DISSERTATIONES MEDICINAE UNIVERSITATIS TARTUENSIS

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CHARACTERIZATION OF ANTIBODIES TO COAGULATION FACTOR VIII

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TARTU 2002

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TARTU UNIVERSITY
PRESS

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Dissertation is accepted for the commencement of the degree of Doctor of Pharmaceutical Sciences on April 24, 2002 by the Council of the Faculty of Medicine, University of Tartu.

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Commencement: June 17, 2002

Publication of this dissertation is granted by the University of Tartu.

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Tiigi 78, Tartu 50410
Tellimus nr. 353

"Blut ist ein ganz besonderes Saft"
Goethe, Faust

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

This dissertation is based on the following publications and some unpublished data:

I Kallas A, Talpsep T. Von Willebrand factor in factor VIII concentrates protects against neutralization by factor VIII antibodies of haemophilia A patients. *Haemophilia* 2001; 7 (4): 375–380

II Kallas A, Pooga M, Benhida A, Jacquemin M, Saint-Remy J-M. Epitope specificity of anti-FVIII antibodies during immune tolerance therapy with FVIII preparation containing von Willebrand factor. *Submitted for publication*

III Kallas A, Talpsep T, Everaus H. Changes in epitope specificity and in distribution of IgG subtypes of FVIII antibodies during immune tolerance therapy (ITT) in hemophilia A patients with FVIII antibodies – a case report. In Scharrer I., Schramm W. (eds.) *31st Hemophilia Symposium.* Springer Verlag Berlin Heidelberg, 2001, 23–40

IV Kallas A, Talpsep T. The von Willebrand factor collagen-binding activity assay: clinical application. *Annals of Hematology* 2001; 80 (8): 466–471

Related papers

Kallas A, Everaus H. Ülevaade desmopressiini kasutamisest verehüübimishäirete ravis. *Eesti Arst* 1998; 2: 119–126

Kallas A, Viires M, Everaus H, Hinrikus T. Faktor VIII – von Willebrandi faktori kompleks hemofiilia A asendusteraapias kasutatavates erinevates faktor VIII preparaatides. *Eesti Arst* 1997; 5: 417–424

ABBREVIATIONS

aa	amino acid(s)
AP	alkaline phosphatase
APC	activated protein C
APCC	activated prothrombin complex concentrate
Arg	arginine
BCIP	5-bromo-4-chloro-3-indolyl-phosphate
BSA	bovine serum albumin
BU	Bethesda unit(s)
CRM	cross reacting material
DNA	deoxyribonycleic acid
EDTA	ethylenediaminetetreaacetic acid
ELISA	enzyme-linked immunosorbent assay
FII	factor II, prothrombin
FIX	factor IX
FIXa	activated factor IX
FVII	factor VII
FVIIa	activated factor VII
FVIII	factor VIII
FVIII:Ag	factor VIII antigen
FVIII:C	factor VIII coagulation activity
FVIIIa	activated factor VIII
FVIII-VWF preparation	FVIII preparation, which contains VWF
FX	factor X
FXa	activated factor X
GBS	glycine-buffered saline
GP	glycoprotein
GST	glutathione S-transferase
HCh	heavy chain
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulforic acid)
hFVIII	human factor VIII
HIV	human immunodeficiency virus
HLA	human leucocyte antigen
HRP	horseradish peroxidase
IC50%	IgG concentration yielding 50% of inhibition of FXa generation induced by FVIII
ICH	intracranial haemorrhage
ID50%	dilution of plasma sample neutralizing 50% of FVIII:C activity
Ig A, G, M	immunoglobulin class A, G, M
IL	interleukin
IFN-γ	interferon-γ

IP	immunoprecipitation assay
IT	immune tolerance
ITT	immune tolerance treatment
IU	international unit(s)
kb	kilobase
kDa	kiloDalton(s)
LCh	light chain
mAb	monoclonal antibody
n.d.	not determined
OPD	o-phenylene-diaminedihydrochloride
OD	optical density
p	p value
PBS	phosphate-buffered saline
PCB	postcircumcisional bleeding
PCC	prothrombin complex concentrate
pdFVIII	plasma-derived factor VIII concentrate
PL	phospholipid
pNA	p-nitroaniline
pNPP	p-nitrophenylphosphate
PS	L-(-phosphatidyl)-l-serine
PUP	previosly untreated patients
PTP	previosly treated patients
r	correlation coefficient
rFVIIa	recombinant activated factor VII
rFVIII	recombinant factor VIII
rHCh	recombinant heavy chain of factor VIII
rLCh	recombinant light chain of factor VIII
RT	room temperature
RVV	Russell's viper venom
scFv	single chain variable domain
SD	standard deviation
SDS-PAGE	sodium-dodecylsulfate polyacrylamide gel electrophoresis
Ser	serine
TBS	Tris-buffered saline
TGF-β	transforming growth factor β
Th	helper T cell
TNF	tumor necrosis factor
Tris	tris(hydroxymethyl)-aminomethane
U	unit(s)
WFH	World Federation of Haemophilia
V_H	immunoglobulin heavy chain variable domain
WHO	World Health Organisation
VWD	von Willebrand's disease
VWF MM	von Willebrand factor multimers

VWF	von Willebrand factor
VWF:Ag	von Willebrand factor antigen
VWF:CB	von Willebrand factor collagen binding
VWF:RCo	von Willebrand factor ristocetin cofactor activity

1. INTRODUCTION

Haemophilia A is a bleeding disorder caused by congenital deficiency in coagulation factor VIII (FVIII). The disease was first recognised as a familial bleeding disorder observed as excessive bleeding following circumcision already in Talmud in the 2nd century AD. Haemophilia as an inheritable disorder, occurring only in males, was first mentioned in literature in 1803 by J.C. Otto (Otto, 1803). Later two different coagulation disorders were distinguished: haemophilia A (classic haemophilia, caused by FVIII deficiency) and haemophilia B (Christmas disorder, caused by the Christmas factor, FIX deficiency), which was first described by A. Pavlovsky in 1947 (Pavlovsky, 1947). A novel bleeding disorder caused by the FVIII-related protein, now known as the von Willebrand factor (Weiss and Hoyer, 1973), which circulates in plasma in a non-covalent complex with FVIII, was described by Dr. Erik von Willebrand (von Willebrand, 1926). In 1959, the International Committee on Nomenclature systematized the names of all clotting factors and the anti-haemophiliac factor was designated as factor VIII (Wright, 1959).

The care and treatment of haemophilia have improved in parallel with scientific progress yielding the first gene transfer in the 21st century. FVIII gene was cloned in 1984 (Gitschier *et al.*, 1984; Toole *et al.*, 1984), allowing the identification of various mutations, deletions, and inversion within a gene enabling prenatal diagnosis and carrier detection. At present, the substitution of a deficient clotting factor by the administration of FVIII obtained by plasma fractionation or prepared by recombinant DNA technology is available as a treatment option for all haemophilia patients in the world. However, the issues of optimal and cost-effective care of both, the disease and the complications are still unsolved today. The development of antibodies against the administered FVIII is the most common and the most serious complication of haemophilia A replacement therapy. The ability of these antibodies to inhibit FVIII coagulation activity is the main reason for the failure to treat the disease with FVIII preparations. It is generally acknowledged that the development of FVIII antibodies is highly patient-specific and depends on the nature of the administered FVIII preparation. The reasons why some patients develop antibodies and others do not, are still unknown.

The occurrence of anti-FVIII antibodies in healthy persons causing acquired haemophilia, the presence of anti-idiotypic antibodies in healthy individuals and anti-FVIII antibodies in haemophilia A patients provide a unique opportunity to study the relationship between natural autoreactivity, autoimmunity in the disease and antigen-driven immune responses to a single protein. Therefore, this thesis presents a study on the ability of FVIII antibodies to inhibit FVIII coagulation activity and on the changes in immune response to FVIII in the course of immune tolerance therapy.

2. REVIEW OF LITERATURE

2.1. Occurrence of haemophilia A

Haemophilia A occurs with an incidence of approximately 1 in 5,000 male births, and the prevalence is 1 in 10,000 persons (Larsson, 1984). Based on FVIII coagulation activity (FVIII:C) haemophilia patients could be classified as having a severe, moderate or mild form of the disease according to the recommendation by Factor VIII and Factor IX Subcommittee (White *et al.*, 2001) (Table 1). In the year 2000, 34 haemophilia A patients were registered in Estonia, and most of them were diagnosed to have a severe form (n=24, 70%). The expected number of haemophilia A patients based on prevalence studies should be around 150, which is significantly higher than the number of patients diagnosed. The proportion of severe cases among patients (70%) is remarkably higher than in Sweden and in The Netherlands, where 30% and 40% of haemophiliacs, respectively, have a severe form of the disease, and 50% of patients have a mild form (Larsson, 1984; Rosendaal *et al.*, 1991). The difference could stem from the possibility that only severe cases have been diagnosed and patients with mild and moderate forms of the disease manage without a special treatment and care. Haemophilia B caused by the FIX gene defect is four-fold less common than haemophilia A (Rosendaal, *et al.*, 1991). Only 3 patients with FIX deficiency have been diagnosed in Estonia so far, which corresponds to 8% of haemophilia cases, while the expected prevalence is 20% of all haemophilia patients. The number of patients with the von Willebrand disease (VWD) increases every year and 33 cases had been diagnosed already by the year 2000 (Report on the WFH Global Survey 2001, 2001). Three patients out of 33 have Type 3 VWD, which fairly corresponds to the average prevalence of 2.5 to 3 cases per million of population in the Scandinavian countries of Europe and the Middle-East (Mannucci *et al.*, 1984). Since VWD Type 3 has similar clinical symptoms with haemophilia A, all haemophilia A patients are diagnosed according to the laboratory investigations of FVIII coagulation activity and the VWF antigen level. Clinical manifestation of different forms of haemophilia A in relation to the severity of the disease is shown in Table 2.

Table 1. The classification of haemophilia A (White *et al.*, 2001).

Factor VIII activity	**Classification**
<0.01 IU/ml (<1% of normal)	severe
0.01–0.05 IU/ml (1–5% of normal)	moderate
>0.05 –<0.40 IU/ml (5–40% of normal)	mild

Table 2. Clinical manifestation of haemophilia (DiMichele, 1996).

Bleeding manifestation	Severe	Moderate	Mild
Age of onset	≤1 year	1–2 years	2 years–adult
Neonatal symptoms	PCB: usual ICH: occasional	PCB: usual ICH: uncommon	None Rare
Muscle/joint haemorrhage	Spontaneous, requires no trauma	Requires minor trauma	Requires major trauma
CNS hemorrhage	High risk (2–8%)	Moderate risk	Rare
Postsurgical haemorrhage (without prophylaxis)	Frank bleeding, severe	Wound bleeding common	Wound bleeding with FVIII:C lower than 0.3 IU/ml
Oral haemorrhage following trauma, tooth extraction	Usual	Common	Frequent

PCB, postcircumcisional bleeding, ICH, intracranial haemorrhage, CNS, central nervous system

2.2. Phenotype of haemophilia A

The phenotype of haemophilia A is characterized according to the level of FVIII coagulation activity in the patient's plasma. Factor VIII coagulation activity (FVIII:C) can be estimated either by the clotting assay or the chromogenic substrate method. Clotting assay measures the time for the clotting of test plasma, which is diluted in FVIII-deficient plasma in the presence of clotting initiators ($CaCl_2$ and kaolin suspension) by a coagulometer (Kirkwood, 1980; Kirkwood and Barrowcliffe, 1978). In the chromogenic assay the test plasma dilution in buffer is mixed with a reagent that contains purified factor IXa and factor X (Carlebjork *et al.*, 1987; Wagenvoord *et al.*, 1989). In the presence of $CaCl_2$ and phospholipids FX is activated by FVIII. Activated FX cleaves off p-nitroaniline (pNA) from a chromogenic substrate. The amount of the released pNA correlates with the FVIII:C activity of the tested plasma. Factor VIII:C activity is reported in international units (IU/ml), with 1 IU/ml corresponding to 100% of the factor found in 1 ml of normal plasma pool (Barrowcliffe *et al.*, 1983). The clotting assay and the chromogenic method both use the local plasma standard (plasma pool contains plasma samples of at least

20 healthy persons), which is calibrated against the international standard. Coefficient of variance in the clotting assay is rather high (up to 15%) compared to that of the chromogenic assay (below 5%) (Carlebjork *et al.*, 1987; Hubbard *et al.*, 1986; Prowse *et al.*, 1986; Rosen *et al.*, 1985). Up to now, both methods are accepted for the standardized estimation of FVIII:C activity in plasma for the diagnosis of haemophilia A, however, only the chromogenic assay has been recommended as a reference method by the European Pharmacopoeia for measuring FVIII:C activity in FVIII preparations (Hubbard *et al.*, 2001). Compared to the clotting assay, chromogenic assay has been reported to be applicable for measuring the residual FVIII:C activity in the presence of FVIII antibodies by the Bethesda assay. However, Hutton and co-workers reported no significant difference in the results of the Bethesda assay if residual FVIII:C activity was measured by the chromogenic method or by the clotting assay (Hutton *et al.*, 1991).

Factor VIII antigen level as estimated by the ELISA assay yields additional information about FVIII protein in patients that enables researchers to determine correlation of the phenotype and genotype more precisely (Girma *et al.*, 1998). This assay, in general, is in routine use only in a few haemophilia centres. According to the level of FVIII:Ag haemophilia A patients are diagnosed as cross reactive material positive (CRM+, FVIII:Ag normal), reduced (VWF:Ag lower than normal), or negative (FVIII:Ag undetected) (Amano *et al.*, 1998b; Hoyer, 1993).

2.3. Structure and function of FVIII

Factor VIII protein consists of three homologous A domains and two C domains and a unique B domain (Figure 1). In the FVIII molecule domains are arranged in the order A1-*a1*-A2-*a2*-B-*a3*-A3-C1-C2 (Toole *et al.*, 1984; Vehar *et al.*, 1984; Wood *et al.*, 1984). FVIII is cleaved intracellularly at the B-a3 junction to yield several Me^{2+} linked heterodimers (Fay *et al.*, 1986). The initial cleavage is followed by a numerous of additional cleavages within the B domain (Vehar *et al.*, 1984) (Figure 1A). These cleavages lead to the generation of the heavy chain (HCh) consisting of the A1-A2 and B domains and the light chain (LCh) composed of A3, C1 and C2 domains. The C-terminal regions of the A1 domain (amino acids, aa 337–373) and the A2 domain (aa 711–740) as well as the N-terminal region of the light chain (aa 1649–1689) contain a high number of negatively charged residues and are therefore called acidic regions (*a1, a2, a3*) (Eaton and Vehar, 1986).

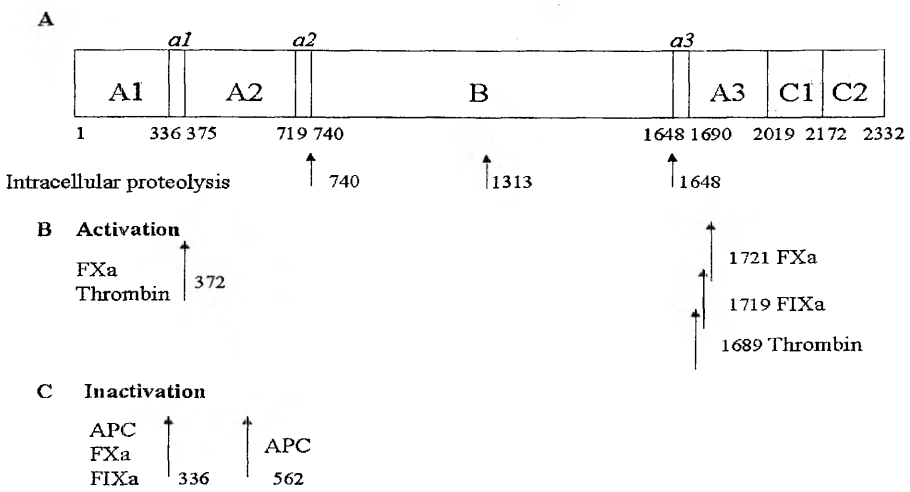

Figure 1. Structure of factor VIII. Factor VIII domains defined according to amino acid homology are indicated. Major part of factor VIII circulates in plasma as a set of heterodimers, consisting of the light chain (a3-A3-C1-C2) and the heavy chain (A1-a1-A2-a2-B) **(A).** Activation **(B)** and inactivation of FVIII **(C)** by different serine proteases (Lenting *et al.*, 1998).

