Monoclonal gammopathy of clinical significance: \textit{In vivo} demonstration of the anti-thrombotic effect of an acquired anti-thrombin antibody

Monoclonal gammopathy of clinical significance (MGCS) is a novel concept that has been suggested to describe an abnormal plasma cell clone producing a monoclonal immunoglobulin (MIg) associated with pathological manifestations.\(^1\) One of the MGCS-related complications includes autoantibody activity. To date, MIg-associated bleeding disorders have been essentially reported with auto-antibodies directed against von Willebrand factor (VWF) or factor VIII (FVIII).\(^2,3\) To our knowledge, no MGCS associated with an auto-anti-thrombin antibody (ATA) has yet been described. ATA have been reported in several cases of bleeding\(^4\) or thrombosis\(^5\) but can also be asymptomatic.\(^6\) This is a rare disorder that may develop after bovine thrombin exposure\(^7\) or in association with anti-phospholipid syndrome, liver cirrhosis\(^8\) or viral infection.\(^10\) Due to their versatile clinical presentation and the absence of specific diagnostic tests, ATA constitute a heterogeneous group of disorders that are difficult to characterize.

Here we present the case of a patient with a MIg (IgG\(_k\)) and an ATA. The use of a diverse array of \textit{in vitro} and \textit{in vivo} models were applied to establish the causal relationship between MIg, ATA and bleeding tendency, sustaining the concept of MGCS-related bleeding disorder.

The case concerns a 40-year-old man with no previous personal or family history of bleeding until he first presented with a psoas hematoma and hematuria. A prolonged activated partial thromboplastin time (aPTT) associated with an IgG\(_k\) monoclonal gammopathy (MIgG, monoclonal component evaluated at 12 g/L) was discovered on this occasion. Bone marrow plasma cell percentage was below 5\%, \(\beta\)2-microglobulin and light chains were found normal and the patient did not present any CRAB symptoms as assessed by imaging (\textit{data not shown}) and blood tests (Online Supplementary Data). All these elements allowed to exclude the diagnosis of myeloma. The prolonged aPTT was not corrected by the addition of normal pooled plasma (NPP) and prothrombin time (PT), fibrinogen as well as FVIII/VWF were within the normal range. No further hemostatic investigations were performed, and the bleeding tendency was related to an unspecified acquired disorder. Over the next 6 years, bleeding episodes (rectal bleeding, retroperitoneal hemorrhage, muscular hematomas) were managed by

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure1.png}
\caption{In \textit{vivo} patient’s laboratory evaluation. (A) Routine coagulation tests were performed on platelet poor plasma (PPP) obtained from citrated blood after double centrifugation using APTT SP HemosIL (IL), Innovin (Siemens), Dade Thrombin (Siemens) and using a STAR®-MAX (Stago) according to manufacturer’s instructions. The detection of lupus anticoagulant was performed using a diluted Russel’s Viper Venom Time (dRVVT, LAC screen and LAC confirm, Siemens) and using CS SI100 (Sysmex) according to manufacturer’s instructions. Thrombin time (TT) mixing studies were performed on patient’s PPP and a normal pooled plasma (NPP) obtained from 30 healthy volunteers. Mixing studies (vol/vol) were performed by measuring TT of serial dilutions from 1:1 to 1:64 of patient’s PPP in NPP, and in NPP diluted in Owren Koller buffer as control (NPP+OK, vol/vol); (B) Thrombin generation measurement using calibrated automated thrombogram (CAT). Thrombin generation test (TGT) was studied on PPP obtained from citrated blood. Coagulation was initiated using low concentration of tissue factor 1 (TF1) (1 pM) in the presence of phospholipid (4 \(\mu\)M) and using CAT according to manufacturer’s instructions (PPP Reagent Low, Stago). TGT was performed in patient’s PPP and normal pooled plasma (NPP) obtained from 30 healthy volunteers. Mixing studies (vol/vol) were performed by measuring TGT of serial dilutions from 1:1 to 1:32 of patient’s PPP in NPP, and in NPP diluted in Owren Koller buffer as control (NPP+OK, vol/vol); Insert: TGT was performed in patient’s and control platelet rich plasma (PRP) obtained from citrated blood. Coagulation was initiated using low concentration of tissue factor (TF) (1 pM) and using CAT according to manufacturer’s instructions (PRP Reagent, Stago). aPTT: activated partial thrombin time; TT: prothrombin time.}
\end{figure}
recombinant activated factor VII (rFVIIa) and several courses of dexamethasone, that allowed to temporarily normalize aPTT. Both azathioprine and rituximab were unsuccessful in reducing the bleedings and normalizing aPTT.

