BIOCHEMICAL AND MOLECULAR GENETIC CHARACTERIZATION OF INHERITED AND ACQUIRED PARAHAEMOPHILIAS

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INTRODUCTION

Blood coagulation

The haemostatic system is designed to maintain blood in fluid state under physiologic conditions but primed to react to vascular injury in an explosive manner to stem blood loss by sealing the defect in the vessel wall. This complex function of the haemostatic system is provided by humoral, cellular and vascular mechanisms.

The blood coagulation process is initiated when subendothelial tissue factor (TF) is exposed or expressed to the blood flow following either the damage or activation of the endothelium. The TF can bind both zymogen (FVII) and active FVII (FVIIa), which are already present in tiny amounts in the circulation, forming TF-FVII and TF-FVIIa (extrinsic factor tenase) complexes. Both forms of the complex have enough catalytic activity to cleave small amount of factor X (FX) into active enzyme (FXa) but the catalytic activity of the TF:VIIa complex is much higher and its substrate specificity involves factor IX (FIX). In the initiation phase of coagulation, the complex can generate picomolar concentrations of thrombin, which partially activates platelets and cleaves the procofactors factor V (FV) and factor VIII (FVIII), generating the active cofactors, FVa and FVIIIa, respectivelly. FVIIIa forms the intrinsic tenase complex with serine protease, factor IXa (FIXa), on a membrane surface and activates FX also at a 50-100-fold higher rate than the extrinsic tenase complex. The FXa, forms the prothrombinase complex with the cofactor FVa on the membrane surface, which cleaves prothrombin into thrombin. This high rate thrombin formation is the characteristic feature of the propagation phase of coagulation. The soluble thrombin further amplifies its own generation by activating factor XI and completing activation of platelets, FV and FVIII. Thrombin also cleaves fibrinogen into soluble fibrin monomers with the removal of fibrinopeptide A and B. The fibrin monomers then polymerize to form a fibrin mesh. The thrombin activated factor XIII stabilizes the clot by covalent crosslinking of fibrin.

In the termination phase of coagulation the thrombin generation is inhibited by anticoagulant systems, either by inhibition of enzymes or by modulation of the activity of the cofactors. The tissue factor pathway inhibitor (TFPI) inhibits the FXa and FVIIa-TF-FXa complex. Main physiological function of the serin protease inhibitor antithrombin is to inhibit thrombin and FXa. Antithrombin is, in itself, an inefficient serine-protease inhibitor,

but the heparin-like molecules (heparan-sulphate) that are present on the surface of endothelial cells stimulate its activity. Protein C (PC), a zymogen to an anticoagulant protease, is activated on the surface of intact endothelial cells by thrombin that has bound to the membrane protein thrombomodulin. Activated protein C (APC) can inactivate the phospholipid membrane-bound cofactors FVa and FVIIIa by limited proteolysis.

Circulating platelets are activated by exposed subendothelial filaments and soluble activating agents and adhere to the subendothelium, aggregate and release their granular contents, resulting in further aggregation and formation of primary thrombus. Activated platelets provide procoagulant surfaces upon which the complex-dependent reactions of the blood coagulation cascade are localized. Additionally activated platelets release their α-granule content that are important procoagulant and regulatory proteins of blood coagulation.

The blood coagulation factor V (FV)

Human coagulation factor V (FV) is a single-chain glycoprotein with a M_r of 330 kD. Its plasma concentration is approximately 20 nmol/L (7μg/mL). Approximately 25% of whole blood FV is compartmentalized in the α-granules of platelets and becomes secreted during activation. The principal site of FV biosynthesis is the liver, but it is still unclear whether platelet FV originates from the uptake of exogenous FV via endocytosis by megakaryocytes or megakaryocytes themselves can synthesize FV. FV is a single-chain molecule, undergoes multiple posttranslational modifications, including N-glycosylation, phosphorylation and sulfation, before being secreted.