The half-life of FVIII in plasma is increased by the ability to form a complex with VWF. At least two binding sites within the light chain, the a3 and the C-terminal region (aa 2303–2332) of the C2 domain are the main determinants of binding to VWF (Saenko and Scandella, 1997; Saenko *et al.*, 1994; Shima *et al.*, 1993; Shima *et al.*, 1992). The light chain of FVIII has a 10-fold lower binding affinity to VWF in the absence of the heavy chain, which points to an important role of the heavy chain of FVIII in retaining the proper conformation of the light chain (Saenko *et al.*, 1999a). Recombinant C2 domain itself has an even lower binding affinity to VWF compared to the full length of the FVIII light chain (Saenko and Scandella, 1997). VWF hinders the FVIII binding to phospholipids of the membrane and also to FXa-complex components (FX; FIXa) (Lenting *et al.*, 1994). The binding sites for phospholipids and VWF in the C2 domain partially overlap and therefore VWF prevents FVIII from interacting with phospholipids exposed on the membrane of cells at the site of vascular injury (Foster *et al.*, 1990b; Saenko *et al.*, 1994). Even though the binding sites for VWF and FIXa within the light chain are different, the VWF-FVIII complex cannot bind with FIXa (Lenting *et al.*, 1994). Factor IXa possesses comparable affinity for both non-activated and activated FVIII, and only VWF can prevent premature binding to FIXa (Duffy *et al.*, 1992).

However, VWF cannot protect FVIII from activation by thrombin. Cleavage by thrombin results in the dissociation of VWF from the complex and the conversion of FVIII into active conformation. Thrombin cleaves FVIII at one specific site within the light chain, (Arg1689) and at two sites in the heavy chain (Arg372 and Arg740, Figure 1B) (Eaton *et al.*, 1986). Processing of FVIII by FXa involves cleavage at two FXa-specific sites (Arg336, Arg1721) and three thrombin-specific sites (Arg372, Arg740, and Arg1689) (Eaton *et al.*, 1986). Factor VIII activation by FXa yields FVIIIa with a 4-fold lower activity as compared to the thrombin-activated product (Eaton *et al.*, 1986; Lollar *et al.*, 1985; Neuenschwander and Jesty, 1992), which could be caused by some inactivation due to the FXa specific cleavage at Arg336 in the heavy chain. Cleavage sites within both the heavy and the light chains of FVIII are equally important for activation. Recombinant mutant FVIII, which contains substitution of either Arg372 or Arg1689 cannot be processed correctly and therefore is unable to correct or normalize the reduced clotting time in FVIII-deficient plasma (Pittman and Kaufman, 1988). Missense mutations of FVIII activation sites Arg372, Ser373 and Arg1689, found in the FVIII gene of patients are associated with haemophilia (Kemball-Cook and Tuddenham, 1997). The activated FVIII associates with FIXa when the coagulation cascade has been activated. Factor tenase-complex binds to the membrane surface of cells, which serves two different functions: first the induction the association of FVIIIa and FIXa at the membrane and secondly the assembly of the active complex via structural re-arrangements (Gilbert *et al.*, 1990). In the presence of phospholipids FIXa has a 2,000-fold higher affinity for FVIIIa (Gilbert *et al.*, 1990), and FVIIIa is able to induce conformational changes in a FIXa active site (Lamphear and Fay, 1992a). Three sites of FVIII are involved in FIXa binding: amino acids 558–565, 698–710 and 1811–1818 (Fay *et al.*, 1994; Fay and Scandella, 1999; O'Brien *et al.*, 1995; Lenting *et al.*, 1996; Mertens *et al.*, 1999). Several mutations have been found in the haemophilia A patient's gene coding these sites of FVIII that lead to bleeding probably due to the impaired complexing with FIXa (Kemball-Cook and Tuddenham, 1997). Recombinant FVIII mutants with replacements of amino acids at positions 558 and 566 cannot bind FIXa either (Fay *et al.*, 1994). The activity of the tenase-complex is suppressed by the inhibition of enzyme FIXa or cofactor VIIIa. Inactivation of FVIIIa proceeds by two distinct pathways: proteolytic degradation or spontaneous dissociation. Activated FVIII has a very short half-life, 10 min (Curtis *et al.*, 1994; Lollar *et al.*, 1992). Instability is caused by the weakness of interaction between the A2 domain and the metal ion-linked A1/A3-C1-C2 dimer. Proteolytic degradation involves cleavage of the heavy chain at positions 336 and 562 by various enzymes, e.g. FIXa, FXa and activated protein C (Figure 1C) (Fay *et al.*, 1991a; Fay and Walker, 1989; Koedam *et al.*, 1988; O'Brien *et al.*, 1992; Walker *et al.*, 1987). The cleavage at position 336 leads to a release of acidic sequences, which cause a rapid release of the A2 domain from FVIIIa heterotrimer (Fay *et al.*, 1993). This acidic region is responsible for

the binding of substrate FX into the tenase-complex. Dissociation of the A2 domain from FVIIIa impairs both intermolecular (A2 domain and heterodimer) and intramolecular interactions (FVIIIa-FX). Activated protein C (APC) cleaves the FIXa binding site of FVIIIa at Arg562 (Fay *et al.*, 1991c). It is not clear yet whether the spontaneous dissociation or proteolysis dominates in FVIIIa inactivation. Some reports support the idea that dissociation predominates (Lamphear and Fay, 1992b), however, two different roles found for FIXa in the assembly of the FVIIIa-FIXa complex complicate the understanding of this process even more. Factor IXa stabilises FVIIIa by linking the A2 domain to the A3 domain (Fay *et al.*, 1994; Lenting *et al.*, 1996; Lollar *et al.*, 1984), and protects FVIIIa against inactivation by APC (Bertina *et al.*, 1984). However, in certain conditions FIXa can inactivate FVIIIa by cleaving at position 336, a motif that is cleaved by FXa and APC (Lamphear and Fay, 1992b; O'Brien *et al.*, 1992).

2.4. FVIII gene and common mutations

FVIII gene of 186 kb is one of the largest genes and accounts for 0.1% of the whole X-chromosome (Gitschier *et al.*, 1984; Toole *et al.*, 1984; Vehar *et al.*, 1984; Wood *et al.*, 1984). The coding region is distributed over 26 exons. Factor VIII mRNA is 9 kb long and encodes a 2,351-amino-acid-long polypeptide, from which a hydrophobic signal peptide of 19 amino acids is removed during secretion (Gitschier *et al.*, 1984; Toole *et al.*, 1984). 5% of all severe haemophilia A patients carry a unique mutation in the FVIII gene (Kemball-Cook and Tuddenham, 1997). Changes in intron 22 were identified by failure of polymerase-chain–reaction amplification across the boundary of exon 22 and exon 23 (Antonarakis, 1995). This inversion is mediated by the presence of three copies of a particular DNA sequence (termed sequence A), with one copy being located within intron 22 of the FVIII gene and the other two being 400 kb telomeric to the first (Levinson *et al.*, 1992a; Levinson *et al.*, 1992b; Freije and Schlessinger, 1992). Unequal crossing-over between two of these sequences leads to the inversion of a portion of the FVIII gene (exons 1 to 22) so that no intact FVIII protein is produced. The crossing-over with the most distal or the most proximal sequence A and its homologue in intron 22 results in Type 1 or Type 2 inversion, respectively. Some individuals have more than two copies of the extragenic sequence A outside the FVIII gene, whose crossing-over leads to Type 3 inversions (3A, 3B) (Lakich *et al.*, 1993; Rossiter *et al.*, 1994). All these inversions can be detected by Southern blotting. Type 1 inversion is the most common gene defect found in 35% of haemophilia A patients with a severe form of the disease (Antonarakis *et al.*, 1995). Type 2 inversion accounts for a reason for a severe form in 7% of patients (Antonarakis *et al.*, 1995). Deletions

in the FVIII gene ranging from 1000 to several hundred thousand nucleotides are quite rare and account only for 5% of the severe form of haemophilia (Kemball-Cook and Tuddenham, 1997). In the year 2000 the updated database of FVIII gene defects described 309 different mutations, 264 of them were single amino-acid substitutions resulting in various severity forms of haemophilia. In addition, 28 insertions ranging from 1 kb to 21 kb have been identified in the FVIII gene (Kemball-Cook and Tuddenham, 1997).

2.5. Treatment with FVIII preparations

Treatment of haemophilia aims at correcting the haemostatic defect in order to treat or prevent haemorrhage. Early treatment at the onset of symptoms decreases both the amount of bleedings and the extent of the ensuing tissue damage. The dosing regimen for factor VIII replacement in haemophilia A is based on three general pharmacological and therapeutic principles: (1) the volume of distribution of the clotting factor within intravascular or extravascular compartments, which affects *in vivo* factor recovery in plasma following infusion, (2) factor clearance and half-life in plasma, (3) the minimal level of the haemostatic factor required to control a particular type of haemorrhage. One unit of FVIII raises the plasma level by approximately 0.02 IU/ml (2%). The mean elimination half-life of FVIII is 12 h, however, it has to be measured in each particular patient because of a large variation in half-lives (Bjorkman *et al.*, 1992; Morfini *et al.*, 1992); and the minimum haemostatic plasma factor level is 20–30%. The average annual consumption of FVIII preparations for one patient with a severe form of the disease is 20,000 IU. The number of annual consumption of FVIII preparation per capita reflects the level of haemophilia care in the country. According to World Federation of Hemophilia (WFH) guidelines, adequate treatment is guaranteed when the number is more than 2.0 (Jones, 1995). In many countries (Denmark, Sweden, Canada, Ireland) the annual consumption of FVIII preparations per capita has reached 4 to 8 (Report on the WFH Global Survey 2001). Estonia reached the level 1.0 in 1998 and retained the consumption level slightly higher than 1 for more than 3 years.

Several FVIII preparations fractionated from plasma or produced by recombinant DNA technology are available for treatment nowadays. B-domain-deleted recombinant FVIII preparation is the newest on the market, however, it possesses comparable efficacy and safety with other recombinant FVIII preparations (Charlebois *et al.*, 2001; Courter and Bedrosian, 2001a; Courter and Bedrosian, 2001b; Pittman *et al.*, 1993). Plasma-derived FVIII preparations are still used in significant amounts, however, several additional measures have been applied in order to increase the safety of the product and to minimise the

risk of transmitting viruses and transmissive agents (e.g. prions), which may be present in plasma. Plasma-derived FVIII preparations can be divided into three main groups according to their purity and the manufacturing process: intermediate purity (specific activity 1–50 FVIII IU per mg of protein), high purity (50–200 FVIII IU per mg of protein), and monoclonal-antibody-purified concentrates (>1000 FVIII IU per mg of protein before adding stabilizing albumin, Berntorp, 1994; Berntorp, 1996). Compared to other plasma-derived products, the immuno-affinity purified concentrates contain only a trace amount of the von Willebrand factor, the physiological stabilizer of FVIII (Berntorp and Nilsson, 1988).

2.6. Structure and function of the von Willebrand factor

The von Willebrand factor (VWF) is a glycoprotein with an essential role in primary haemostasis and in blood coagulation. A VWF subunit consists of 2,050 amino-acids after the cleavage of the signal peptide (22 residues) and large propeptide (741 residues, D1-D2 domains) from the primary translation product, pro-VWF. The domains of VWF are arranged in the following sequence: D1-D2-D'-D3-A1-A2-A3-D4-B1-B2-B3-C1-C2-CK (Figure 2) (Sadler, 1998). The mature subunit is extensively glycosylated, containing 12 N-linked and 10 O-linked oligosaccharides (Matsui *et al.*, 1992). N-linked oligosaccharides of VWF are different from other plasma glycoproteins, because they contain ABO blood group oligosaccharides (Matsui *et al.*, 1992). After glycosylation in the endoplasmatic reticulum, pro-VWF dimerizes by forming disulphide bonds near the C-terminus (Marti *et al.*, 1987; Wagner and Marder, 1984). Dimers of pro-VWF are transported to the Golgi, and there additional disulphide bonds form between the N-terminus of subunits. The molecular weight of VWF multimers ranges from 500 kDa to 20 000 kDa (Haberichter *et al.*, 2000; Jorieux *et al.*, 2000). The removal of the VWF propeptide, completion of glycosylation and sulfation of N-linked oligosaccharides all take place in the Golgi complex. VWF is secreted constitutively (95%) and the remainder of it is stored in cytoplasmic granules, Weibel-Palade bodies that are specific of endothelium (Bonthron *et al.*, 1986; Wagner and Marder, 1984). In endothelial cells VWF pool consists of large multimers of mature VWF subunits, while constitutively secreted VWF consists mostly of small multimers and also of a high amount of unprocessed pro-VWF. Pro-VWF half-life *in vivo* is rather short (2 h) compared to the half-life of mature VWF (12 h, Borchiellini *et al.*, 1996).

VWF mediates the adhesion of platelets to subendothelium at the site of vascular injury and stabilizes factor VIII by forming a non-covalent complex (Sadler, 1998). Healthy persons with blood group O possess a lower VWF activity and antigen level (Moeller *et al.*, 2001; O'Donnell and Laffan, 2001;

Shima *et al.*, 1995a), which is accompanied by a reduced level of FVIII:C activity (McLellan *et al.*, 1988). The importance of the pre-infusion level of the VWF antigen in haemophilia A patients for the FVIII half-life has been studied and patients with blood group O had a lower FVIII half-life as compared to patients with blood group A (Vlot *et al.*, 2000). Although each subunit of VWF possesses a high affinity binding site for FVIII, in plasma only 20% of the sites are occupied by FVIII (Leyte *et al.*, 1989). In addition to the stabilization of FVIII in plasma, VWF can alter the intracellular trafficking of FVIII from the constitutive to the regulated secretory pathway by producing an intracellular storage pool of both proteins (Rosenberg *et al.*, 1998).

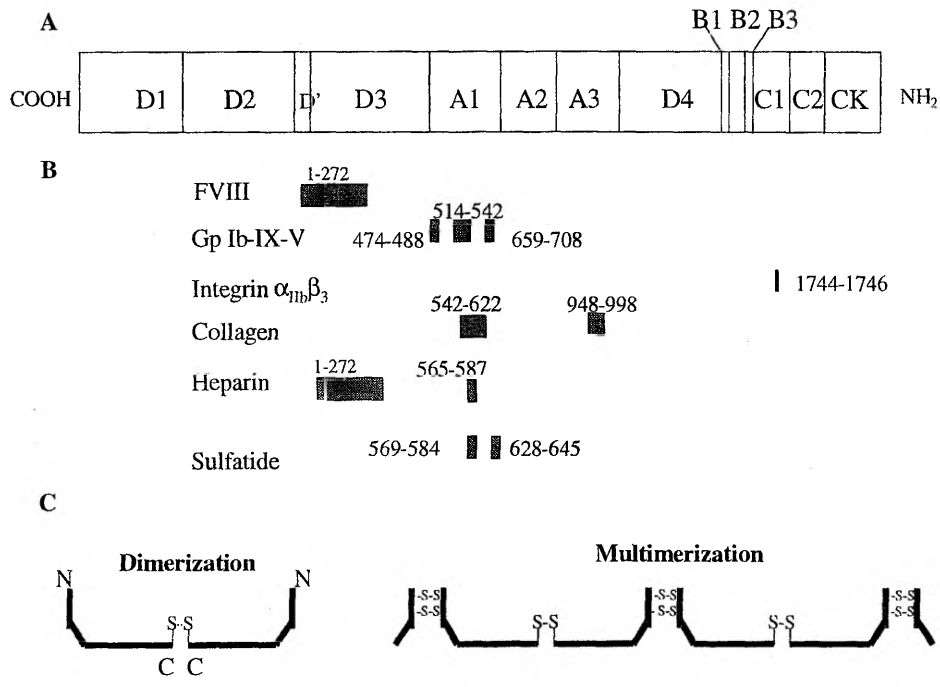

Figure 2. Structure of the von Willebrand factor. The localization of five types of conserved structural domains (**A**). Binding sites of various macromolecules within the subunit (**B**). Dimerization by formation of a disulphide bond between the C-termini and multimerization by disulphide bonding near the N-terminal regions of VWF monomers (**C**) (Sadler, 1998).

FVIII dissociates from VWF to participate in coagulation, while VWF mediates the formation of a platelet plug. It has been shown that VWF binds to FVIII less avidly after the VWF association with collagen and other matrix structures such as proteoglycans that cause the conformational change of the FVIII binding site

of VWF (Bendetowicz *et al.*, 1999). Subendothelium-bound VWF supports platelet adhesion by a dual step mechanism (Savage *et al.*, 1996). The first interaction between the A1 domain of VWF and the GP Ib-IX-V receptor complex on platelets is rapid and VWF attacks platelets near the surface at a high shear stress. Rolling platelets make new interactions as different regions of the platelet membrane come into closer contact with the vascular surface. Signal transduction leads the activation of integrin $\alpha_{IIb}\beta_3$ on platelets, which mediates the secondary adhesion and aggregation of platelets and binding via integrin $\alpha_{IIb}\beta_3$ to the C-terminus of the C1 domain of VWF. This interaction has a lower dissociation constant compared to VWF interaction with the GP Ib-IX-V receptor complex and therefore causes an irreversible adhesion of platelets (Savage *et al.*, 1996).