He was referred to us for precise diagnosis of the hemostatic disorder. Blood count was normal and monoclonal component was stable. A thrombin time (TT) was performed along with aPTT, both were markedly prolonged (>120 and 76 seconds [sec], respectively vs. 16 and 35 sec for control). The prolonged TT was observed using bovine and human thrombin. PT was slightly modified (11.9 vs. 9.5 sec for control, international normalized ratio [INR]: 1.36) (Figure 1A). Coagulation factors, including fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI and reptilase time were normal. Any presence of anticoagulant treatment was excluded (data not shown). Mixing studies (vol/vol) were performed by measuring TT of serial dilutions of patient’s platelet poor plasma (PPP) in NPP, to quantitate the inhibitor level (modified Bethesda assay). The results showed prolonged TT from dilutions 1:1 to 1:16 (from >120-28 sec), and normalization from 1:32 (21 sec) suggesting the presence of an ATA estimated at 32 Bethesda Units. A lupus anticoagulant was also detected without any other autoantibody or dysimmune abnormality, except the patient’s MIgG (no anticardiolipin or anti-beta2GPI antibodies).

In order to highlight the acquired plasmatic ATA-induced hemorrhagic tendency ex vivo, a thrombin generation test (TGT) was performed (CAT, Stago®) (Figure 1B). Compared to control, TGT in patient’s platelet rich plasma (PRP), showed a prolonged lag time (LT) of 15.5 minutes (min) associated with a decreased endogenous thrombin potential (ETP) of 834 versus 1,730 nM/min corresponding to a 300% increase of LT and a 50% decrease of ETP, respectively. In patient’s PPP, no peak of thrombin generation could be detected within 1 hour (h). Similarly to TT, TGT was performed using several dilutions of the patient’s PPP in NPP. With each dilution, the LT was shortened, ranging from more than 60-6 minutes (min) versus 5.15 min in NPP. ETP never exceeded 575 nM/min for the 1:32 dilution compared to 1,182 nM/min in NPP. These results equal to an increased LT ranging from 660-20% and a decreased ETP ranging from 75-51% for dilutions from 1:1 to 1:32 respectively.

In order to confirm that the ATA belong to the same clone as the MIgG, the electrophoresis pattern of the antibodies was studied using isoelectrofocusing on agarose gel followed by immunofixation with an anti-IgG antiserum (Hydragel CSF Isofocusing®, Sebia). The patient’s ATA was isolated from patient’s plasma by affinity chromatography using activated agarose beads (Aminolink®, ThermoScientific) covalently coupled to human α-thrombin (2 mg). The patient’s total IgG was purified using protein A agarose (ThermoScientific). The immunofixation of plasmatic IgG, purified total IgG and ATA showed a similar monoclonal banding, suggesting that the ATA was indeed part of the MIgG, and not a coincidental autoantibody (Figure 2). The diagnosis of MGCS-related bleeding disorder due to a MIgG and an ATA was retained. In order to eradicate the ATA, a combination of bortezomib and dexamethasone was initiated (at doses used in myeloma patients), but four cycles of this combination did not decrease the MIgG. Continuous thalidomide and dexamethasone treatment was also unsuccessful. The patient’s TT was only temporarily shortened 24-48 h after intravenous infusion of IgG. Over the years, the patient continued to present bleeding episodes (hematospermia, psoas hematoma). Given the failure of the previous therapies and the efficacy of “on demand” hemostatic treatment, the patient was finally managed by close follow-up, self-initiated combination of steroids and rFVIIa in case in case of hemorrhage.

In order to illustrate the anti-coagulant effect of the
In vitro, aPTT and TT were performed after addition of the patient’s purified ATA IgG (2.45 mg/mL). Compared to control IgG (2.5 mg/mL), addition of the patient’s ATA IgG to NPP led to a prolonged aPTT (59 versus 33 sec) and a prolonged TT (>120 vs. 19 sec).