The gene for human FV has been localized to chromosome 1q21-25. It spans approximately 80 kilobases of DNA and consists of 25 exons and 24 introns. FV has a mosaic-like structure, with domain organization A1-A2-B-A3-C1-C2 that is similar to that of FVIII. Overall, the two coagulation cofactors share approximately 40% sequence identity in their A and C domains.

Development of FXa-cofactor activity of FVa is regulated by limited proteolysis of FV mediated by procoagulant enzymes such as thrombin and FXa. As a result, the large connecting B-domain dissociates from FVa, which is formed by the noncovalently associated

heavy (A1-A2) and light (A3-C1-C2) chains. The full procoagulant FVa contains a heavy chain (M_r: 105 kD) and a light chain (M_r: 72 or 74 kD) associated via calcium binding.

FVa inactivation is accomplished by APC-mediated proteolysis of FVa at positions Arg306, Arg506, and Arg679. Cleavage at Arg306 is necessary for the complete inactivation of FVa activity. The rate of this cleavage is potentiated 20-fold by protein S (PS).

FV has a dual function in maintaining normal haemostasis. FVa is the cofactor to FXa in the prothrombinase complex, which cleaves prothrombin to thrombin. Circulating single-chain FV is an inactive procofactor, expressing less than 1% of the procoagulant enzyme-activated FXa-cofactor activity that it can maximally obtain. FVa is considered an essential FXa cofactor, inasmuch as its presence in the prothrombinase complex enhances the rate of prothrombin activation by several orders of magnitude. The nonactivated intact FV or APC cleaved FV molecules function as an APC cofactor in synergy with PS in the regulation of FVIIIa activity. The full procoagulant activation of FV results in lost anticoagulant APC-cofactor activity of FV. Results indicate that a delicate balance exists between the procoagulant and anticoagulant properties of FV, with activation leading to a procoagulant protein that is devoid of anticoagulant properties. In contrast, APC-catalyzed proteolysis of intact FV generates an anticoagulant protein that has no procoagulant properties. FVa has essential procoagulant functions, without which severe bleeding tendencies can occur. The nonactivated FV functions as an APC cofactor in the regulation of FVIIIa activity. Failure of this function (FV R506Q, FV Leiden) may lead to thrombosis. The better understanding of this dual function of the FV molecule can help us in a better interpretation of the clinical symptoms of FV-deficient patients and in the establishment of their future treatment.

FV deficiency

The first patient with haemorrhagic FV deficiency (parahaemophilia) and FV itself was discovered by Paul Owren in Norway. Since that time FV defects associated with both haemorrhagic and thrombotic conditions have been described. FV defects with haemorrhagic conditions include classical and combined forms of parahaemophilias. Thrombotic FV defects involve isolated homo- and heterozygous FV Leiden mutations and

pseudohomozygous FV Leiden mutation (heterozygous FV Leiden mutation combined with heterozygous type I FV deficiency).

Parahaemophilias can be classified by pathomechanism as inherited and acquired FV deficiencies. Both types can occur in isolated or combined form. Patients with isolated FV deficiency have only the FV activity decreased, while in the second group the parahaemophilia is combined with other coagulation factor defects (combined FV, FVIII deficiency).

The classification by FV activity was taken from the system used in haemophilia practice. Severe parahaemophilia is associated with less than 1% of normal plasma activity of FV, whereas moderate and mild forms have plasma activity of 1-5% and 5-30% of normal, respectively.

The plasma and platelet pools of FV are not always equally affected in different forms of parahaemophilia. In platelet type FV deficiency only the platelet FV pool is decreased, while in combined FV deficiency both plasma and platelet FV are affected.

The parahaemophilia associated with proportional decrease of both FV activity and antigen levels is called type I deficiency. The FV deficiency caused by the presence of dysfunctional FV molecule is the type II deficiency, which is detected in 17% of FV-deficient patients. In those cases the FV antigen level is much higher than the FV activity.

