed unlabeled GPIb and A3 domain could compete for the immunoprecipitation of metabolically labeled rvWF and ΔA1-rvWF (missing aa 478-717) by the inhibitor. These domains seem to be the only parts of vWF interacting with the inhibitor(s). To investigate the monoclonality of the in-
Acquired von Willebrand Disease

Inhibitor, we performed additional immunoprecipitations of $^{125}$I-vWF with serum of the patient. As shown in Fig 5, an excess of either unlabeled recombinant GPIb fragments or unlabeled recombinant A3 fragments was able to compete for binding of the inhibitor to $^{125}$I-vWF to the same extent as unlabeled vWF, thereby excluding the presence of two anti-vWF antibodies. The culture medium, unlabeled recombinant A2 domain, and unlabeled recombinant D4 domain were unable to compete for binding of the inhibitor to $^{125}$I-vWF.

**DISCUSSION**

The adhesion of platelets to the exposed subendothelium or perivascular collagen at sites of vascular injury requires the binding of vWF to these tissues. 21 The high molecular weight multimers of vWF are known to play an important role in this initial phase of hemostasis by expressing numerous binding sites for collagen 22 and other subendothelial constituents. 23 In the described patient, an IgM autoantibody was found to be involved in the pathogenesis of acquired vWD that had severe consequences in vivo.

In the last 3 years, the patient developed a progressive bleeding tendency consisting of recurrent epistaxis, easy bruising, and severe gastrointestinal blood loss, requiring almost weekly hospitalization. To achieve adequate hemostasis at times of bleeding, intensive replacement therapy with DDAVP and FVIII/vWF concentrates was instituted with only minor improvement. This finding suggested the presence of a circulating inhibitor to the FVIII/vWF complex.

Inhibitors of vWF in plasma are normally shown in mixing experiments with normal pooled plasma by their ability to inhibit an immunologic (vWF:Ag) or functional activity (vWF:RCoF) of vWF. In this patient, the mixing experiments did not indicate the presence of an inhibitor. However, the vWF:CBA activity, which reflects the in vitro ability of vWF to bind to collagen type I, was selectively inhibited. This suggests that the inhibitor was directed against an epitope involved in the binding of vWF to collagen type I or located near these collagen binding domains, causing steric hindrance of the vWF-collagen interaction.

The results of the infusion study are in agreement with this hypothesis. After Haemate P infusion, a discrepancy between the functional activities of vWF, the vWF:RCoF, and vWF:CBA activity, was noted. The vWF:RCoF and vWF:Ag activities normalized after infusion, indicating the presence of sufficient amounts of functional vWF. Nevertheless, the bleeding time remained prolonged. In contrast, the vWF:CBA activity remained decreased. This finding may indicate a correlation between the collagen-binding activity and the bleeding time in this patient.

There was a strikingly rapid clearance of the vWF-related activities vWF:Ag, vWF:RCoF, and vWF:CBA and the high molecular weight multimers of vWF after infusion. As increased proteolysis of vWF and binding of vWF to lymphoma cells were ruled out, the decrease of high molecular weight multimers of vWF is probably explained by a preferential complex formation between the inhibitor and the high molecular weight multimers of vWF, as observed by others. 3, 6 These multimers are known to possess the ristocetin cofactor activity and have numerous collagen-binding domains. Subsequently, these vWF-inhibitor complexes are rapidly cleared from the circulation by the mononuclear phagocytic system.

vWF has two domains involved in the interaction with collagen type I and III. 24 They reside on part of the GPIb-binding domain (aa 422-826) known as the A1 domain and the A3 domain (aa 909-1112). With the use of several separate vWF fragments in the immunoprecipitation experiments, we could show that the inhibitor was reactive with the GPIb and A3 domains of vWF. Despite a considerable heterogeneity between the two collagen-binding domains of vWF, the inhibitor recognized an epitope occurring on both domains. Several mouse monoclonal antibodies (MoAbs) that also inhibit the binding of vWF to collagen have been described. 25-27 Most of them were reactive either with the A1 25 or the A3 domain. 25-27 Our autoantibody resembles the characteristics of the mouse MoAb described by Roth et al 24 that was also reactive with both collagen-binding domains.

To our knowledge, this is the first antibody of human origin inhibiting the binding of vWF to collagen, resulting in a virtual absence of functional vWF and a concomitant bleeding tendency. In contrast to severe congenital vWD, in which there is a comparable absence of functional vWF, our patient did not suffer from muscle or joint bleedings. Apparently the amount of FVIII:C present was sufficient.
Fig 4. Immunoprecipitation of metabolically labeled culture supernatant of recombinant vWF fragments with patient serum. Precipitates were separated using 4% to 15% SDS-PAGE and autoradiographed for 20 days. (A) Lane 1, immunoprecipitation of patient’s serum with labeled GPIb domain supernatant (the arrow indicates the position of the precipitated GPIb domain); lane 2, labeled A2 domain supernatant; lane 3, labeled A3 domain supernatant (the arrow indicates the position of the precipitated A3 domain); lane 4, labeled D4 domain supernatant. (B) Competition immunoprecipitation experiment with excess unlabeled GPIb, A3 domains, or excess BHK culture medium. Lane 1, immunoprecipitation of labeled A1-rvWF, a mutant lacking the A1 domain of vWF, in the presence of an excess BHK culture medium; lane 2, same as lane 1, but labeled ΔA1-rvWF was precipitated in the presence of an excess mixture of unlabeled GPIb and A3 domain fragments; lane 3, labeled rvWF precipitated in the presence of an excess BHK culture medium; lane 4, same as lane 3, but labeled rvWF was precipitated in the presence of an excess of unlabeled GPIb and A3 domain fragments. The two visible bands in lanes 1 and 3 represent the propolypeptide and the mature subunit of vWF, which are due to inefficient processing in the BHK cells. Note the size difference between the deletion mutant ΔA1-rvWF, missing 239 amino acids, in lane 1 and the rvWF in lane 3.

enough to prevent these hemophilia-like bleeding complications.

In conclusion, in this study a case of acute vWD is described caused by an IgM autoantibody that inhibits the binding of vWF to collagen and that reacts with both the GPIb and A3 domains of vWF. Although several investigators using cultured endothelial cells have recently shown that it is unlikely that subendothelial collagen is the binding site
ACQUIRED VON WILLEBRAND DISEASE

Fig 5. Immunoprecipitation of $^{125}$I-vWF with the patient’s serum, in competition with the four recombinant fragments of vWF and cold rvWF, showing that both the GPIb and A3 domain of vWF, as well as full-length rvWF, were able to compete with the binding of the inhibitor to vWF, whereas the A2 and D4 domain of vWF and excess BHK culture medium were not. Precipitates were analyzed using 5% PAGE, scanned using a Phospholmager and analyzed using Imagequant software. Lane 1, excess BHK culture medium (CM); lane 2, 20-fold excess of cold vWF (vWF); lane 3, excess GPIb (GPIb) culture medium; lane 4, excess A2 (A2) culture medium; lane 5, excess A3 (A3) culture medium; lane 6, excess D4 (D4) culture medium.

This patient shows that disturbing the interaction of vWF with collagen type I in vivo results in a severe bleeding tendency. Apparently, collagen located elsewhere in the vessel wall plays a pivotal role in maintaining proper hemostasis by providing a binding site for vWF.

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Acquired von Willebrand disease caused by an autoantibody selectively inhibiting the binding of von Willebrand factor to collagen

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