In order to demonstrate the anti-prothrombotic effect of the patient’s ATA in vivo, infusion of the patient’s ATA IgG was carried out in a mouse model of laser-induced arteriolar thrombus formation using intravital microscopy as previously described.

Figure 3. In vivo effect of the infusion of patient’s purified immunoglobulin G on thrombus formation and fibrin generation using intravital microscopy and a mouse model of laser-induced arteriolar injury. Intravital videomicroscopy of the cremaster muscle microcirculation was performed as previously described. Wild-type mice were pre-anesthetized with intraperitoneal ketamine (Fort Dodge) and xylazine (Lloyd). A canulus was inserted in the jugular vein to maintain anesthesia with pentobarbital (Sigma-Aldrich) and infuse platelet and fibrin labeling as well as patient’s purified Immunoglobulin G (IgG). Digital images were captured with a C9300 Digital Camera (Hamamatsu) connected to a VS4-185 Image Intensifier Gen III (VideoScope International). Laser injury: vessel wall injury was induced with a micropoint laser system (Photonics Instruments) focused through the microscope objective. Multiple thrombi were studied in a single mouse, with new thrombi formed upstream of earlier thrombi. Image analysis: fluorescence data were captured digitally over 3 minutes following vessel injury. Image analysis was performed with Slidebook Version 5.5 (Intelligent Imaging Innovations) as previously described. In order to account for the variability of thrombus formation in any given set of experimental conditions, the data from 10-15 thrombi in two separate mice were used to determine the median value of the integrated fluorescence intensity before and 10 minutes after intravenous infusion of 690 μg of patient’s purified IgG. Platelet and fibrin labeling were performed using anti-CD42 antibody conjugated with DyLight488 (0.1 μg/g mouse, green) (Emfret) and with anti-fibrin antibody conjugated with AlexaFluor647 (0.5 μg/g mouse, red) (Sekisui). The kinetics of platelet accumulation and fibrin formation at the site of injury were determined by calculating median fluorescence values at 649 and 488 nm over time. (A-B) Thrombus formation at the site of injury. Representative images of fluorescence signal associated with platelets (green) and fibrin (red) over 120 seconds (T0-T120) following vessel injury (white arrow) are shown within the context of brightfield histology in the absence (A, left panel) or 10 minutes after infusion of patient’s IgG (690 μg) (B, right panel). (C-D) Platelet accumulation (C) and fibrin generation (D) at the site of injury. The median integrated platelet fluorescence (C, F platelet) or fibrin fluorescence (D, F fibrin) as a function of time in the absence (blue line = 10 thrombi, two mice) or 10 minutes after infusion of patient’s IgG (black line = 15 thrombi, two mice).
induces an inhibition of the initial (low concentration) TF-triggered thrombin formation as well as further amplification of thrombin generation in PPP. In PRP, the ATA-induced inhibition might not be sufficient to prevent thrombin-induced-platelet activation. Therefore, the release of platelet-stored coagulation factors, and the residual traces of thrombin, might be sufficient to partially overcome the action of ATA at the surface of activated platelets, leading to a delayed and decreased but detectable thrombin generation. We further demonstrate the anti-thrombotic potency of the patient’s purified ATA in vitro and in vivo. Indeed, purified the patient’s ATA IgG retained the capacity to reproduce the biological abnormalities observed in the patient’s PPP, such as prolongation of aPTT and TT. Concomitantly, using a murine model of laser-induced arteriolar injury, we showed that infusion of the purified patient’s ATA IgG was specifically responsible for a significant inhibition of thrombus formation at the site of injury, illustrating the ATA-induced impairment on thrombus formation in vivo.

In conclusion, we report a severe acquired hemorrhagic disorder secondary to an ATA in a patient presenting with a MIgG. We demonstrated the anti-thrombotic property of the MIgG through in vitro, ex vivo, and in vivo experiments that allowed establishing a link between the biological and clinical presentation. This is the first description of the direct responsibility of a MGCS-associated ATA in the occurrence of a patient’s hemorrhagic tendency.