One can still find in the literature the old terminology: CRM positive and negative FV deficiency. CRM positive meant that FV antigen was detectable, while, in CRM negatives it was not. As the sensitivity of FV antigen determinations increased, the CRM reduced terminology was introduced instead which meant decreased but still detectable amounts of FV.

Inherited parahaemophilia

The incidence of congenital FV deficiency is about 1 in 10^6, and its inheritance is autosomal recessive. More than 200 cases have been reported in the literature. The molecular basis for FV deficiency has been established only in a few patients. 21 mutations associated with FV deficiency have been published up till August 2002. 14 patients with described mutations presented severe FV deficiency and 12 of them carried the causative mutation of FV deficiency in homozygous form while the remaining two patients (one of them is the male

infant described in this thesis) were compoud heterozygous. Although many mutations were found in the extremelly large exon 13 of FV gene, there is no hot spot of mutations can be detected. Nonsense, frameshift, splicing and missense mutations of FV gene have been so far identified. With the exception of 5 mutations, which have been found in more than one unrelated family, the majority of the mutations have been found in unique families.

Acquired parahaemophilia

Antibodies directed against FV can result in acquired isolated FV deficiency. Anti-factor V antibodies have generally been considered to be infrequent. However, anti-factor V antibodies are more common in patients exposed to topical bovine thrombin containing bovine FV. These antibodies raised against bovine FV in the preparations used in treatment but cross-react with human FV. Allo-antibodies can develop in FV-deficient patients after treatment with fresh frozen plasma. FV auto-antibodies have been detected in patients following surgery, blood transfusions or antibiotic administration, but the mechanism of auto-anti FV antibody formation often remains unexplained. Although FV inhibitors may occur at all ages, the majority of cases have been observed in patients older than 65 years. The overall prognosis of FV inhibitors is good, but acute bleeding related to the appearance of the inhibitors can be a life-threatening condition. In many cases anti-factor V antibodies are transient, usually lasting for 4-22 weeks.

Clinical symptoms of patients with parahaemophilia

The clinical manifestations associated with parahaemophilia vary to a great extent. Patients are often asymptomatic or have mild to severe haemorrhagic manifestations, there are also reports of thrombotic complications. The type of bleeding is often mucocutaneous including epistaxis, oral cavity bleeding and menorrhagia. Postoperative and muscle or joint bleeding are common. Bleeding episodes in the central nervous system are rare, but frequently fatal. It is noteworthy that while gastrointestinal bleeding and haematuria occur in approximately one third of the patients with haemorrhagic acquired parahaemophilia, it is a very uncommon bleeding manifestation in patient with congenital FV deficiency.

It is an unexplained discrepancy between mice and man, that while FV null mice die either in utero before the 9-10 days of embryonal development or of fatal perinatal haemorrhage, human patients with undetectable FV levels survive.

Laboratory diagnostics of parahaemophilias

In general, patients with parahaemophilia present with prolonged prothrombin time (PT) and activated partial thromboplastin time (APTT). Although patients with bovine thrombin-induced FV-antibodies often manifest a prolonged thrombin time (TT) because of cross reaction not only with human FV but also with thrombin content of the reagent, usually FV inhibitors do not prolong TT. PT, APTT-based one-stage factor activity determinations should be performed in order to differentiate parahaemophilia from other isolated or combined coagulopathias resulting in the similar screening test pattern of blood coagulation. Less than 30% FV activity is the main diagnostic criteria of FV deficiency. Further characterization of FV deficiency (inhibitor type or lack of functional FV) is important for appropriate treatment. Given that plasma FV activities do not correlate well with haemorrhagic tendency, and several authors have suggested that the severity of bleeding symptoms may be more closely related to platelet rather than plasma FV activities, determination of platelet FV activity can be also recommended.

PATIENTS AND GOALS

A male infant presented at birth with subdural haematoma, later suffering from easy bruising and severe haematoma following intramuscular injection. Consanguinity in the family was excluded. No bleeding disorder was detected in other members of the family.