The malproduction of the VWF causes the most common inherited bleeding disorder – the von Willebrand's disease (VWD) with the prevalence of 0.83% in general population (Mannucci *et al.*, 1984). The simplified classification of VWD distinguishes between three groups i.e. partial quantitative deficiency (Type 1), qualitative deficiency (Type 2) and total quantitative deficiency (Type 3) (Sadler, 1994). A highly variable clinical picture and the presence of many different defects in the molecule of VWF complicate the diagnosis of VWD (Mazurier and Rodeghiero, 2001; Meyer *et al.*, 1991). To date no single test giving simultaneously sufficient information about various functions of VWF is available. Several analyses like the ristocetin cofactor aggregation method, the VWF antigen assay, the factor VIII coagulation activity assay and others are required for diagnosing VWD and for the characterization of the defect in a particular patient. Factor VIII coagulation (FVIII:C) activity indirectly reflects the level of its stabilizing protein VWF (Sadler, 1998). The VWF antigen level is estimated by the ELISA method using polyclonal VWF antibodies and thus it does not reflect the functional activity of VWF. The assay of the ristocetin cofactor (VWF:RCo) activity has been widely used for measuring VWF ability to mediate platelet aggregation. The dimeric form of antibiotic ristocetin induces platelet aggregation via binding to both VWF and platelets (Scott *et al.*, 1991). The drawbacks of this test include poor sensitivity (Rodeghiero and Castaman, 1990), difficulties with the standardization (Casonato *et al.*, 1999) and the lack of a physiological analogue. The monoclonal VWF antibody based commercial ELISA test for evaluating RCof–like activity is less efficient in discriminating between VWD subtypes as compared to the collagen binding assay (Favaloro, 2000). The binding of VWF to collagen in the subendothelium is the first step in initiating primary haemostasis. The binding sites of fibrillar collagens Type I and III localize within the A3 domain (Pareti *et al.*, 1987; Ribba *et al.*, 2001; van der Plas *et al.*, 2000), while the collagen Type VI binding site is in the A1 domain of the VWF molecule (Cruz *et al.*, 1995; Hoylaerts *et al.*, 1997; Romijn *et al.*, 2001; van der Plas *et al.*, 2000). The native conformation of VWF is essential for interaction with collagen, and even a

limited reduction of multimers to their dimeric form results in a decreased binding to collagen (Fischer *et al.*, 1996; Pareti *et al.*, 1987). A recently introduced simple ELISA test for collagen binding (VWF:CB) is sensitive and seems to be helpful for screening VWD patients (Casonato *et al.*, 1999; Ramasamy *et al.*, 1998; Siekmann *et al.*, 1998). Accordingly, in the present study the VWF content in FVIII preparations was characterized using the collagen binding assay in addition to the other tests for the quantification of VWF.

2.7. Factor VIII antibodies

2.7.1. Prevalence and incidence of FVIII antibodies among haemophilia A patients

Mutated FVIII gene of haemophilia A patients results in the expression of protein with an impaired functional activity or lack of FVIII protein. Therefore, administered FVIII can be recognized by organism as a foreign protein that induces the immune system response. The development of FVIII antibodies is associated with a risk of dangerous bleeding episodes with possible life-threatening or neurological complications, muscle and/or joint impairment and consequently increased morbidity and mortality (Kasper, 1989).

The first reports describing the so-called anticoagulant effect in a haemophilia patient were published already in 1942 and 1943 (Craddock and Lawrence, 1947). These case reports described neither correction in bleeding time nor improvements in clinical symptoms after repeated infusions of blood to haemophilia A patients (Craddock and Lawrence, 1947). Development of the "anticoagulant effect" was also recognized as a reason for acquired bleedings in healthy persons. In 1954, Houge and Fearnley showed that "the anticoagulant effect" was caused by an antibody and not by an enzyme as it had been suggested earlier (Hougie and Fearnley, 1954). They also found that autoantibodies and alloantibodies have a similar property of inhibiting FVIII, and the titer of antibodies fluctuated largely due to the treatment with blood infusions (Hougie and Fearnley, 1954). Nowadays FVIII preparations of different purity rather than blood is used. Haemophilia A patients who have developed FVIII antibodies are divided into groups of high responders (>5 Bethesda units per ml, BU/ml) and low responders (<5 BU/ml) according to their response to FVIII administration (White *et al.*, 2001). One Bethesda unit is defined as an amount of FVIII antibodies, which neutralizes FVIII:C activity by 50% after 2 h of incubation at 37 °C (Kasper *et al.*, 1975). Generally antibodies develop after relatively few exposure days to the FVIII preparation (a day on which at least one dose of the FVIII preparation was administered), the medium being 9–15 days (Scharrer *et al.*, 1999). Therefore, most of the antibodies are diagnosed at

the young age of a patient (0.08–5.2 years). Patients with FVIII:C activity lower than 1 IU/ml need more frequent replacement therapy, and antibody development is also more frequent among this group of patients (up to 50%) (Scharrer *et al.*, 1999). Now it is known that the defect in the FVIII gene is one of the main reasons for a FVIII antibody response to occur. According to the updated database, up to 60% of patients with intron 22 inversion and 38% of patients with large deletions of the FVIII gene develop anti-FVIII antibodies (Kemball-Cook and Tuddenham, 1997; Oldenburg *et al.*, 2000; Schwaab *et al.*, 1995a). Another patient-specific determinant is the HLA genotype. However, no relationship between the FVIII antibody development and the HLA-genotype has been demonstrated until now in Caucasians (Aly *et al.*, 1990; Frommel *et al.*, 1981; Hay *et al.*, 1997; Lippert *et al.*, 1990; Oldenburg *et al.*, 1997). In Japanese haemophilia A patients the absence of HLA-A24 has been demonstrated to be one determinant for the FVIII antibody development, and HLA-DR4.1, DQ4 and DQA1*0301=2 were also positively associated with patients possessing FVIII antibodies compared with normal subjects (Ohta *et al.*, 1999).

The effect of replacement therapy with the FVIII preparation of different purity on the FVIII antibody development has been intensively studied in both previously untreated (PUP) and previously treated patients (PTP). Factor VIII antibodies in all these studies have been measured by the Bethesda assay, which estimates the amount of inhibitory antibodies. The antibody frequency of published PUP studies is shown in Table 3. Studies conducted on PUPs have several methodological differences, but the prevalence of high responder patients can be compared. Most of these studies are retrospective and possess a lower frequency of the testing of FVIII antibodies compared to prospective studies. Therefore, the prevalence of low titer antibodies and transient antibodies may have been overlooked. Furthermore, it was not specified how many patients of the study group carried intron 22 inversion in the FVIII gene. The administration of plasma-derived concentrates of higher purity and the application of recombinant DNA technology for FVIII production did not lead to any significant change in the incidence of FVIII antibody formation.

Whether the change of the FVIII preparation leads to a higher risk of antibody development has been studied in previously treated patients (PTP). Generally patients extensively treated with the pdFVIII preparation developed no FVIII antibodies after a change to the rFVIII preparation (Table 4), except a single patient due to the treatment with Kogenate (Schwartz *et al.*, 1990) and another patient who was treated with ReFacto (Courter and Bedrosian, 2001a). Among Canadian haemophilia A patients, the frequency of FVIIII antibody development was also low, 2–3% (Giles *et al.*, 1998). However, several case reports have shown that the patient treated extensively with an intermediate or high purity concentrate has responded to the administration of monoclonal-antibody-purified concentrate or the recombinant FVIII preparation by anti-FVIII antibody production (Baglin and Beacham, 1998b; van den Berg *et al.*,

1999). In two patients the development of FVIII antibodies was reported after the change to a high purity FVIII preparation Replenate® (Bio Products Laboratory, Elstree, UK) (Baglin and Beacham, 1998a). The use of the recombinant FVIII product for the first time in continuous infusion during surgery caused the development of high titer antibodies (van den Berg *et al.*, 1999). Generally antibody response is rather rare in previously treated patients and is probably influenced by other simultaneously occurring processes, like continuous infusion of FVIII during surgery, infection or medication.

Table 3. Comparison of frequency of development of inhibitory FVIII antibodies in different studies on previously untreated patients (PUP).

Reference	FVIII preparation	Study period	n	Patients with severe haemophilia	FVIII antibodies	
					Total	HR
Courter and Bedrosian, 2001b	ReFacto	1994–99	101	54	32%	16%
Bray *et al.*, 1994	Recombinate™	1990–93	73	55	23%	9%
Gruppo *et al.*, 1997	Recombinate™	1990–96	72	72	31%	10%
Lusher *et al.*, 1993	Kogenate®	1989–92	81	49	20%	9%
Lusher, 1991	Monoclate®	1986–89	38	25	18%	16%
Ehrenforth *et al.*, 1992	Various pd	1976–91	63	27	24%	17%
Addiego *et al.*, 1993	Various pd	1975–85	89	89	28%	21%
de Biasi *et al.*, 1994	Various pd	1975–92	64	48	20%	16%
Peerlinck *et al.*, 1993b	Lyophilised cryoprecipitate	1971–90	67	47	6%	2%

Pd - plasma derived; HR - high responders

Table 4. FVIII antibody development in patients switched from treatment with pdFVIII preparations to rFVIII preparation.

Reference	rFVIII preparation	n	FVIII antibodies %
Courter and Bedrosian, 2001a	ReFacto	113	0.9
Schwartz *et al.*, 1990	Kogenate®	72	1.3
Aygoren-Pursun and Scharrer, 1997	Kogenate®	39	0
Abshire *et al.*, 2000	Kogenate FS	71	0
Giles *et al.*, 1998	Kogenate®	478	2–3
White *et al.*, 1997	Recombinate™	69	0

Only two concentrates (Bicinact® in Belgium and OctaviSDPlus) which were produced by using the treatment with a solvent-detergent and heat pasteurization for virus inactivation have been exceptions, causing a remarkably higher frequency of antibody response in a large group of patients (Gilles *et al.*, 1997; Peerlinck *et al.*, 1997). On the other hand, discontinuous use of these concentrates was accompanied by the disappearance of FVIII antibodies in treated patients. Epitope mapping showed that FVIII antibodies were targeted at the C2 domain of FVIII (Laub *et al.*, 1999; Sawamoto *et al.*, 1998), and it was suggested that the insufficient quality of starting plasma pools with the combination of solvent-detergent treatment and pasteurization altered the structure of the C2 domain (Saenko *et al.*, 2001), which led to an enhanced binding to phospholipids (Raut *et al.*, 1998). FVIII purification by controlled-pore silica adsorption and pasteurization (60°C, 10 h) for viral inactivation (FVIII CPS-P in the Netherlands and FVIII P in Belgium) also caused changes in the FVIII structure and a higher FVIII antibody response in previously treated patients (Mauser-Bunschoten *et al.*, 1994; Peerlinck *et al.*, 1993a). However, none of these concentrates is in clinical use any more.

Different purity FVIII preparations have various effects on the immune system of haemophilia A patients as shown by *in vitro* and *in vivo* studies. Results of *in vitro* studies revealed the decreased levels of IL-2, TNF, IFN-γ and increased levels of IL-4, IL-10 in each different leukocyte subset after stimulation with the FVIII preparation of intermediate purity (Hodge *et al.*, 1999a). The immuno-modulatory effect of these concentrates was explained by the presence of TGF-β (Hodge *et al.*, 1999a). *In vivo* investigations in HIV-negative haemophiliacs without FVIII antibodies have shown an increased number of CD8+ cells and a decreased number of CD4+ cells, suggesting that the immuno-modulatory effect of treatment was probably caused by the presence of TGF-β

in FVIII preparations (Hodge *et al.*, 1999b). TGF-β is suggested to have some Th2-like effects, analogous to the co-stimulatory effect on CD8+ T-cells. The FVIII preparations of intermediate purity in general are more prone to decrease the number of CD4+ cells compared to immuno-affinity purified concentrates used for the treatment of HIV–positive haemophilia A patients (de Biasi *et al.*, 1991; Seremetis *et al.*, 1993; Varon, 1995). HIV-negative haemophiliacs who developed FVIII antibodies have been investigated only in one multicentre study so far (Kaplan *et al.*, 2000). This study showed no difference in IFN-γ and IL-10 production by the FVIII-induced lymphocytes in haemophilia A patients who developed FVIII antibodies and who did not (Kaplan *et al.*, 2000).

2.7.2. Treatment of haemophilia A patients possessing FVIII antibodies

Treatment of haemophilia A patients possessing anti-FVIII antibodies has two distinct purposes: to prevent or treat bleeding episodes and to induce tolerance for FVIII. Bleeding episodes of low responder patients can be treated by increased dosages of the FVIII preparation, but this is not an optimal treatment for high responders. Factor VIII bypassing agents have been used for almost over 25 years. Prothrombin complex concentrates (PCC), activated prothrombin complex concentrates (APCC) as well as recombinant FVIIa (rFVIIa) theoretically overcome the need for FVIII in achieving effective haemostasis. Administration of APCC increases the level of factors II, VII, VIIa, X, and Xa, all of which are responsible for the generation of a significant amount of thrombin in the absence of FVIII (Sultan and Loyer, 1993). In animal models the administration of a higher amount of factors Xa and prothrombin, the main components in APCC, leads to the generation of a significant amount of thrombin analogously with the administration of APCC (Turecek *et al.*, 1999). So far the administered dose of APCC has been based on empirical observations. Unfortunately, APCC also contains trace amounts of FVIII and FIX, therefore some patients may respond with an elevated level of FVIII antibodies after treatment episodes (Hilgartner and Knatterud, 1983; Kantrowitz *et al.*, 1987; Laurian *et al.*, 1984; Mannucci *et al.*, 1976; Negrier *et al.*, 1997). Compared to prothrombin complex concentrates, APCC is not more thrombogenic, probably because the coagulation defect is never fully corrected due to the deficiency of FVIII or FIX in haemophilia patients. Very rarely has the treatment of a patient resulted in myocardial infarction (Chavin *et al.*, 1988; Negrier *et al.*, 1997) or disseminated intravascular coagulation (Fukui *et al.*, 1981; Negrier *et al.*, 1997; Stenbjerg and Jorgensen, 1977).

An alternative possibility for treatment is the application of recombinant FVIIa (NovoSeven, Novo Nordisk, Denmark). The amount of the administered

rFVIIa is usually given as protein concentration in micrograms per kilogram of body weight. Due to the short half-life (2.5–3 h), rFVIIa has to be regularly administered after every 2–4 hours (Brinkhous *et al.*, 1989). FVIIa forms a complex with the tissue factor and activates FX and FIX on the surface of platelets, which also become activated. In the absence of FVIII or FIX, rFVIIa is also able to activate FXa if the platelet surface is provided (Hoffman *et al.*, 1998). At concentrations considerably exceeding the normal level rFVIIa can also act independently from the tissue factor (Monroe *et al.*, 2000) and overcome the inhibiton of FX generation by physiological factor VII (van't Veer and Mann, 2000). It has been reported recently that the administration of rFVIIa in high doses induces thrombin generation in healthy volunteers even after pre-treatment with a specific inhibitor, recombinant nematode anticoagulant protein c2, which inhibits the tissue factor-FVIIa complex activity (Freiderich *et al.*, 2001). A prospective study to compare *in vivo* efficiency of both bypassing agents, APCC and rFVIIa, is in preparation now and hopefully will add complementary information as to which concentrate has to be preferentially used for particular types of bleedings and for preventing bleeding episodes in haemophilia A patients possessing anti-FVIII antibodies.

Factor VIII preparation fractionated from porcine blood is also available for the treatment of patients with FVIII antibodies. However, it can only be used in patients possessing antibodies with low cross-reactivity to porcine FVIII (Fiks-Sigaud *et al.*, 1993). Generally, antibodies recognizing the A2 domain of FVIII have a lower affinity to porcine FVIII as compared to human FVIII (Sawamoto *et al.*, 1997).

Among various immune tolerance treatment protocols the Bonn regimen recommends the administration of high doses of FVIII twice a day and a regular dose of APCC (Brackmann and Gormsen, 1977). The overall success rate of the Bonn protocol for the desensitisation of haemophilia A patients was 86% (52/60), while 13.3% failed (8/60) as reported by Oldenburg (Oldenburg *et al.*, 1999). The interruption of treatment remarkably prolonged the duration of treatment: medium 39.9 months versus 11.6 months by continuous treatment. There was also a tendency that patients with intron 22 inversion in the FVIII gene needed a longer treatment period as compared to patients with other gene defects. The best success rate was achieved by using FVIII dosage >200 IU/kg/day (Oldenburg *et al.*, 1999). In a retrospective study of paediatric patients good correlations were found between the outcome and length of immune tolerance treatment and also between the outcome and interval from the first FVIII antibody detection till the onset of treatment (Kreuz *et al.*, 1995b). The interruption of therapy was the main reason for the failure of treatment, because a subsequent increase of the antibody titer was much more difficult to suppress. Antibody elimination was achieved in 19/21 patients during medium time of 4 months in high responders and 1.5 months in low responders. As a conclusion, Kreuz and colleagues recommended the use of at least 100 IU/kg of FVIII per day and the starting of treatment as soon as possible after the

detection of FVIII antibodies in order to achieve immune tolerance during a short treatment period (Kreuz *et al.*, 1995b).

No correlation between the purity of the FVIII preparation used for treatment and the effectiveness of the treatment has been established. In most cases the patients have been treated with pdFVIII preparations (Oldenburg *et al.*, 1999), while rFVIII preparations have also been reported to be effective in making patients tolerant to FVIII (Batlle *et al.*, 1999; Rothschild *et al.*, 1998). However, Kreuz *et al.* have reported 4 therapy-resistant patients, who were successfully tolerated after a switch from the immuno-affinity-purified FVIII preparation of a very high purity to the one of with intermediate purity (Kreuz *et al.*, 1995a). Despite of the importance of VWF as a physiological stabilizer of FVIII in concentrates, it is still unknown how the presence of VWF could protect FVIII by FVIII antibodies *in vivo* and benefit in achieving tolerance.