Berenice Schell,1 Celine Desconclois,1 Xavier Mariette,1,2 Cecile Gouyard,1,3 Peter J. Lenting,1,4 Cecile V. Denis1,5 and Valerie Proulle1,6

1Service Hematologie Biologique, Hopital Bicetre, AP-HP, Le Kremlin-Bicetre; 2Service Rhumatologie, Hopital Bicetre, AP-HP; INSERM UMR_S_1176, Le Kremlin-Bicetre; 3Universit´e Paris-Saclay, Paris; 4Service Medicine Interne, Hopital Bicetre, AP-HP, Le Kremlin-Bicetre and ´INSERM UMR_S_1484, Le Kremlin-Bicetre, France

Correspondence: VALERIE PROULLE - valerie.proulle@aphp.fr
doi:10.3324/haematol.2019.242370
Disclosures: no conflicts of interests to disclose.
Contributions: VP designed the study, made the diagnosis, performed experiments, wrote the manuscript and is taking primary responsibility for the article. BS analyzed the data and wrote the manuscript. CD performed experiments. XM and CG managed the patient. CD, XM, CG, PL and CD revised the manuscript.

References

1. Fermand JP, Bridoux F, Dispensieri A, et al. Monoclonal gammopathy of clinical significance: a novel concept with therapeutic implications. Blood. 2018;132(14):1478-1485.
2. Tiede A. Diagnosis and treatment of acquired von Willebrand syndrome. Thromb Res. 2012;130(Suppl 2):S2-6.
3. Kruse-Jarres R, Kempston CL, Baudou F, et al. Acquired hemophilia A: updated review of evidence and treatment guidance. Am J Hematol. 2017;92(7):695-705.
4. La Spada AR, Skalbegg BS, Henderson R, Schmer G, Pierce R, Chandler W. Brief report: fatal hemorrhage in a patient with an acquired inhibitor of human thrombin. N Engl J Med. 1995;335(8):494-497.
5. Costa JM, Fiesseing JN, Capron L, Aiach M. Partial characterization of an autoantibody recognizing the secondary binding site(s) of thrombin in a patient with recurrent spontaneous arterial thrombosis. Thromb Haemost. 1992;67(2):193-199.
6. Baglin TP, Langdown J, Frasson R, Huntington JA. Discovery and characterization of an antibody directed against exosite I of thrombin. J Thromb Haemost. 2016;14(1):137-142.
7. Lawson JH, Pennell BJ, Olson JD, Mann KG. Isolation and characterization of an acquired antithrombin antibody. Blood. 1990;76(11):2249-2257.
8. Hwang KK, Grossman JM, Visvanathan S, et al. Identification of anti-thrombin antibodies in the antiphospholipid syndrome that interfere with the inactivation of thrombin by antithrombin. J Immunol. 2001;167(12):7192-7198.
9. Bartheis M, Heimburger N. Acquired thrombin inhibitor in a patient with liver cirrhosis. Haemostasis. 1985;15(5):395-401.
10. Chung YC, Lin YS, Liu HS, Wang JR, Yeh TM. Antibodies against thrombin in dengue patients contain both anti-thrombotic and pro-fibrinolytic activities. Thromb Haemost. 2013;110(2):358-365.
11. Proulle V, Furie RA, Merrill-Skoloff G, Furie BC, Furie B. Platelets are required for enhanced activation of the endothelium and fibrinogen in a mouse thrombosis model of APS. Blood. 2014;124(4):611-622.
12. Arad A, Proulle V, Furie RA, Furie BC, Furie B. Beta(2)-glycoprotein-I autoantibodies from patients with antiphospholipid syndrome are sufficient to potentiate arterial thrombus formation in a mouse model. Blood. 2011;117(12):3455-3459.
13. Zhang S, Wu Z, Li J, et al. Clinical performance of antibodies to prothrombin and thrombin in Chinese patients with antiphospholipid syndrome: potential interest in discriminating patients with thrombotic events and non-thrombotic events. Rheumatol Int. 2017;37(4):579-584.
14. Matsumoto T, Nomori K, Shimura M. A combined approach using global coagulation assays quickly differentiates coagulation disorders with prolonged aPTT and low levels of FVIII activity. Int J Hematol. 2017;105(2):174-183.
15. Millet A, Graveleau J, Guert P, et al. Thrombin generation in patients with acquired haemophilia and clinical bleeding risk. Br J Haematol. 2011;158(1):136-139.