A 78-year-old female with melena and severe anemia was admitted to hospital. She had undergone oophorohysterectomy 3 years before the apparent admission, when she had received fresh frozen plasma and red blood cell (RBC) concentrate because of intra and postoperative bleeding. There was no known history of haemorrhagic diseases in her family. Gastrointestinal investigations revealed atrophic antrum gastritis and bile reflux with cholelithiasis, but there was no apparent anatomical reason for the bleeding. Beside substitution therapy with fresh frozen plasma, platelet and RBC concentrates, the patient was treated with high dose human immunoglobulin, plasmapheresis and immunosupressive therapy (combination of cyclosporin A and cyclophosphamide). During the following three years the patient had several relapses and had to be hospitalized on four different occasions.

Our goals:

1. Characterization of the type of haemorrhagic diathesis presented by these patients.
2. The investigations used in the characterization of haemorrhagic diathesis verified severe FV deficiency in both patients. In further studies, we investigated the molecular genetic background and biochemical mechanism of their parahaemophilias.

MATERIALS AND METHODS

Preparation of plasma and platelet specimens

Following informed consent blood samples from the patients and the infant's family members were collected in 1:10 volume of 0.105 mol/L sodium citrate. Blood samples used for Western blotting or immunoprecipitation were immediately treated with a cocktail of protease inhibitors. Plasma was separated by centrifugation at 2,000 g for 20 min and aliquots were stored at –70°C. The washed platelet suspension was prepared by differential centrifugation in the presence of platelet antagonists.

Coagulation tests

PT, APTT and TT determinations were performed to screen coagulopathies. Measurement of the inhibitor to FV was attempted by a modified, PT-based Bethesda method. The presence of lupus type inhibitor was tested by functional assays.

FV coagulant activity

FV coagulant activity in plasma was measured by one-stage clotting assay based on prothrombin time. Standard human plasma was used for calibration. FV activity in platelet lysate samples was determined by the same assay system, but in this case the assay was calibrated against pooled normal platelet lysate.

Determination and characterization of factor V by ELISA

Total FV, FVHC and FVLC antigen levels were determined by sandwich ELISA. The specificity of different ELISA tests was achived on using different capture antibodies such as a human FV polyclonal antibody, a monoclonal antibody directed to epitope on the 150 kD activation peptide in the connecting B domain and a monoclonal antibody directed to epitope on C2 domain of FVLC. All potentially catched normal and abnormal FV molecules were detected by rabbit anti-human FV antiserum on the other side of the sandwich. Standard human plasma was used for the calibration of plasma assays, while in platelet FV activity and

antigen assays platelet lysate from healthy donors was used as calibrator. FV antigen levels were expressed as a percentage of normal average.

Immunoprecipitation, SDS-PAGE and immunoblotting

FV was isolated from the plasma by immunoprecipitation. Immunoprecipitates and whole plasma samples were analyzed by SDS-PAGE and by Western blotting. The polyclonal antibody against FV used for immunoprecipitation was used as primary antibody. To strenghten the results from the analysis of the male infant other primary antibodies were also used (rabbit anti-FV antiserum, monoclonal antibody against the B domain of FV).

PCR amplification and sequencing

The whole coding and splicing regions of FV gene with its promoter have been amplified by PCR. PCR products purified by ultrafiltration were sequenced on an ABI 310 Genetic Analyzer.

Immunoglobulin G isolation

The total IgG fraction was isolated from the patient suspected having FV inhibitor and from normal volunteers on a Protein G Sepharose column and concentrated using a microconcentrator.

The binding of patient's IgG to factor V and Va

A solid-phase ELISA was developed to detect the binding of anti-FV IgG to purified FV and FVa. Serial dilutions of plasma and isolated IgG preparations were added to purified FV or FVa coated on polystyrene plates. Bound IgG was detected by peroxidase labeled rabbit anti-human IgG.