Slight modifications of the Bonn protocol are also efficient in inducing tolerance to FVIII. Nowadays the administration of APCC is skipped and it is included only in cases when a patient has a high bleeding tendency. The recommended FVIII dosage is 150 IU/kg/twice a day to shorten the time period necessary for successful toleration.

The Malmö protocol is the most radical treatment of high responders to rapidly decrease the titer of FVIII antibodies (Carlborg *et al.*, 2000; Nilsson and Berntorp, 1990). It comprises the extracorporal adsorption of IgG antibodies on a protein A column (removing IgG from a patient's blood), followed by the administration of a high dose of FVIII, cyclophosphamide (immunosuppressive effect) and intravenous immunoglobulin (replacement of the removed IgG). So far 15 haemophilia A patients have been treated according to the Malmö protocol (Berntorp, 2001). Although by using intermediate purity FVIII preparations in the Malmö protocol successful tolerance was achieved in 10 patients, 3 patients failed to achieve it. Only 2 patients were treated with the monoclonal antibody-purified FVIII preparation and the treatment of both patients was unsuccessful. The overall success rate of this treatment was 67% (Berntorp, 2001).

In addition to sepharose-bound staphylococcal protein A for removing FVIII antibodies (Immunosorb™, Sweden), another type of affinity resin has been reported to be effective (Knobl *et al.*, 1995). This contains the sepharose-bound polyclonal sheep antibody to human immunoglobulins, which removes all subclasses of IgG, IgM, and IgA from the patient's blood (Ig-Therasorb™, Germany). Until now, this treatment protocol has been used to reduce the FVIII antibody titer in ten patients, however, without long-lasting tolerance (Jansen *et al.*, 2001).

Administration of prednisone (Aznar *et al.*, 1983) or cyclophosphamide and intravenous immunoglobulin (Nilsson *et al.*, 1988) in addition to FVIII has also been successfully applied to achieve tolerance in haemophilia A patients and patients with acquired hemophilia.

In order to facilitate the comparison of the clinical efficacy of different immune tolerance treatment (ITT) protocols, three ITT registries have been established: international (Mariani and Kroner, 1999), North-American (DiMichele and Kroner, 1999) and German (Lenk, 1999). These registries are retrospective and in 1999 data of 295 patients, 68% from Europe and 28% from North America were included in the international registry. The remaining 4% covered the data of patients from Japan and Australia (Mariani and Kroner, 1999). Multivariate analysis was performed to evaluate the variables determining the success of ITT. A high dose of FVIII as well as the low FVIII antibody titer at the beginning of treatment was significant for the successful elimination of FVIII antibodies. Two other parameters, the maximal historical titer and the time interval from the first detection of the antibody until the start of treatment were not decisive in the success rate (Mariani and Kroner, 1999).

2.7.3. Properties of FVIII antibodies

In 1968 anti-FVIII antibodies of anticoagulant activity were found to be of IgG and IgM subclasses. These antibodies did not precipitate after the forming of the antigen-antibody complex, and the formed complex was not able to activate the complement system (Lusher *et al.*, 1968). Further studies revealed the prevalence of the IgG4 subclass antibody in a haemophilia A patient (Andersen and Terry, 1968). Other investigators have reported predominantly the IgG4 subclass, however, a study on the heterogeneous mixture of antibodies has indicated that the isotypic distribution of FVIII antibodies followed the general physiological profile of IgG subclasses (Gilles and Saint-Remy, 1994). Accordingly, in the present study IgG subclasses of FVIII antibodies of the haemophilia A patient during the immune tolerance treatment have been analysed.

Anderson and Troup studied the neutralization of FVIII:C by antibodies developed in haemophilia A patients (Andersen and Troup, 1968). They found that there was a linear relationship between the residual FVIII:C activity (on a logarithmic scale) and time (linear scale) at the higher tested dilutions of the anticoagulant, while at lower dilutions linearity was lost. The neutralisation of FVIII by antibodies did not follow the first type of kinetics over 2 h of incubation. When the residual FVIII:C activity on a logarithmic scale was plotted against antibody dilution on a linear scale, the curves of only a few antibody samples were linear. It was explained by the heterogeneity of antibodies with anticoagulant properties and by a different mechanism of inhibiting FVIII (Andersen and Troup, 1968). Now two types of antibodies can be distinguished by the neutralizing character of the FVIII:C activity (Gawryl and Hoyer, 1982; Ling *et al.*, 2001). Type 1 antibodies cause a complete inhibition of FVIII:C activity and a linear relationship between the residual FVIII:C activity (on a

logarithmic scale) and antibody concentration is observed (Biggs *et al.*, 1972b). Type 2 antibodies show a non-linear pattern of this relationship and incomplete inactivation of FVIII:C even at very high antibody concentrations (Biggs *et al.*, 1972a). Type 2 inactivation pattern of FVIII:C was also suggested to be characteristic of FVIII-light-chain-specific antibodies that are capable of inhibiting the FVIII binding to VWF (Peerlinck *et al.*, 1999). Further a more precise epitope mapping showed that the C2 domain was the main target of these antibodies (Peerlinck *et al.*, 1997; Sawamoto *et al.*, 1998).

2.7.4. Epitope specificity of FVIII antibodies and relation to the mechanism of inhibition of FVIII biological activity

Immunoblotting analysis using intact or thrombin-digested FVIII enabled the researchers to reveal the preferential binding of FVIII antibodies to the light chain of FVIII and the A2 domain in up to 68% of haemophilia A patients (Foster *et al.*, 1990a; Fulcher *et al.*, 1987; Scandella *et al.*, 1989). However, antibodies against conformational epitopes cannot be detected by immunoblotting. Therefore, amino acid residues 373–740 of the A2 domain of FVIII were expressed from a baculovirus vector in insect cells (Scandella *et al.*, 1993; Scandella *et al.*, 1992). Resulting A2 fragment was soluble and suitable for use in an immunoprecipitation assay. The immunoprecipitation (IP) assay is sensitive for the detection of a low amount of antibodies and also those A2-domain antibodies, which were not detected by immunoblotting. The development of the A2-domain-specific antibodies in patients was significantly higher than previously estimated by the Western blot (Scandella *et al.*, 1992). Later, in addition to the recombinant A2 domain, C2 domain and A3-C1 fragments were used in the IP assay (Prescott *et al.*, 1997; Scandella *et al.*, 1995a; Scandella *et al.*, 1995b). The immunoprecipitation assay demonstrated that the most immunogenic domains were the C2 and A2 domains, as most of the antibodies were targeted at one or both of these regions of FVIII, while some of FVIII-light-chain specific antibodies recognized the A3-C1 fragment (Scandella *et al.*, 2001). The respective FVIII fragments that were used in the IP assay are rather large, but antibodies recognize the epitope containing 6–12 amino-acid residues (Palmer *et al.*, 1997). Therefore, the fine specificity of antibodies developed in patients cannot be assessed with these recombinant FVIII fragments. Taking advantage of synthetic peptide arrays, a wide repertoire of antibody epitopes has been discovered within all FVIII domains (Palmer *et al.*, 1997). This more precise technique has demonstrated the presence of binding sites of rare antibodies within the A1 domain in addition to the localization of most immunogenic regions within the C2, A2, A3 and C1 domains (Palmer *et al.*, 1997). However, this method has also limitations. Firstly, antibody epitopes are

quite often discontinuous rather than continuous, and secondly, the epitopes that are presented by synthetic peptides may actually not be accessible on the native FVIII. With synthetic peptides no antibodies targeted at important cleavage sites on FVIII were detected (Palmer *et al.*, 1997). The inhibitory activity of FVIII antibodies depends largely on the accessibility of the antibody binding site on FVIII. Random fragments of FVIII displayed on λ phage particles were selected to characterize the specificity of antibodies directed to conformational epitopes within the C2 domain (Kuwabara *et al.*, 1999). This study showed the importance of the disulphide bridge in the C2 domain for being recognized by antibodies (Kuwabara *et al.*, 1999).

Antibodies recognizing different domains can neutralize FVIII:C activity by independent mechanisms (Scandella, 2000). The light chain of FVIII contains the binding sites for VWF within the C2 domain (aa 2303–2332) and the a3 domain (aa 1649–1671) (Saenko and Scandella, 1997; Saenko *et al.*, 1994; Shima *et al.*, 1995b; Shima *et al.*, 1993; Vlot *et al.*, 1995). Therefore, it can be expected that antibodies against those regions could interfere with the FVIII binding to VWF. Indeed, human antibodies directed against epitopes within amino acids 2170–2327 of the C2 domain or within amino acids 1670–1684 of the a3 domain are capable of inhibiting the FVIII binding to VWF (Foster *et al.*, 1990b; Healey *et al.*, 1998; Shima *et al.*, 1993). Human anti-FVIII antibody 2E9, which is C1-domain specific, obtained by the immortalization of B-lymphocytes of a patient with mild haemophilia A, is also able to interfere with the FVIII binding to VWF (Jacquemin *et al.*, 2000). On the contrary, some FVIII antibodies targeted at amino acids 2218–2307 of the C2 domain have the highest inhibitory activity in the presence of VWF (Saenko *et al.*, 1996). These antibodies delay the release of FVIII from the FVIII-VWF complex and thereby inhibit the biological activity of FVIII (Saenko *et al.*, 1996).

Since the C2 domain of FVIII contains a highly hydrophobic region, which contains partially overlapping binding sites for VWF and phospholipids, usually C2-domain-specific antibodies are able to interfere with the FVIII binding to PS and the FVIII binding to VWF (Foster *et al.*, 1990b). Recently it has been shown that C2 domain inhibitory antibodies, such as BO2C11, frequently recognize the region containing amino acid residues 2199/2200 and 2251/2252 of the C2 domain (Barrow *et al.*, 2001) and interfere with the FVIII binding to VWF and PS (Barrow *et al.*, 2001; Jacquemin *et al.*, 1998).

The A2 domain of the heavy chain of FVIII is a site at which antibodies in haemophilia A patients preferentially develop. Theoretically these antibodies may interfere with the interaction of the A2 domain with the A1 domain or with the A1/A3-C1-C2 dimer or block thrombin activation at Arg391 via steric hindrance or the conformational effect. A2-domain antibodies could also block the interaction of FVIII with FIXa or FX (Fay *et al.*, 1991b). The A2 domain and A3 domain of FVIII contain FIXa binding sites (Fay *et al.*, 1994; Lenting *et al.*, 1996; Mertens *et al.*, 1999). The binding of FVIII to FIXa is impaired in the presence of VWF (Lenting *et al.*, 1994). Human A2-domain-specific antibodies

specially recognizing region 484–509 inhibit FVIII:C activity by disrupting the A2 domain binding to FIXa (Fay *et al.*, 2001; Fay and Scandella, 1999). Human A3-domain specific antibodies with binding sites within amino-acids 1804–1819 (Zhong *et al.*, 1998) and 1778–1823 (Fijnvandraat *et al.*, 1998) and the murine monoclonal antibody targeted at amino acids 1778–1840 (Lenting *et al.*, 1994) are also able to inhibit the interaction of FVIII with FIXa. Zhong with co-authors has investigated the epitope specificity of 18 FVIII-light-chain antibodies developed in haemophilia A patients (Zhong *et al.*, 1998). These antibodies were partly neutralized by the recombinant C2 domain, and the epitope of antibodies was localized within the A3 domain (aa 1804–1819). Three out of the 18 tested samples were able to interfere with the FVIII binding to FIXa and to abolish the generation of Xa (Zhong *et al.*, 1998).

The development of A1-domain-specific antibodies is rather rare (Palmer *et al.*, 1997; Scandella *et al.*, 2001). The a1 domain has been shown to contain a FX binding site within amino acids 337–372 (Lapan and Fay, 1997). Using synthetic peptide arrays a small amount of antibodies recognizing 1–2 different FVIII peptides from region 20–349 has been found in haemophilia A patients (Palmer *et al.*, 1997). Inhibitory activity of the A1 domain antibodies may be caused by interfering with the A1 domain binding to A2 and/or to the A3-C1-C2 fragment by abolishing thrombin cleavage at Arg391 or by blocking the binding to other tenase complex components. Human antibodies towards regions aa 351–365 and aa 336–372 and the murine monoclonal antibody with an epitope within aa 338–362 of the A1 domain have been described to inhibit FVIII:C activity (Foster *et al.*, 1988; Ware *et al.*, 1988). The mechanism of the inhibition of the FVIII:C activity might be due to the inhibition of cleavage by activated protein C (cleavage site at Arg336) or by thrombin (cleavage site at Arg372).

The N-terminal and C-terminal regions of the B-domain (aa 760–1667) are also targets of several FVIII antibodies. However, the B-domain deleted FVIII has a normal FVIII:C activity and possesses a normal binding to VWF (Sandberg *et al.*, 2001), obviously antibodies against the B-domain have little or no influence on FVIII:C activity.

2.7.5. V_H repertoire of human immunoglobulins directed to FVIII

Limited information is available about the primary structure of human antibodies directed to FVIII. Van den Brink with the co-workers have investigated which germline gene is responsible for encoding the variable domains of the heavy chain of immunoglobulins directed towards the most immunogenic regions of FVIII, namely the C2, A2, and A3-C1 domains (van den Brink *et al.*,

2000a; van den Brink *et al.*, 2001; van den Brink *et al.*, 2000b). The single chain variable domain antibody fragment (scFv) directed to the C2 domain of FVIII was derived from the antibodies of a patient with acquired haemophilia. Sequence analyses revealed that the heavy chain of scFv was encoded by V_H genes, the most homologous ones to germline gene segments DP-10, DP-14 and DP-88 belonging to the V_H1 gene family (van den Brink *et al.*, 2000b). An earlier report using the Epstein-Barr virus for the immortalization of lymphocytes, a monoclonal IgG4 antibody (BO2C11) was derived from the B-cell repertoire of a haemophilia A patient with inhibitory FVIII antibodies (Jacquemin *et al.*, 1998). Gene segment DP-5, which also belongs to the V_H1 gene family, encoded the heavy chain of the C2-domain-specific antibody (Jacquemin *et al.*, 1998). These two studies have reported that the antibodies of C2-domain specificity are assembled by using V_H gene segments derived from the V_H1 family (Jacquemin *et al.*, 1998; van den Brink *et al.*, 2000b).

Several scFv of the A2 domain antibodies derived from a haemophilia A patient have also been characterized (van den Brink *et al.*, 2000a). One A2-domain-specific antibody scFv (aa 484–508) was encoded by germline gene segment DP-10 of the V_H1 gene family. Another A2 domain antibody scFv with the binding site within amino acids 712–736, but non-inhibitory to FVIII, was encoded by germline gene DP-47 of the V_H3 gene family. DP-47 is the most frequently rearranged germline gene segment in the human repertoire, which is observed in 12% of IgG positive B-cells (van den Brink *et al.*, 2000a).

The third most common immunogenic region lies within the A3-C1 domains. Six human monoclonal scFv fragments directed towards the A3-C1 domains were isolated by using the phage display technology (van den Brink *et al.*, 2001). Epitope mapping of scFv by using hybrid FVIII/FV light chain fragments showed that five out of six scFv were directed towards residues 1778–1840 within the A3 domain, while one recognized the independent region. Sequence analyses revealed that the V_H domains of two scFv were encoded by germline gene segments of the V_H1 gene family and those of four by germline gene segments belonging to the V_H3 gene family. The authors concluded that anti-A3 antibodies are composed of the V_H domains derived from multiple germline gene segments of the V_H1 and V_H3 families (van den Brink *et al.*, 2001).

2.7.6. Immune response to human FVIII in murine model of haemophilia A

Mice with targeted disruption of exon 16 or 17 of the FVIII gene are deficient in FVIII, and the clotting assay estimates FVIII:C activity to be less than 1% of normal. These mice have impaired haemostasis, severe bleeding after a minor

trauma like tail snipping, subcutaneous and intramuscular bleeding after routine handling and spontaneous bleeding (Bi *et al.*, 1995; Bi *et al.*, 1996; Qian *et al.*, 1999; Qian *et al.*, 2000). FVIII-deficient mice have been used as an animal model to study the mechanism of the anti-FVIII antibody response to the administered human FVIII (Wu *et al.*, 2001). After four or five intravenous infusions of human FVIII, mice developed FVIII antibodies and after 6 injections FVIII antibodies with inhibitory activity. Most of the antibodies were of the IgG1 and to a lesser extent of the IgG2 subclass, indicating that both Th1 and Th2 cells were involved in antibody response. CD4+ enriched splenocytes from treated mice proliferated in response to FVIII and secreted IL-10 mostly. A human FVIII-specific T-cell line derived from the treated mice secreted both IL-4 and IFN-γ, suggesting that it included both Th1 and Th2 cells. CD4+ enriched splenocytes of the hFVIII-treated mice recognised all the domains of FVIII, when the library of 233 overlapping synthetic peptides (each 20 amino-acids long) was used to study epitope specificity (Wu *et al.*, 2001). The authors concluded that the development of FVIII antibodies in mice was analogous to what happens in haemophilia A patients and especially that IL-10 may drive antibody synthesis. However, CD4+ cells specific of FVIII produced both Th1 and Th2 cytokines. Using a similar animal model, Qian with the co-authors have reported, that the FVIII antibody response is T-cell-dependent since haemophiliac mice deficient also in the T-cell co-stimulatory ligand B7-2 showed no such response to hFVIII injections (Qian *et al.*, 1999). In another experiment using CTLA4-Ig, the effect of the blocking interaction of CD28 with B7 on FVIII antibody development was investigated *in vivo* (Qian *et al.*, 2000). CTLA4-Ig is a soluble fusion protein in which an extra cellular domain CTLA4 binds to B7-1 and B7-2 ligands and thereby blocks B7 interaction with CD28 and CTLA4. In haemophilic mice CTLA4-Ig completely blocked a primary response to FVIII, and furthermore, it even prevented or diminished a further increase in the antibody titer when administered to mice with low titer antibodies (Qian *et al.*, 2000). Blocking of another interaction, CD40-CD40L, has shown to be also effective in order to prevent FVIII antibody production in haemophiliac mice (Rossi *et al.*, 2001), but without a long-lasting immune tolerance effect as expected (Reipert *et al.*, 2001).