Mapping the epitope for the anti-FV auto-antibody

A blocking ELISA was developed for mapping the epitope of the auto-antibody on FV. Monoclonal antibodies recognising epitopes on the heavy chain, the B-domain, the C2 domain on the light chain (HV1) and on a non-characterized region of the light chain were incubated with FV coated on polystyrene microplates. After washing serial dilutions of patient's IgG

were added. The amount of patient's IgG bound to FV was determined by peroxidase-labeled anti-human IgG.

The effect of patient's IgG on the activity of factor Va

Various amounts of the patient's IgG were added to pooled normal plasma, and then PT, APTT and TT were measured. In control experiments IgG isolated from healthy donors was used to replace the patient's IgG. The results were compared to PT, APTT and TT measured from mixtures of patient plasma and control plasma pool.

The effect of patient's IgG on the activity of FVa was tested using the prothrombinase-based FVa assay. Recombinant thrombin activated FV was incubated with various dilutions of control and patient IgG preparation. The remaining FVa activity was determined by using kinetic assay with chromogenic thrombin substrate.

Detection of factor V-anti factor V immune-complexes in the patient's plasma and platelet lysate

An ELISA was developed for the detection of FV specific immune-complexes in plasma and platelet lysate. Anti-human FV polyclonal antibody was used as capture antibody and peroxidase labeled anti-human IgG recognizing the Fc-portion of human IgG was used as detection antibody.

RESULTS

Severe inherited FV deficiency; characterization of coagulopathy in the male infant

Screening tests for coagulopathy

The proband had highly prolonged PT (58.1 sec; control: 8.7-11.5 sec) and APTT (198.8 sec; control: 29.5-42.7 sec).

FV activity

FV activity was undetectable in the proband's plasma and platelet lysate (<0.4% in each). The mother, the father and one grandmother had moderately decreased (approximately 50%) FV activity, while the remaining tested family members presented normal FV phenotype.

FV gene sequence analysis

DNA sequence analysis revealed two causative mutations in heterozygous form: one base deletion (thymine) in exon 13 at nucleotide position 2952, and one base insertion (guanine) in exon 16 at nucleotide position 5493. The proband's mother was heterozygous for 5493insG while his father was heterozygous for 2952delT. In the proband's DNA there were two further non-causative mutations, a homozygous silent A327→G substitution in exon 2 and a heterozygous A6250→T substitution in exon 22.

Detection of the mutant protein

Both causative mutations would theoretically lead to the synthesis of truncated FV molecules. FV antigen level in the patient's plasma was severely reduced (1.03%), but detectable. In ELISA systems using B domain or LC specific monoclonal antibodies (<0.5%) only the N-terminal portion of FV containing HC plus B domain could be detected (1.7%). The parents and the affected grandmother had antigen values around 50 %. No FV was detected in the patient's plasma by Western blotting, however when FV antigen was concentrated by

immunoprecipitation a faint band with a M_r of 236 kD reacted with monoclonal anti-FV B domain antibody or polyclonal anti-FV antibodies. No intact FV could be seen.

Acquired FV deficiency; characterization of coagulopathy in the elderly lady

Demonstration and characterization of the acquired factor V deficiency

The PT (81.3 sec; control: 8.7-11.5 sec) and APTT (147.2 sec; kontroll: 29.5-42.7 sec) of the patient were significantly prolonged on admission and remained prolonged during the following 3 years. TT was always normal. In mixing studies a concentration-dependent correction of the prolonged clotting times were observed. However, even in the 1:1 mixture, the normal pooled plasma failed to correct the prolongation of PT and APTT completely. Incubation of the mixture for 1 hour at 37°C did not change the situation. FV activity was severely decreased both in the plasma (0.48%; reference interval: 80-120%) and in the platelet lysate (1.12% of normal average) of the patient.

On admission 2.0 BU FV inhibitor was measured by the modified Bethesda assay, and the antibody titer remained low (1.25-2.0 BU) during the 3-year follow-up period. Titration of purified FV into patient plasma resulted in normalization of PT and APTT at 7.5-10 μg/mL supplemented FV concentration. Tests for lupus anticoagulant were negative.

In the patient's plasma, total FV:Ag and FVHC:Ag levels were normal (106% and 139%, respectively), while FVLC:Ag was severely decreased (7.9% of normal avarage). Similarly, FV:Ag was normal (80%), and FVLC:Ag was low (4.8%) in platelets. The decrease of FVLC:Ag, however was not due to the presence of a truncated molecule, since FV immunoprecipitated from the patient's plasma showed a single FV specific band of ~330kD on the immunoblot.

DNA sequence analysis of FV gene did not identify any disease-causing mutations. A few known polymorphisms and a so far unidentified nucleotide change in exon 13 were detected in heterozygous form. The patient was heterozygous for R506Q FV (Leiden) mutation.

Demonstration of the Factor V-binding auto-antibody in the patient's IgG fraction

The presence of FV-specific auto-antibody was demonstrated in the patient's plasma and in its IgG fraction by ELISA technique.

Both the plasma and the platelet lysate contained immune-complexes that were captured by FV-specific antibodies and could be detected by an antibody against human IgG. Such FV-IgG immune-complexes could be recognized even at 100-fold plasma dilution.

A blocking ELISA was used to assess the effect of four anti-FV monoclonal antibodies with known epitopes on the binding of the patient's IgG auto-antibodies to FV. Although all four monoclonals recognized FV, only the HV1 monoclonal antibody directed to a specific region of C2 domain of FVLC inhibited the binding of the patient's antibody.

Neutralization of the procoagulant activity of factor V by the patient's auto-antibody

The addition of the patient's IgG to normal plasma prolonged both PT and APTT to an extent similar to the effect of unfractionated patient's plasma, while the addition of normal plasma IgG did not. The inhibitor had no effect on TT. The inhibitory activity of the patient's IgG seemed to be weak because effective inhibitory action appeared only at IgG concentration above 8 mg/mL.

Increasing concentration of the patient's IgG inhibited the FXa-cofactor function of FVa, while control IgG had no effect. In this system at a concentration of 1 mg/mL IgG, the inhibitory effect exceeded 70% and at physiological IgG concentration (~10 mg/mL) only 2.5% of the original FV activity remained.

CONCLUSIONS

Results of the screening tests and factor determinations in both patients clearly indicated that both of our patients had severe forms of FV deficiency. The age and history suggested inherited parahaemophilia in the case of the male infant and acquired FV deficiency in the old lady. However, in her case the results of mixing studies and the Bethesda assay did not unequivocally exclude the presence of an allo-antibody that had developed on the basis of a genetic FV disorder after exposure to foreign plasma proteins.

FV antigen level in the male neonate's plasma was severely reduced, but detectable (approximately 1%), which corresponds to the phenotype of type I FV deficiency. In ELISA systems using B domain or LC specific monoclonal antibodies only the N-terminal portion of FV containing HC plus B domain could be detected. DNA sequence analysis of the whole coding and splicing regions of FV gene and its promoter revealed two causative mutations in heterozygous form. Both causative mutations introduced a frameshift and predicted novel stop codons at position 930 and 1776, respectively, that would lead to the synthesis of truncated FV molecules. The predicted protein resulting from the exon 13 mutation would lack part of the B domain and the entire LC. The exon 16 mutant protein would lack a significant part of the LC and this 1775 amino-acid long polypeptide would have a M_r of 200 kD. Considering that it contains the heavily glycosylated B domain, it is very likely that the 236 kD protein, that we now designate $FV_{Debrecen}$, represents this larger truncated protein. The absence of the smaller truncated protein and the highly reduced amount of $FV_{Debrecen}$, could be due to reduced synthesis of mutant mRNAs, to the instability and intracellular degradation of mutant proteins and to the accelerated plasma clearance of truncated FV.