3. AIMS OF THE STUDY

The main objective of the study was to investigate the ability of FVIII antibodies developed in haemophilia A patients to inhibit FVIII coagulation activity and changes in the immune response to FVIII in the course of the immune tolerance therapy.

Accordingly, the study had the following aims:

1. To investigate the protective effect of VWF on the inhibition of the FVIII coagulation activity by FVIII antibodies *in vitro*.

2. To study the dynamics of FVIII antibody characteristics of a haemophilia A patient during the immune tolerance treatment with the FVIII preparation containing VWF
   a. To characterize the epitope specificity of FVIII antibodies and the changes in epitope specificity during treatment.
   b. To characterize the changes in the IgG subclass distribution of FVIII antibodies.

3. To investigate the mechanism of FVIII antibody action on the inhibition of FVIII coagulation activity.

4. To characterize the von Willebrand factor in FVIII preparations that were used in *in vitro* and *in vivo* experiments.

4. MATERIALS AND METHODS

4. Study subjects

4.1. Haemophilia A patients with persistent FVIII antibodies (Papers I and III)

Plasma samples from 12 haemophilia A patients having FVIII antibodies have been characterized in the current study. Since the FVIII light chain contributes to the FVIII binding to VWF (Saenko *et al.*, 1999b; Saenko and Scandella, 1997; Saenko *et al.*, 1994) only those samples were selected, which contained antibodies targeted at the FVIII light chain and the A2 domain as detected by using the immunoblotting assay. Anti-FVIII antibodies had developed in all 12 tested patients (samples from 6 patients from the Department for Coagulation Disorders of Malmö University Central Hospital, Malmö, Sweden, and 6 patients from Children's Hospital of Tartu University Clinicum, Tartu, Estonia) in response to the on-demand treatment with plasma-derived FVIII preparations. The Bethesda titer of antibodies ranged from 1 to 300 BU/ml. Plasma samples were taken in a stable clinical situation before any immune tolerance induction.

The study protocols were approved by the Ethics Committee of the University of Tartu, Estonia.

4.1.2. Patient undergoing immune tolerance treatment (II, III)

High responder patient TK (FVIII antibodies > 5BU/ml) was tolerated to FVIII by the administration of the FVIII preparation that contains VWF (Haemoctin-SDH, Biotest, Dreieich, Germany) and the epitope specificity of FVIII antibodies was investigated during treatment. A three-year-old severe haemophilia A patient carrying the intron 22 inversion (Type 1) developed FVIII antibodies as a response to the treatment with the plasma-derived FVIII preparation with the maximal antibody titer of 26 BU/ml. At the beginning of the immune tolerance treatment (ITT) the Bethesda titer was 17.5 BU/ml. Before the ITT the patient had had 23 exposure days to FVIII. Haemoctin SDH was used since it was the only available FVIII preparation in Estonia at that time. The immune tolerance therapy comprised continuous infusion (40 IU/kg/h for 8 days via vascular line) followed by bolus injections of 100 IU/kg/day for 1 month and treatment was continued with a dose of 100 IU/kg twice a week for 10 months. Treatment was temporarily interrupted after 3 months due to the problems with venous access. Therefore, the second continuous infusion was applied for 8 days with a dose 150 IU/kg/h. After that treatment was continued with a dose 100 IU/kg twice a week until *in vivo* recovery had normalized in 13

months and prophylactic treatment was started (a dose of 100 IU/kg twice a week). During ITT the patient had no bleeding episodes.

4.2. Reagents

Recombinant FVIII (rFVIII, Recombinate™ and albumin-free rFVIII provided for laboratory use by Baxter, Glendale, CA, USA) was used throughout the study. Recombinate™ contains full-length FVIII produced by the recombinant DNA technology and a large amount of human albumin as a stabilizer, but it is free of VWF. As a source of the FVIII-VWF complex, a plasma-derived FVIII preparation Haemoctin SDH (Biotest, Dreieich, Germany) was used in *in vitro* and *in vivo* experiments. Recombinant FVIII heavy chain and light chain were kindly provided by Dr. Mirella Ezban. Bovine serum albumin (BSA), casein, p-nitrophenylphosphate (pNPP), thrombin, 5-bromo-4-chloro-3-indolyl-phosphate (BCIP), 4-nitro-blue-tetrazolium-chloride (NBT), L-(-phosphatidyl)-l-serine, ristocetin, and avidin-peroxidase were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Biotin derivate (EZ Link sulfo-NHS-LC-Biotin) was purchased from the Pierce company (Rockford, IL, USA). Polyclonal anti-VWF antibody and its conjugate with HRP, rabbit anti-human IgG conjugated to alkaline phosphatase, and substrate o-phenylene-diaminedihydrochloride (OPD) were obtained from Dako (Glostrup, Denmark). Rabbit anti-human IgG and the anti-human IgG-peroxidase conjugate were purchased from Bio Rad (Hercules, California, USA). IgG subclass-specific antibodies (sheep anti-human IgG1, IgG2, IgG3, IgG4) were obtained from CLB (Amsterdam, the Netherlands). Sheep anti-rabbit IgG-biotin conjugate, the donkey anti-sheep IgG-biotin conjugate, and the ECL™ kit were obtained from Amersham Pharmacia Biotech (Piscataway, NJ, USA). Human monoclonal anti-human FVIII antibodies 2E9 and BO2C11 as well as mouse monoclonal anti-human FVIII antibodies Mab15, F29B10 and F7B4 (Figure 3) were kindly provided by Dr. J.-M. Saint-Remy from the University of Leuven (Leuven, Belgium). Mouse monoclonal FVIII antibody ESH4 was obtained from American Diagnostica (Greenweich, CT, USA). The test kit for measuring the FVIII:C activity (Coatest FVIII:C), purified bovine factor X, factor IXa with factor X (FIXa+FX reagent), phospholipid emulsion, Russell's viper venom and substrates S-2288 and S-2337 were purchased from the Chromogenix Instrumentation Laboratory SpA (Milano, Italy). International standards, the WHO 3rd standard for FVIII and VWF in plasma (code 91/666) and 4th international standard for the blood coagulation factor VIII:C concentrate (code 88/804) were obtained from NIBSC (Potters Bar, UK). The *in vitro* assays were carried through in Tris-buffered saline (TBS, 0.02 M Tris, 0,15 M NaCl, pH 7.2), phosphate-buffered saline (PBS, 0.08 M, pH 7.2), carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$,

pH 9.6) or glycine-buffered saline (GBS, 0.02 M glycine, 0.034 M NaCl, pH 9.2). All chemicals were of an analytical grade if not stated otherwise.

4.3. Assays for studying the functional activity of FVIII antibodies

4.3.1. Bethesda assay (I–III)

FVIII antibody level in a patient's plasma samples was measured by the Bethesda method as described by Kasper *et al.* (Kasper *et al.*, 1975), except that TBS buffer containing 1% of BSA was used instead of imidazole buffer for preparing the test plasma dilutions. The normal plasma pool (made from 22 plasma samples of healthy persons) was used as a source of FVIII. The resulting residual FVIII:C was determined by the chromogenic method according to the manufacturer's instructions (Coatest FVIII:C) in 96-well microplates (Micro-Well™, Nunc, Denmark). The coefficient of variance between the assays was 3.0% at FVIII:C activity 1 IU/ml (6.4% at FVIII:C activity 0.5 IU/ml and 11.2% at FVIII:C activity 0.2 IU/ml). The local plasma pool of 22 donors' plasmas calibrated against the WHO 3rd standard for FVIII and VWF in plasma (code 91/666) was used as a standard throughout the study.

4.3.2. Inhibition of FVIII:C activity by antibodies in the presence and absence of VWF (I–III)

The standard Bethesda method was slightly modified in order to investigate the protective effect of VWF on the inhibition of FVIII:C activity by FVIII antibodies. Plasma-derived FVIII-VWF concentrate (Haemoctin SDH) and recombinant FVIII (Recombinate™) were diluted to 1 IU/ml of FVIII:C in TBS buffer containing 1% of BSA (TBS-BSA) and incubated with an equal volume of a serially diluted (1:2 to 1:1000 in TBS-BSA) FVIII antibody plasma sample for 1 h at 37°C. The residual FVIII:C was determined by the chromogenic method. Nearly the maximal inhibition of FVIII:C coagulation activity was obtained already during 1st h of incubation with FVIII antibodies, while the prolongation of incubation to 2 h had a minimal supplementary effect (0.1–2%). Percentage of neutralization was calculated in relation to a control. The control contained a mixture where the test plasma sample was replaced by a buffer (TBS with 1 % of BSA) or by plasma from a haemophilia A patient without FVIII antibodies and a detectable level of FVIII:Ag (CRM negative). The test plasma sample

dilution yielding 50% of the neutralization of FVIII:C activity was determined for both concentrates. The results were used to calculate the ratio between the dilutions resulting in 50% neutralization of FVIII:C with rFVIII and FVIII-VWF, respectively.

4.3.3. Purification of IgG from plasma samples (II)

IgG was purified from the plasma samples of patient TK in order to eliminate several proteins present in plasma, which could interfere with the detection of FVIII antibodies in various assays, e.g. VWF which is able to compete with FVIII antibodies in interfering with the FVIII binding to phospholipids. IgG was purified from plasma samples by affinity chromathography using Protein-G Sepharose (HiTrap™ Protein G, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions. The eluted IgG fraction was dialysed against PBS overnight at 4°C. The concentration of the obtained IgG was measured by the ELISA assay using the rabbit anti-human IgG antibody (Bio Rad, USA) coated onto microplates (2 μg/ml in GBS) and the bound IgG from the investigated sample was detected by using the rabbit anti-human IgG antibody conjugated with peroxidase. The standard curve was built by using the human IgG standard with the known concentration. Antibodies specific of FVIII were detected by an analogous ELISA assay using rFVIII-coated (Recombinate™) microplates.

4.3.4. Tagging of recombinant FVIII with biotin (II)

Recombinant full-length carrier-free FVIII (specific activity >4000 IU/mg of protein) was labelled with biotin under mild conditions by using EZ Link sulfo-NHS-LC-Biotin (Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Biotin can easily be attached to the free amino groups of protein, but a great care has to be taken to retain the functional activity of FVIII during biotinylation. The water-soluble biotin derivative was used in order to perform the biotinylation reaction in the absence of organic solvents such as dimethyl sulfoxide, which could cause the denaturation of protein. This biotin derivative has an approximately 22.4 Å long spacer arm that facilitates biotin recognition by streptavidin and avidin. Briefly, rFVIII (100 μg/ml) was dialysed against HEPES buffer (0.01 M HEPES, 0.15 M NaCl, 0.01 M $CaCl_2$, pH 8.5). The biotin derivative was dissolved in water to a concentration of 0.01 mg/ml. Nine volumes of rFVIII and 1 volume of the biotin solution were mixed and incubated for 2 h at RT. The free residual biotin derivative was removed by dialysis

against HEPES buffer (HEPES 0.01 M, NaCl 0.15 M, $CaCl_2$ 0.01 M, pH 7.2). Biotinylation in such conditions did not impair FVIII:C activity as estimated in the chromogenic assay, neither the binding to phosphatidylserine nor binding to VWF in the respective ELISA assays.

4.3.5. Inhibition of FVIII binding to phospholipids by the antibodies of the patient (II)

To study whether the FVIII antibodies of patient TK were able to interfere with the FVIII binding to phospholipids, the IgG fraction of plasma samples was incubated with the FVIII-biotin conjugate and added to phosphatidylserine-coated microplates. Briefly, the wells of polystyrene microplates (MaxiSorp™, Nunc, Denmark) were coated with L-(-phosphatidyl)-l-serine (100 µl of 5 µg/ml solution in methanol), air-dried and blocked with 5% BSA in TBS for 1 h at room temperature (RT). The wells were rinsed with washing buffer TBS containing 0.1% of Tween 20 (TBS-T) and a mixture containing an equal volume of biotin-labelled rFVIII 2 µg/ml in TBS-BSA (1% of BSA in TBS buffer), and a serially diluted IgG sample of the patient (from 500 µg/ml to 15 µg/ml) was added and incubated for 2 h at RT. The amount of the bound FVIII-biotin was determined by using avidin-peroxidase (1 µg/ml in PBS with 1% of BSA). The reaction was visualized with the OPD substrate solution (o-phenylenediamine in 0.1 M citrate-phosphate buffer, pH 5.0, containing 0.03% H_2O_2). The reaction was stopped by adding 1M H_2SO_4 to the wells. Absorbance was read at 492 nm on a microplate reader Labsystem Multiscan Plus (Labsystems Oy, Finland). A control was included in each test where buffer was used instead of the test plasma sample dilution. The human monoclonal C2-domain-specific antibody BO2C11 was used as a positive control.

4.3.6. Inhibition of FVIII binding to the von Willebrand factor by the antibodies of the patient (II)

In order to study whether FVIII antibodies were able to interfere with the FVIII binding to VWF, the IgG fraction from plasma samples was incubated with the FVIII-biotin conjugate and added to VWF-immobilized microplates. First, the microplate wells (MaxiSorp™, Dako, Denmark) were coated with 100 µl of the mouse anti-human VWF polyclonal antibody (2 µg/ml) in GBS buffer by overnight incubation at 2–8°C. The wells were blocked with 0.5% of casein in TBS (TBS-casein) for 30 minutes. The immobilized polyclonal antibody was saturated with VWF in complex with FVIII by using the normal plasma pool

(plasma pool of 22 plasmas of healthy persons) diluted to 1:20 in TBS-BSA by incubating for 1 h at RT. Factor VIII was dissociated from VWF by incubating with 0.4 M $CaCl_2$ for 30 min at RT. After washing with TBS-T, a mixture was added containing the test IgG sample dilution and an equal volume of rFVIII-biotin conjugate diluted to 2 μg/ml in TBS-casein (preincubated for 30 min at 37°C before adding). The reaction was carried through for 2 h at RT and the amount of the bound FVIII was determined as described above. Monoclonal antibody BO2C11 was again used as a positive control.

4.3.7. Chromogenic method for measuring FXa generation (II)

In order to characterize the mechanism of the inhibition of FVIII:C activity by the antibodies of patient TK, we used the FXa generation assay. The rate of the conversion of FX to FXa was monitored in a reconstituted system. Recombinant FVIII (2 IU/ml in TBS-BSA) was preincubated with various concentrations (500 to 4 μg/ml) of the tested IgG for 30 min at 37°C. Then the FVIII-antibody complex was reacted with purified bovine FIXa+FX (final concentration 2.4 IU/ml according to labelled activity) in the presence of phospolipids for 10 min at 37°C. FXa generation was initiated by adding the $CaCl_2$ solution to the mixture (final concentration 6.25 mM). Aliquots were removed at appropriate times to assess the initial rates of product formation and were added to microplate wells containing the EDTA solution (final concentration 11 mM) to stop the reaction. The amount of the generated FXa was determined by the activity of cleaving chromogenic substrate S-2288 (final concentration 1.1 mg/ml) by reading absorbance at 405 nm. Each assay included 2 controls, namely, in the first mixture the tested IgG was replaced by the IgG prepared from the plasmas of healthy donors (a positive control), and in the second the tested IgG and FVIII were substituted by buffer (TBS-BSA, a negative control). The amount of the generated FXa in the sample was expressed as a percentage of the positive control. The IgG fractionated from healthy donors was checked for the absence of FVIII antibodies by the ELISA assay. In another experiment, rFVIII was substituted by the recombinant FVIII light chain diluted to 12 IU/ml in TBS-BSA. Preliminary experiments showed that in the presence of the FVIII light chain FXa generation was lower than in the presence of rFVIII. Using the recombinant FVIII heavy chain instead of rFVIII, led to the generation of a negligible amount of FXa.

4.3.8. Inhibition of FX binding to FVIII by the antibodies of the patient (II)

In order to assess whether the antibodies of patient TK were able to inhibit the FIXa and FX binding to FVIII, we used the ELISA assay. The microplates were coated with PS (5 μg/ml in methanol) and air-dried. The wells were blocked with 5% of BSA in TBS for 1 h at RT. An equal amount of rFVIII (2 IU/ml in buffer A, 0.05 M Tris, 0.015 M NaCl, 0.015 M $CaCl_2$, BSA 1%) and the tested IgG at indicated concentrations (7 to 500 μg/ml) were preincubated for 30 min at 37°C and transferred to PS-coated wells of the microplate. After 2 h of incubation, the non-bound material was removed by washing the wells with buffer B (0.05 M Tris, 0.015 M NaCl, 0.05 M $CaCl_2$, Tween-20 0.1%, pH 7.2). Purified bovine FX diluted to 0.1 U/ml in buffer A according to the labelled activity, was added to the wells and incubated for 15 min at RT. The wells were rapidly washed 3 times with buffer B. Subsequently substrate S-2337 (final concentration 0.6 mg/ml) was added to the wells and FX was activated by the FX-activating enzyme from Russell's viper venom (21.5 μg/ml) in the presence of $CaCl_2$ (0.025 M). The inhibition of FX binding was calculated relative to the control, which contained the IgG preparation from healthy donors instead of the tested IgG sample (100% of binding). Control experiments confirmed that the RVV enzyme by itself did not hydrolyse the chromogenic substrate.

4.4. Assays for characterization of epitope specificity of FVIII antibodies

4.4.1. Immunoblotting analysis (I-III)

The immunoblotting assay was used for the estimation of the FVIII antibody binding to the FVIII light chain and heavy chain. Plasma-derived FVIII preparation Haemoctin SDH (Biotest, Germany) was separated on sodium dodecyl sulfate containing polyacrylamide gradient gel (6–15%). In addition to intact FVIII, the thrombin-digested FVIII and the recombinant FVIII light chain and heavy chain were separated on the respective lines of SDS-PAGE. The optimal thrombin concentration and suitable incubation time were chosen according to pilot experiments. FVIII was cleaved by thrombin (0.01 IU thrombin per 1 IU of FVIII) for 30 minutes at 37°C. After electrophoresis, the proteins were transferred to a nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The membrane was blocked with 3% (w/v) fat-free milk powder in PBS and incubated with plasma samples diluted 1:4 in PBS. Antibody binding to FVIII fragments was detected with the rabbit anti-human IgG-AP

conjugate and bands were developed by using BCIP/NBT as a substrate. After each incubation step, the membrane was extensively washed with TBS-T. If the sample contained a low amount of FVIII antibodies, antibody binding was detected after subsequent incubations with the sheep anti-rabbit IgG-biotin conjugate and the streptavidin-HRP solution by using more sensitive chemiluminescence-based Western blotting detection reagents, the ECL™ kit.

4.4.2. ELISA for the quantification of antibodies recognizing the light chain or heavy chain of FVIII (I–III)

In order to estimate a relative amount of FVIII antibodies that bind to the FVIII light chain or heavy chain, respectively, the tested plasma samples were incubated with increasing concentrations of the respective recombinant FVIII fragments, and the amount of remaining FVIII antibodies was determined by the ELISA assay. Microplates (PolySorp™, Nunc, Denmark) were coated with 8 IU/ml rFVIII (Recombinate™) in carbonate buffer overnight at 2–8 °C. The sites of non-specific binding in the wells of the microplate were blocked with 3% of BSA in TBS for 1 hour at room temperature and the unbound BSA was washed off with TBS-T. Plasma samples with FVIII antibodies were diluted to 4 BU/ml, except the plasma samples with the FVIII antibody titer ≤4 BU/ml, which were used undiluted. In order to neutralize FVIII antibodies, the plasma samples were incubated for 1 h at 37°C with an equal volume of the recombinant FVIII heavy chain or light chain, serial dilutions ranging from 0.2 to 12.6 IU/ml according to the labelled activity. After incubation, aliquots of the incubation mixture were transferred to the wells of the microplate precoated with rFVIII and incubated for 2 h at 37°C. The amount of bound FVIII antibodies was determined by using the rabbit anti-human IgG conjugated with alkaline phosphatase (1:1000, Dako, Glostrup, Denmark) and visualised with AP-substrate pNPP (p-nitrophenyl phosphate), recording absorbance at 405 nm after stopping the reaction with 2M NaOH. Two controls were included in each assay: a mixture that contained buffer instead of the antibody plasma sample (minimal binding), and the other where buffer instead of recombinant FVIII fragments was used (maximal binding). The percentage of neutralization was calculated as follows: 100 — [(binding after incubation with fragment — minimal binding) / (maximal binding — minimal binding)] x 100. The plateau concentration was defined as the minimal concentration of the FVIII fragment, which yielded the maximum optical density (OD), and all OD values within the plateau region were averaged.

4.4.3. Chromogenic method for the quantification of antibodies recognizing the light chain or heavy chain of FVIII (I–III)

To estimate a relative amount of FVIII inhibitory antibodies neutralized by the FVIII light chain or heavy chain, the tested plasma samples were incubated with increasing concentrations of the respective recombinant FVIII fragments and the amount of remaining FVIII antibodies was estimated as the ability to inhibit FVIII:C activity by the chromogenic method. Plasma samples were diluted to obtain neutralization activity of 4 BU/ml, except the plasma samples containing ≤4 BU/ml of FVIII antibodies, which were used undiluted. The resulting samples were incubated with an equal volume of serially diluted recombinant FVIII fragments as described above, and an aliquot was removed for the estimation of the amount of antibodies by an ELISA assay. To the remaining solution an equal volume of rFVIII (2 IU/ml in TBS containing 2% of BSA) was added and incubated for 1 h at 37°C. Residual FVIII coagulation activity (FVIII:C) was measured by using the chromogenic method. Percentage of neutralization was calculated as above using FVIII:C activity instead of binding capacity. Maximum FVIII:C activity was measured in the mixture of the respective recombinant fragment and rFVIII without any antibody sample. If the tested plasma contained FVIII antibodies less than 10 BU/ml, a plasma sample taken from a haemophilia A patient (CRM-negative, without FVIII antibodies) instead of buffer was used in order to measure the maximal FVIII:C activity. Minimal FVIII:C activity was measured for each test sample by incubating rFVIII without any competitive FVIII fragments. The plateau value was calculated analogously to the previous assay.

In order to obtain comparable results, all neutralisation experiments for the testing each particular sample were carried out simultaneously under identical conditions. In principle, the total amount of the FVIII light chain and FVIII heavy chain recognizing antibodies has to be approximately 100%, but also slightly higher or lower values were obtained analogously to previous studies, where a similar methodology had been used (Scandella *et al.*, 1995b).

4.4.4. Recombinant FVIII fragments (II)

The respective FVIII DNA fragments for cloning were generated by PCR using primers with the restriction enzyme sites. The fragments were cloned into the pGEX4T-2 expression vector (Pharmacia, Uppsala, Sweden) and controlled by sequencing in both directions with the T7 sequencing kit. Glutathione S-transferase (GST) fusion proteins were expressed in the DH5 α *Escherichia coli*

strain. Fusion protein aggregates were dissolved by using 1.5% Sarkosyl (Sigma) and 2% Triton X-100 and purified on the glutathione column.
The FVIII domains were expressed so that each domain contained an additional 8 to 97 amino-acid residues of the neighbouring domains at both ends in order to ensure the intactness of cleavage sites for thrombin, FXa and activated protein C (Lenting *et al.*, 1998).

The recombinant FVIII fragments were produced and purified by Abdellah Benhida at the Centre for Molecular & Vascular Biology, University of Leuven, Leuven, Belgium.

4.4.5. Binding of FVIII antibodies to recombinant FVIII fragments (II)

In order to more precisely characterize the epitope specificity of patient TK's antibodies, we studied the antibody binding to the following recombinant FVIII fragments: A1, a1, A2, a3, A3, C1, C2 by using an ELISA assay (Figure 3). Microplates were coated with the respective recombinant FVIII fragments (2 μg/ml in GBS buffer) for 2 h at RT. The wells were blocked with TBS-casein (0.5% of casein) for 30 min. Investigated IgG fractions with concentrations from 15 μg/ml to 500 μg/ml in TBS-casein were added to the wells of the microplate and incubated for 2 h at RT. The fraction of bound FVIII antibodies was determined by using the rabbit anti-human IgG peroxidase conjugate. After washing the reaction was visualized with the OPD substrate solution. Absorbance was read at 492 nm after stopping the reaction with 1M H_2SO_4. As a positive control, the monoclonal antibody specific of a particular recombinant FVIII fragment was included in each experiment. The results were expressed as OD values corrected by the subtraction of the background value (non-specific binding to GST fusion with irrelevant protein).

This assay was performed in the Centre for Molecular & Vascular Biology, University of Leuven, Leuven, Belgium.

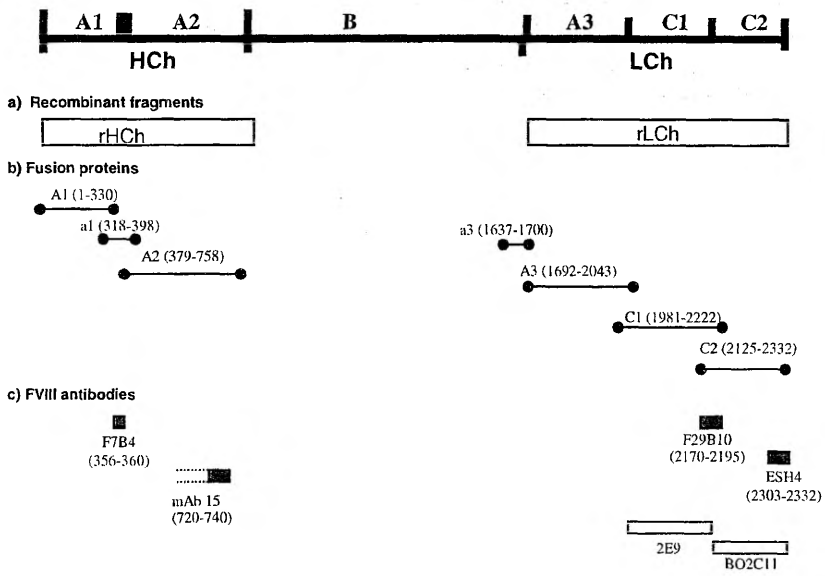

Figure 3. FVIII recombinant fragments (**a**) used in neutralization experiments and GST-fusion proteins (**b**) used for characterization of epitope specificity in ELISA assays. Binding sites of monoclonal FVIII antibodies used in a competitive binding assay (**c**).

4.4.6. Competitive binding to FVIII between the antibodies of the patient and monoclonal antibodies with the known epitope (II)

Monoclonal FVIII antibodies with the known epitope were used in the competitive binding assay to characterize the specificity of FVIII antibodies of patient TK (Figure 3). Human monoclonal C2-domain-specific antibody BO2C11, C1-domain-specific antibody 2E9, and mouse monoclonal antibodies F29B10 (amino-acids 2170–2195), and ESH4 (aa 2303–2332) were applied to characterize the FVIII-light-chain-specific antibodies of the patient. Mouse monoclonal antibodies F7B4 (aa 356-360) and mAb 15 (A2 –domain-specific) were selected to characterize the epitopes of the patient's antibodies targeted at the FVIII heavy chain. Microplates were coated with different human or mouse monoclonal antibodies to human FVIII (diluted to 2 μg/ml in GBS buffer) for 2 h and blocked with TBS-casein for 30 min at RT. A mixture containing an equal volume of the tested IgG sample (concentrations from 15 μg/ml to 500 μg/ml) and the recombinant FVIII-biotin conjugate (2 μg/ml) was applied and incubated for

2 h at RT. The amount of the bound rFVIII-biotin was determined by using avidin-peroxidase as described above. The validity of the assay was confirmed by using the same monoclonal antibody that was used to coat microplates instead of the tested antibody. The background value (no antigen in the coating solution) was subtracted from the respective OD values. The degree of competition was calculated relative to the maximal binding obtained when no competitor (buffer instead of the test IgG sample) was used.

4.5. Characterization of IgG subclasses of FVIII antibodies

4.5.1. IgG 1-4 subclasses of FVIII antibodies (III)

In order to characterize the IgG subclassess of FVIII antibodies, the antibody binding to the recombinant FVIII (Recombinate™) that was immobilized onto microplates was detected by using the IgG-subclass-specific antibodies. Immunoplates (PolySorp™, Nunc, Roskilde, Denmark) were coated with rFVIII (8 IU/ml in carbonate buffer) overnight at 2–8°C and blocked with 5% of BSA in TBS for 1 h at RT. Plasma samples containing FVIII antibodies were diluted from 1:10 to 1:5000 in TBS and reacted with immobilized FVIII for 2 h at 37°C. After washing with TBS-T, the bound FVIII antibodies were detected by incubating with the respective sheep anti-human IgG1, IgG2, IgG3 or IgG4 antibodies (1:1000 dilution in TBS) for 1 h at 37°C followed by the donkey anti-sheep IgG-biotin conjugate (diluted 1:1000) and incubated for 1 h at RT. The wells were washed thoroughly and streptavidin–AP conjugate (1:1000) was added to the wells and incubated for 30 min at RT. After washing, the substrate pNPP solution was added and absorbance was read at 405 nm after stopping the reaction with 2M NaOH (maximum absorbance was achieved in 30 min). FVIII antibody-free plasma from a severe haemophilia A patient was used instead of the investigated plasma sample as a negative control. The cut-off value was defined as a mean value plus 3 standard deviations of optical densities measured for controls. The ELISA titer was defined as a maximal dilution of the sample yielding OD exceeding the cut-off value. The estimated cut-off values of optical density for IgG1, IgG2, IgG3, and IgG4 FVIII antibodies were 0.113, 0.103, 0.089 and 0.089, respectively.

4.5.2. IgG1-4 subclasses of FVIII-light-chain-specific antibodies (III)

Immunoplates (PolySorp™, Nunc, Roskilde, Denmark) were coated with the recombinant FVIII light chain diluted in TBS at 6 IU/ml and incubated over night at 2–8°C. The next steps of the assay were identical to those described in the previous section. However, the cut-off values were higher for the FVIII light chain than for the full-length FVIII. The cut–off values for IgG1, IgG2, IgG3 and IgG4 were 0.222, 0.217, 0.167 and 0.166, respectively.

4.6. Characterization of VWF content in FVIII preparation (IV)

4.6.1. FVIII preparations (IV)

Four different FVIII preparations were characterized as follows, Haemoctin SDH 250 IU (Biotest, Dreieich, Germany), Koate-HP 310 IU (Bayer Miles, Elkhart, IN, USA), lyophilized cryoprecipitate KRYO-AHG 400 IU (North Estonian Blood Centre, Tallinn, Estonia) and Haemate 1000 IU (Aventis Behring, Bridgewater, NJ, USA). FVIII preparations were diluted according to the manufacturer's instructions, and stored at –70°C until assayed.

4.6.2. ELISA for the quantification of the VWF antigen (IV)

The concentration of the VWF antigen in FVIII preparations was determined by using an ELISA. The microplates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with the rabbit polyclonal anti-human VWF antibody (1:1000 in carbonate buffer) overnight at 2–8°C. The samples were diluted to yield FVIII:C activity from 0.02 to 0.05 IU/ml in PBS, transferred to the wells and incubated for 2 h at RT. The bound VWF was quantified by using the rabbit polyclonal anti-human VWF antibody conjugated with peroxidase (1:1000 dilution, incubation for 1 h at RT), by using OPD as a substrate, and reading absorbance with the ELISA reader after stopping the reaction with 1M H_2SO_4. The standard graph was constructed by using the normal plasma standard (made from plasmas obtained from 22 healthy persons) that was calibrated against the international standard (the 3rd standard for FVIII and VWF in plasma, code 91/666) in an analogous ELISA assay. The coefficient of variance between the days was 4.2 % at the VWF:Ag level of 1 IU/ml.

4.6.3. Estimation of FVIII coagulation activity (IV)

The FVIII:C activity in FVIII preparations was measured by the chromogenic method in microplates using the test kit Coatest FVIII:C according to the manufacturer's instructions. FVIII preparations were pre-diluted to yield the FVIII:C activity of 1 IU/ml in TBS-BSA (1% BSA). The 4th International standard for blood coagulation factor VIII:C preparation (88/804) was used for building a standard graph. The coefficient of variance between the days was 3.5% for FVIII:C activity 1 IU/ml.

4.6.4. Ristocetin cofactor assay (IV)

Ristocetin cofactor activity of VWF (RCof) was determined by using the formaldehyde-fixed washed platelets for the aggregation test (Macfarlane *et al.*, 1975). The assay is based on the ability of antibiotic ristocetin to dimerize and initiate the aggregation of platelets, which is dependent on the presence of VWF. The formaldehyde-fixed platelets from a healthy donor were prepared as described previously (Evans and Austen, 1977). Briefly, the platelets were fixed by incubating 1 part of platelet-rich plasma with an equal volume of formaldehyde in PBS buffer (4% formaldehyde) overnight at 2–8°C. The platelets were washed and resuspended in TBS. The investigated samples were diluted to yield FVIII:C activity from 0.2 to 0.5 IU/ml in TBS containing 1% of BSA and incubated with an equal volume of formaldehyde-fixed platelets for 10 min at 37°C in an aggregometer (Jelena Laboratories) under continuous agitation. The aggregation of platelets was initiated by adding ristocetin (1.6 mg/ml final concentration) and recording the slope of the aggregation curve. The normal plasma standard (made from plasmas obtained from 22 healthy persons) calibrated against the international standard (the WHO 3rd standard for FVIII and VWF in plasma, code 91/666) was used to construct the standard curve. The coefficient of variance between the days was 19%.