FV activity was below the sensitivity limit of our assay in both FV compartments (<0.4%) of the neonate with inherited FV deficiency and this resulted in severe bleeding tendency. Complete FV deficiency is lethal in knock-out mice, however they can be rescued by a very low level (<0.1%) of transgene FV expression. Although we were unable to detect intact FV in the patient's plasma it cannot be excluded that the patient expresses very low level of FV as a result of ribosomal slippage or somatic reversion. Such low levels of FV might be undetectable on the Western blot. Alternatively, the truncated protein might possess some residual procoagulant activity.

The results of FV antigen determinations of the old lady's plasma and platelet samples raised the possibility of a truncated or abnormal protein. With polyclonal capture antibody in a sandwich ELISA plasma FV concentration was normal, while using HV1 monoclonal anti-FV antibody against the C2 domain of FVLC as capture antibody the FV antigen level was severely depressed. However, Western blotting of FV immunoprecipitated from the patient's plasma verified that the M_r of the FV molecule was normal and DNA sequencing excluded a genetic FV defect leading to the synthesis of an abnormal molecule. As the FV molecule was proved to be intact, these results can only be explained by the decreased binding of HV1 monoclonal antibody to FV being in complex with the auto-antibody. To strengthen this hypothesis four monoclonal antibodies, including HV1 were tested for blocking the binding of the auto-antibody to FV. Two of the antibodies were directed against different epitopes on FVLC and two further antibodies had epitopes on FVHC and on the B domain. Only the HV1 antibody directed against the region of amino acids 2060-2069 interfered with the binding of the patient's auto-antibody. These findings suggest that the epitopes for HV1 and for the auto-antibody are, at least partially, overlapping. The N-terminal portion of C2 domain is involved in the binding of FVa to the phospholipid surface, which would explain the inhibition of this function by the auto-antibody in the clotting tests and the prothrombinase assay.

Anti-FV antibodies reacting with both FV and FVa were detected in the old lady's plasma and IgG fraction, and functional assays clearly showed that the IgG preparation isolated from the patient's plasma inhibited the procoagulant activity of FVa. The presence of FV-anti-FV IgG immune-complexes in the patient's plasma was also demonstrated. The low BU values and the finding that relatively low amount of supplemented FV normalized PT and APTT of the patient's plasma suggest a relatively low titer of the auto-antibody. The global clotting assays and the clinical symptoms indicated that, despite its low concentration, the antibody exerted its inhibitory effect in vivo as well.

To our knowledge the presence of FV inhibitor in platelets has not been investigated. The results received by the analyisis of washed platelets from the old lady - very low FV activity, normal total FV:Ag, low level of FV:LC Ag, presence of FV-anti-FV immune-complexes - clearly suggested the presence of the inhibitor in our patient's platelets. IgG-type inhibitors can get into platelet alpha granules using the physiological IgG transport mechanism of platelets in vivo. The ingestion of FV-anti-FV immune-complexes by platelets

and megakaryocytes and their packaging into alpha granules may represent an alternative mechanism for the uptake of anti-FV IgG.

The gastrointestinal bleeding of our patient with FV inhibitor was successfully stopped by the therapeutic regimen but the prolonged clotting tests and low FV activity remained unchanged during the 3 years of follow-up. The clinical symptoms of our patient with FV inhibitor may be influenced by the fact that the inhibitor was also detected in her platelets and she was heterozygous for FV Leiden mutation. The severity of bleeding symptoms in FV deficiency seems to be more closely correlated with platelet rather than with plasma FV levels. The anti-FV antibody in the platelets of our patient might have effectively interfered with the procoagulant action of platelet FV. Heterozygosity for FV Leiden mutation in our patient may mean that half of the small portion of the FV that was activated and not blocked by the auto-antibody must have been FV Leiden molecule that exerts its procoagulant activity longer than its wild type counterpart. Thus the presence of FV Leiden might also have some moderating effect on the severity of her bleeding diathesis.

THIS THESIS IS BASED ON FOLLOWING PAPERS:

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