4.6.5. Characterization of VWF multimers (IV)

The VWF multimerization status was analysed by the SDS-agarose gel electrophoresis in 1.9% agarose gel according to the method of Metzner *et al.*, by applying the discontinuous submarine technique (Metzner *et al.*, 1998). VWF multimers were detected after the transfer to a nitrocellulose membrane by using the rabbit polyclonal anti-human VWF antibody and the goat anti-rabbit IgG-alkaline-phosphatase conjugate. The bands were developed by using BCIP/NBT as a substrate.

4.6.6. ELISA for the quantification of VWF binding to collagen (IV)

Acid soluble collagen type III from the human placenta was dissolved in 3% acetic acid. The collagen solution was rapidly diluted in PBS buffer, pH 7.2 to reach the concentration of 10 μg/ml and then immobilized onto a microplate for 1 h at room temperature. The plate was blocked with 5% of BSA in PBS containing Tween 20 (0.05%) for 1 h at RT and washed with PBS-containing Tween 20 (0.05%). The tested FVIII preparations diluted from 0.1 to 0.01 IU/ml of FVIII:C activity were transferred to collagen-coated wells and incubated for 1 h at RT. After washing with PBS-T, the bound VWF was detected by using the rabbit anti-human VWF antibody conjugated with peroxidase (1:2000 in PBS). After 1 h of incubation, the wells were thoroughly washed and the bound VWF was quantified by using OPD and stopping the reaction with 1M H_2SO_4 after 5 min, and with absorbancies being read at 492 nm. Normal plasma pool of 22-donor plasmas was calibrated against ristocetin cofactor activity which was the only accepted characteristic of the functional activity of VWF in the 3rd international standard for FVIII and VWF in plasma (91/666) and was used as a local standard for building the standard curve. The coefficient of variance of this assay was 4.8% at the VWF:CB level of 1 IU/ml.

4.7. Statistical analyses (I–IV)

All the calculations were done by using the GraphPad Prizm Program Package (GraphPad Software Inc., USA) Correlation was calculated according to the Spearman rank order correlation coefficient and the coefficient of p 0.05 was considered to be significant. The values were analysed by using the paired t-test or one-way analysis of variance (ANOVA). The data are expressed as means ± SD.

5. RESULTS

5.1. Inhibition of FVIII:C activity by FVIII antibodies in the presence of VWF (Paper I)

In order to assess whether the presence of VWF in the FVIII preparation decreases the inhibition of FVIII:C activity by FVIII-specific antibodies, we used a neutralization assay where the plasma samples of haemophilia A patients were incubated either with the rFVIII or FVIII-VWF preparation. All 12 investigated plasma samples contained antibodies which recognised the FVIII light chain and the A2 domain as detected by immunoblotting. To estimate the proportion of antibodies recognizing epitopes within the FVIII light chain or heavy chain, the investigated samples were incubated with the respective recombinant FVIII fragment. After incubation, the residual amount of FVIII antibodies was measured by the ELISA assay or by their ability to inhibit FVIII:C activity by using the chromogenic method. Two groups of plasma samples were identified on the basis of the relative amount of FVIII light chain antibodies and on the neutralization of FVIII in the presence and the absence of VWF (Table 5). The first group, consisting of 9 plasma samples, had a significantly lower neutralization activity when exposed to the FVIII-VWF complex compared to rFVIII. The ratio between the dilutions yielding 50% neutralization with rFVIII and with FVIII-VWF ranged from 1.5 to 18.3 (mean 6.8). The samples classified to Group 1 contained a significantly higher amount of antibodies directed towards the FVIII light chain (53–96%) than the amount of antibodies directed towards the FVIII heavy chain (3–58%). The proportion of FVIII-light-chain-specific antibodies was calculated relative to FVIII heavy chain antibodies (ratio rLCh/rHCh) ranged from 1.6 to 30 as estimated by the ELISA assay and from 1.9 to 26.6 by the chromogenic method. Three samples classified to Group 2 showed no difference in the inhibition of FVIII:C activity when exposed to rFVIII or FVIII-VWF (the mean ratio 1.0). These plasma samples contained an equal or a lower amount of FVIII-light-chain-specific antibodies than FVIII-heavy-chain-specific antibodies. The ratio between the amounts of FVIII-light-chain-specific antibodies and FVIII-heavy-chain-specific antibodies ranged from 0.2 to 1. When the results of all 12 investigated samples were included, the ratio between the sample dilutions resulting in 50% of the inhibition of FVIII:C activity with rFVIII and with FVIII-VWF correlated with the ratio between the amounts of FVIII light chain antibodies and FVIII heavy chain antibodies as estimated by the chromogenic assay (Spearman rank order correlation coefficient r=0.59, p=0.04, Figure 4).

Table 5. Characterization of investigated FVIII antibodies.

Plasma sample	FVIII anti-bodies BU/ml	Epitope specificity	Neutralization of rFVIII and FVIII-VWF			Antibodies targeted to the recombinant light chain (rLCh) and the heavy chain (rHCh) of FVIII					
						ELISA			FVIII:C		
			rFVIII $ID_{50\%}$	FVIII-VWF $ID_{50\%}$	Ratio rFVIII / FVIII-VWF	rLCh %	rHCh %	Ratio rLCh/rHCh	rLCh %	rHCh %	Ratio rLCh/rHCh
Group 1											
1	140	A2, LCh	750	150	5	96	7	13.7	53	8	6.6
2	9	A2, LCh	9	2	4.5	100	7	14.3	78	30	2.6
3	5	A2, LCh	27.5	1.5	18.3	97	25	3.9	82	42	1.9
4	7	A2, LCh	25	1.5	16.7	92	20	4.6	93	3.5	26.6
5	8	A2, LCh	5	2	2.5	62	20	3.1	75	27	2.8
6	37	A2, LCh	30	8	3.7	93	58	1.6	82	21	3.9
7	300	A2, LCh	265	110	2.5	90	7	12.8	95	7	13.6
8	280	A2, LCh	350	50	7	95	6	15.8	96	6	16
9	4	A2, LCh	3	2	1.5	90	3	30	83	15	5.5
Group 2											
10	1	A2, LCh	0.7	0.7	1	n.d.	n.d.	n.d.	21	21	1
11	5	A2, LCh	2	2	1	70	74	0.9	64	65	1
12	150	A2, LCh	28	23	1.2	18	94	0.2	26	74	0.4

n.d. – not determined; $ID_{50\%}$ - dilution of plasma sample neutralizing 50% of FVIII:C activity

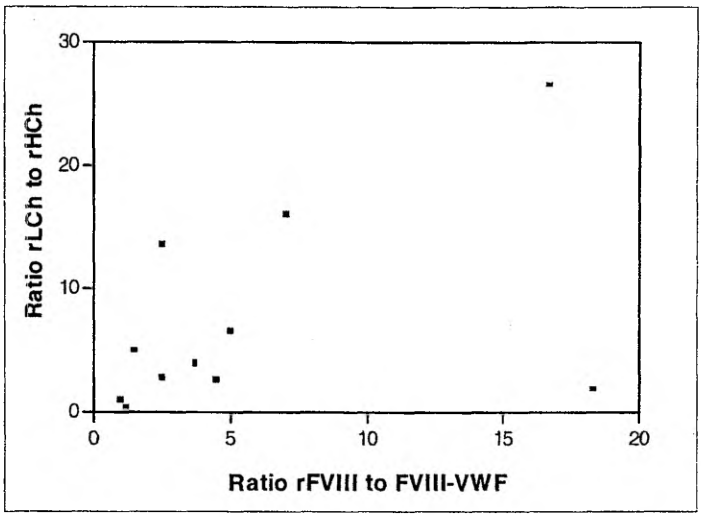

Figure 4. Correlation between the ratio of plasma dilutions yielding 50% of inhibition of FVIII:C activity when incubated with rFVIII or FVIII-VWF (ratio of rFVIII to FVIII-VWF) and the proportion of FVIII-light-chain-specific antibodies relative to FVIII-heavy-chain-specific antibodies as estimated by the chromogenic method (ratio of rLCh to rHCh).

5.2. Characterization of the patient's FVIII antibodies during immune tolerance treatment with VWF containing FVIII preparation (II, III)

5.2.1. The amount of FVIII-light-chain- and FVIII-heavy-chain-specific antibodies (II, III)

Further we characterized the epitope specificity of FVIII antibodies and the changes in epitope specificity during treatment with FVIII-VWF concentrate administered for the immune tolerance treatment (ITT) to a high responder patient TK. The investigated patient's plasma sample taken several months before ITT belonged to Group 1 (Table 4, patient No 2) based on the properties of FVIII antibodies. This sample contained a significantly higher amount of FVIII light chain antibodies than FVIII heavy chain antibodies and the inhibition of FVIII:C activity by FVIII antibodies was lower in the presence of VWF. The results of *in vitro* experiments suggest that the administration of the FVIII-VWF preparation could be more efficient in achieving haemostasis in patient TK. However, FVIII preparation Haemoctin SDH which contains VWF

was actually used for ITT because the choice of concentrates was limited to this one as the only available product at that time in Estonia. Plasma samples for measuring the amount of FVIII antibodies and for characterizing epitope specificity were taken at the beginning and during the immune tolerance treatment. FVIII antibodies in plasma samples were detected by using the Bethesda method (Figure 6D). The relative amount of antibodies targeted at the FVIII light chain or the heavy chain was measured by using both the ELISA and the chromogenic method. At the beginning of treatment (zero point on Figure 6), a significantly higher amount of antibodies were targeted at the FVIII light chain than to the FVIII heavy chain as measured by both, the ELISA assay or the chromogenic method (Figure 5). The neutralization of FVIII inhibitory antibodies by the recombinant FVIII light chain followed Type 2 kinetics as defined by Biggs *et al.* (Biggs *et al.*, 1972a; Biggs *et al.*, 1972b). In all samples taken during treatment, the FVIII-light-chain-specific antibodies were predominant, ranging from 50 to 100% of FVIII antibodies. A low amount (2–30%) of FVIII-heavy-chain-specific antibodies were detected during 10 months, after which the concentration of FVIII heavy chain antibodies dropped below the level of detection (Figure 6C).

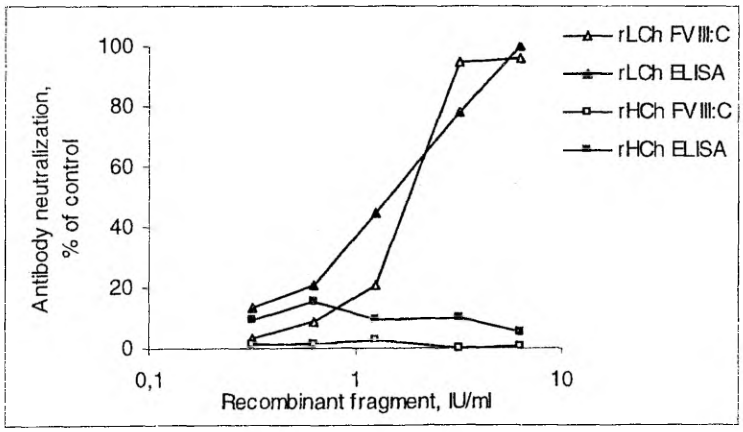

Figure 5. Neutralization of patient TK's anti-FVIII antibodies by the recombinant light chain and heavy chain of FVIII as measured by the chromogenic method (rLCh FVIII:C, rHCh FVIII:C) or the ELISA assay (rLCh ELISA, rHCh ELISA). Tested plasma sample was taken at the beginning of ITT.

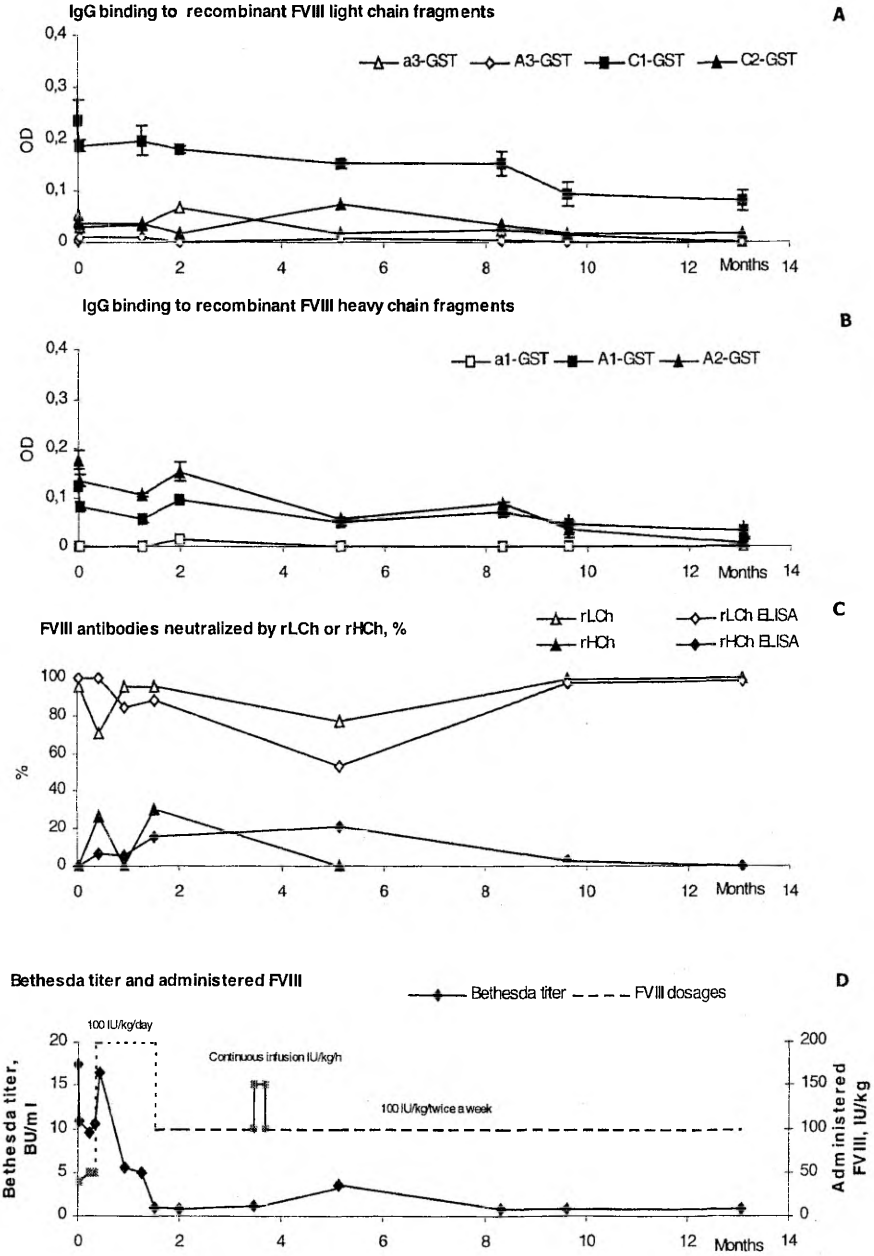

Figure 6. Changes of properties of patient TK's anti-FVIII antibodies during treatment. Binding of patient TK's antibodies to recombinant fragments of FVIII light chain (**A**) and recombinant fragments of FVIII heavy chain (**B**) at 100 μg/ml IgG concentration (mean ± SD). Neutralization of the patient's anti-FVIII antibodies by the recombinant FVIII light chain and the heavy chain as estimated by using the chromogenic method (LCh, HCh) or an ELISA assay (LCh ELISA, HCh ELISA) (**C**). The Bethesda titer and administration scheme of FVIII preparation Haemoctin SDH (Biotest, Germany) (**D**).

5.2.2. Epitope specificity of FVIII-light-chain-specific antibodies (II)

A more detailed characterization of patient TK's antibody epitopes in the FVIII light chain was achieved by using the fragments of the FVIII light chain. We studied the antibody binding to the following recombinant FVIII light chain fragments: a3, A3, C1, C2 by using an ELISA assay. The respective fragments expressed as fusion proteins with glutathione S-transferase (GST) were immobilized onto microplates and incubated with various concentrations of IgG fractions purified from patient TK's plasma samples. At the beginning of treatment of the patient we were able to detect the FVIII-light-chain-specific antibodies directed towards the C1, C2 and a3 domain of FVIII. The amount of antibodies recognizing the C1 domain was significantly higher than the amount of antibodies recognizing the C2 domain of FVIII ($p<0.01$, paired t-test, Figure 6A). The binding of patient TK's antibodies to the recombinant C1 domain immobilized to polystyrene surface was linearly dependent on antibody concentration (Figure 7). A negligible amount of antibodies bound to the a3 domain but no antibody binding to the A3 domain was detected. On the second day of treatment, the amount of C1- and a3- specific antibodies decreased. However, the Bethesda titer also decreased from 17.5 BU/ml to 10.5 BU/ml. Probably, some of FVIII antibodies were neutralized by the administered FVIII. During the following treatment a linear decrease in the concentration of C1-domain- and a3-domain-specific antibodies was detected. The amount of antibodies recognizing different FVIII-light-chain fragments remained rather unchanged, except an observed significant rise in the C2-domain-specific antibody level in the sample taken in 5^{th} month soon after the interruption of treatment ($p<0.05$, ANOVA). The decreases in C2-domain antibody concentration until the interruption of treatment and during the following period were similar.

Since the patient's antibodies were able to bind to the recombinant C1 domain and C2 domain, we studied further whether the antibodies were able to recognize the common region in both these recombinant fragments (amino acids 2125–2222). In a competitive binding assay we used the mouse monoclonal FVIII antibody F29B10, which binds to an epitope within amino acids 2170–2195, present in both, the recombinant C1 and C2 domain. Surprisingly, antibody F29B10 bound to the recombinant C2 domain only, but no binding to the C1 domain was detected. At the beginning of treatment the patient's antibodies inhibited the F29B10 binding to FVIII by 12% (Figure 8A), indicating the presence of antibodies directed also towards this epitope in the C2 domain. During treatment all the tested IgG fractions contained a small amount of antibodies, which interfered with the FVIII binding to mAb F29B10. No correlation was found between the concentration of the C2-domain-specific antibodies and the concentration of antibodies able to compete with mAb F29B10.

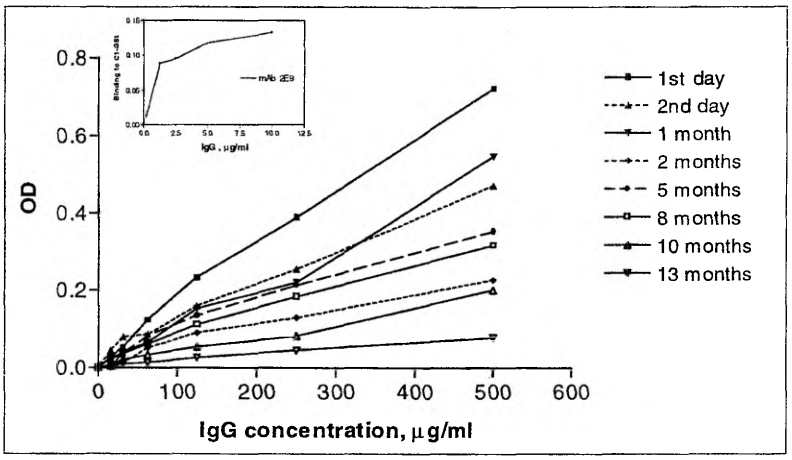

Figure 7. Concentration dependence of the binding of patient TK's antibodies to recombinant C1 domain in the ELISA assay. Monoclonal C1-domain-specific antibody 2E9 was used as a control (insert).

Next we studied whether the tested antibodies could compete with monoclonal antibodies 2E9 and BO2C11 for their binding sites within the C1 domain (Jacquemin *et al.*, 2000) or the C2 domain (Spiegel *et al.*, 2001), respectively. These monoclonal antibodies are known to abolish the interaction of FVIII with VWF. The tested patient's antibodies were mainly targeted at the C1 domain of the FVIII light chain. However, no inhibition of the FVIII–biotin binding to mAb 2E9 was detected in the presence of the patient's IgG fraction in a competitive binding assay. Moreover, no inhibition was detected even when FVIII was pre-incubated with the tested IgG fraction for 30 min before adding it to mAb-2E9-coated microplates. Probably the patient's antibodies and mAb 2E9 were directed to different epitopes of the C1 domain of the FVIII light chain. The patient's antibodies were not able to interfere with the FVIII binding to mAb BO2C11 even when added directly to the wells of the microplate or later, after 30 min of pre-incubation with the FVIII-biotin conjugate.

We used monoclonal antibody ESH4 in a competitive binding assay to study whether the patient's antibodies could bind to the C-terminus of the C2-domain. At the beginning of the treatment of patient TK, antibodies completely abolished the FVIII binding to ESH4 (Figure 8A). The concentration of antibodies capable of interfering with the FVIII binding to ESH4 decreased during treatment. Thus, the tested antibodies were probably targeted at the N-terminus (2170-2190) and C-terminus of the C2-domain, since both ends of the C2 domain are connected close to each other with a disulphide bridge. Obviously, the binding of FVIII to ESH4 was inhibited by the FVIII-light-chain-specific antibodies targeted at the C2 domain and the C1 domain, which could interfere

with this interaction by steric hindrance or by inducing a conformational change of the FVIII light chain so that the ESH4 binding to FVIII was abolished.

5.2.3. Epitope specificity of FVIII-heavy-chain-specific antibodies (II)

The epitope specificity of the FVIII-heavy-chain-specific antibodies was characterized in an ELISA assay by using the recombinant FVIII fragments corresponding to the a1, A1, and A2 domains. At the beginning of treatment of patient TK we could detect antibodies, which bound to epitopes within the A2 domain or A1 domain, but no binding to the a1 domain was detected. The amount of antibodies recognizing the A2 or A1 domains was lower compared to the amount of C1-specific antibodies (Figure 6B). On the second day, the amount of A2- and A1-specific antibodies decreased analogously to the FVIII light chain antibodies. During treatment the concentration of the A1- and A2-domain-specific antibodies decreased.

More precise characterization of the targets of patient TK's antibodies was achieved by the competitive binding assay using FVIII-heavy-chain-specific antibodies. As the first choice, monoclonal antibody F7B4, whose binding site contains amino acids 356–360 of the a1 domain, was used in a competitive binding assay. The result that antibody F7B4 bound with similar affinity to recombinant fragments a1 and A2 was expected, since the epitope of this antibody was present in both these recombinant FVIII fragments. At the beginning of treatment, patient TK's antibodies inhibited mAb F7B4 binding to FVIII by 25% (Figure 8A). All other IgG samples taken during treatment also contained antibodies, which were able to compete with F7B4 for its binding site on FVIII. The inhibition of FVIII interaction with mAb F7B4 correlated with the concentration of antibodies bound to the recombinant A2 domain (Spearman rank order correlation coefficient $r=0.881$, $p=0.007$). Contrary to mAb F7B4, the tested patient's antibodies themselves were not able to bind to the a1 recombinant fragment, thus obviously the epitopes of the patient's FVIII-heavy-chain-specific antibodies located within the C-terminus of A1 domain or the N-terminus of the A2 domain and interfered with the antibody F7B4 binding to FVIII indirectly.

In the competitive binding assay, we also used FVIII-heavy-chain-specific monoclonal antibody, mAb 15, which binds to a conformational epitope in the C-terminus of the A2 domain resulting in a complete inhibition of the FVIII coagulation activity (Gilles *et al.*, 1996; Gilles *et al.*, 1997). The FVIII binding to mAb 15 was interfered with by some of the tested antibody samples, showing that the patient's antibodies were also targeted at this region of the C-terminus of the A2 domain (Figure 8A).

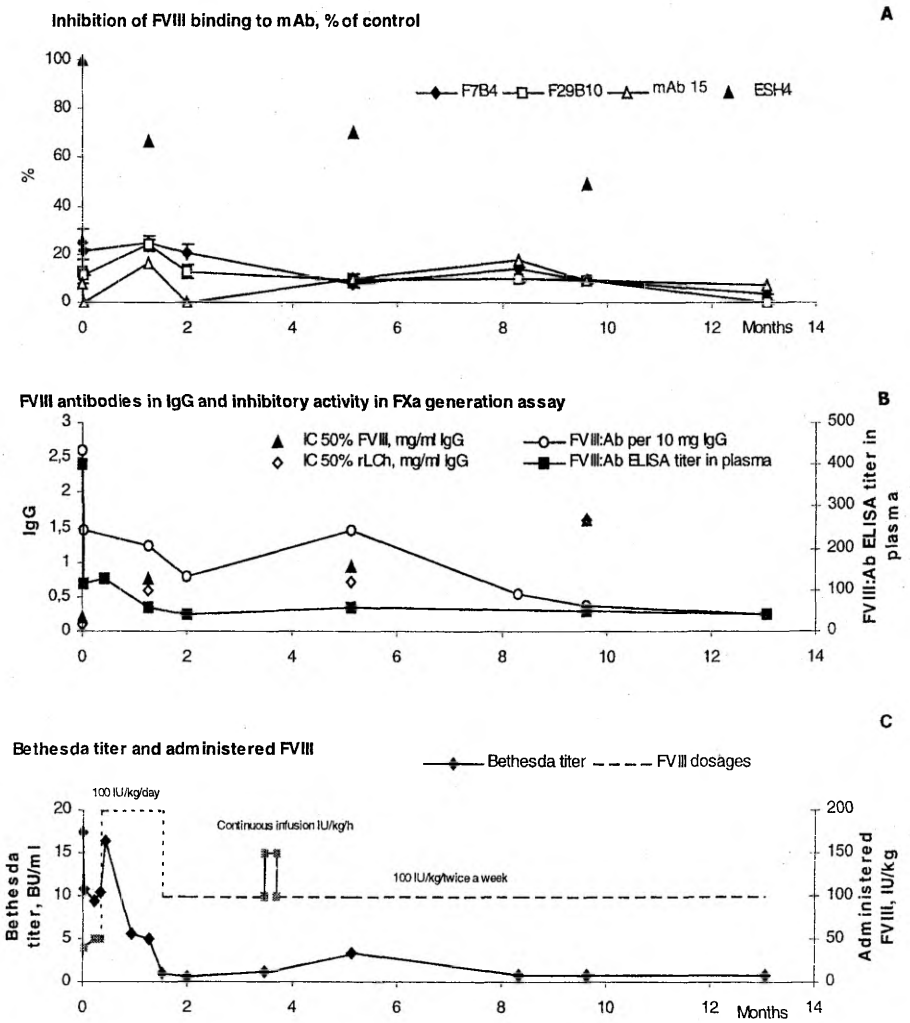

Figure 8. Inhibition of monoclonal anti-FVIII antibody binding to FVIII by patient TK's IgG (mean ± SD) (**A**). The concentration of FVIII antibodies in purified IgG (μg per 10 mg of IgG) and the ELISA titer estimated in plasma. The inhibitory activity of FVIII antibodies in the FXa generation assay is shown as IgG concentration yielding 50% of the inhibition of FXa generation induced by rFVIII (IC 50% FVIII) or the recombinant light chain of FVIII (IC 50% rLCh) (**B**). The Bethesda titer and the administration scheme of FVIII preparation Haemoctin SDH (Biotest, Germany) (**C**).

5.2.4. IgG subclasses of FVIII antibodies (III)

5.2.4.1. IgG subclasses of FVIII antibodies in patients with persistent FVIII antibodies (III)

Antibodies to FVIII are generally of the IgG type immunoglobulins with the prevalence of the IgG4 subclass (Algiman *et al.*, 1992; Fulcher *et al.*, 1987), or according to others, the normal physiological distribution of IgG subclasses is detected as reported by different research groups (Gilles and Saint-Remy, 1994). Since only a negligible amount of FVIII antibodies of the IgM type has been found, we focused on the IgG type antibodies. In order to assess whether the IgG subclass distribution of FVIII antibodies in haemophilia A patients with persistent FVIII antibodies differs from that detected during the immune tolerance treatment, we first investigated the plasma samples of 12 haemophilia A patients. The samples were divided into 2 groups according to the properties of FVIII antibodies as described in section 5.1, except that the first group was represented with 8 samples, because there was not enough plasma of patient No. 9 to perform respective analyses. We used a sensitive ELISA assay for the detection of IgG subclasses of FVIII antibodies directly from the plasma sample. The distribution of IgG subclasses of FVIII antibodies is shown in Table 6. The most abundant in both groups was the IgG4 subclass of FVIII antibodies ($p<0.01$, paired t-test). Results of the Bethesda assay correlated fairly well with the ELISA titers of all four IgG subclasses of FVIII antibodies.

The relative distribution of the IgG subclasses of FVIII-light-chain-specific antibodies was compared to a relative subclass distribution of antibodies against the whole FVIII molecule. The FVIII-light-chain-specific antibodies of Group 1 samples were mainly of the IgG4 subclass. The samples classified into Group 1 showed a similar subclass distribution of antibodies against the FVIII light chain and the whole-FVIII molecule. IgG1 subclass FVIII light chain antibodies and IgG4 subclass FVIII antibodies dominated in the samples classified into Group 2. The difference between the subclass distribution of the FVIII-light-chain-specific and FVIII antibodies could be explained by a higher amount of FVIII-heavy-chain-specific antibodies in these 3 samples. A good correlation was found between the results of the Bethesda assay and the amount of the FVIII light chain antibodies of all IgG subclasses ($p<0.01$).

5.2.4.2. Changes in the IgG subclass distribution of FVIII antibodies during immune tolerance treatment (III)

Further we evaluated the putative changes in the IgG subclass distribution of FVIII antibodies and the FVIII light chain antibodies of patient TK in the course of ITT. At the beginning of the immune tolerance treatment the patient had 35% of IgG4, 23% of IgG1, 29% of IgG2 and 13% of IgG3 subclass FVIII antibodies. Factor VIII-light-chain-specific IgG antibodies were present in the following percentages: 34% of IgG4, 28% of IgG1, 23% of IgG3 and 15% of IgG2 subclasses. Results of the IgG1-4 subclass distribution are shown in Figure 9 (A, B). At the beginning of treatment the concentration of the IgG4 subclass FVIII and the FVIII light chain antibodies was slightly higher than that of other IgG subclasses. During the first continuous infusion the decrease in the concentration of all IgG subclasses except that of IgG1 of FVIII antibodies was identical. During treatment, the distribution of FVIII antibodies between IgG subclasses remained unchanged, retaining a significantly higher level of IgG4 subclass antibodies ($p<0.05$, paired t-test). The curves possessed identical slopes, but significantly different intercepts ($p<0.001$). Antibodies specific of the FVIII light chain had a different IgG subclass distribution. During ITT, factor VIII light chain antibodies of subclasses IgG1, IgG4, IgG3 were almost identically expressed and at a significantly higher level than IgG2 antibodies ($p<0.05$, paired t–test). At the beginning of treatment a fluctuation in the FVIII light chain antibody subclass distribution was found to be analogous to that of FVIII antibodies.

There were two pronounced increases in IgG1 subclass antibody levels ($p<0.05$, ANOVA). The first rise was observed after a one-month treatment with the dose 100 IU/kg per day and the other after 5 months, probably being caused by the second continuous infusion. One of these increased levels was accompanied by an increase of the concentration of FVIII light chain antibodies of the IgG1 subclass and an increase in the Bethesda titer. The other one was associated with a rise in antibodies against the epitopes within the FVIII heavy chain.

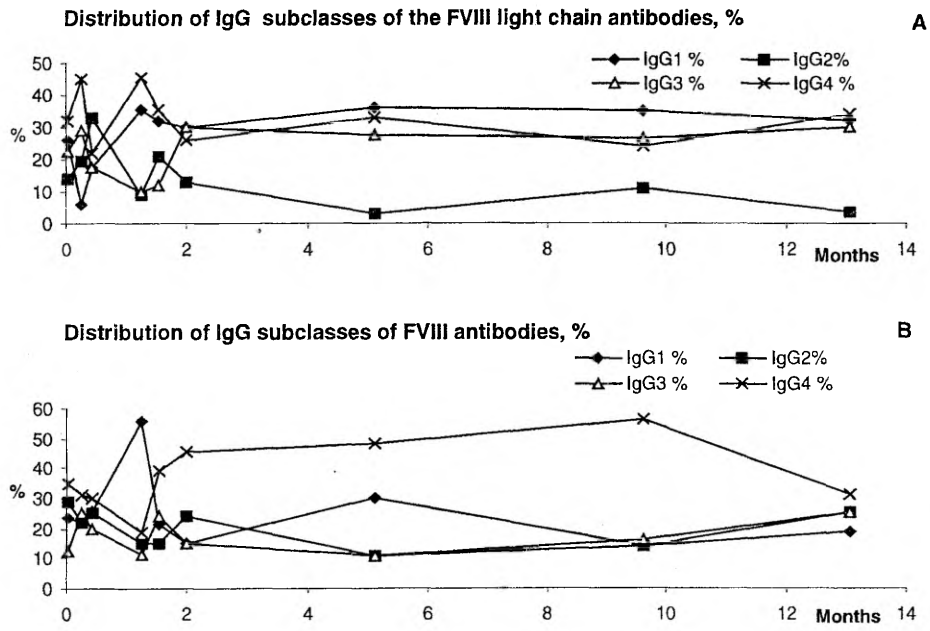

Figure 9. Distribution of IgG subclasses of antibodies targeted at FVIII (**B**) or the FVIII light chain (**A**).

Table 6. Distribution of FVIII antibodies and FVIII light chain antibodies between IgG subclasses.

Plasma sample	BU/ml	Distribution of FVIII antibodies				Distribution of FVIII light chain antibodies				Inversion in intron 22 of FVIII gene
		IgG1 %	IgG2 %	IgG3 %	IgG4 %	IgG1 %	IgG2 %	IgG3 %	IgG4 %	
Group 1										
1	140	22	25.2	21	31.6	29.5	23.2	17	33.5	Type 1
2	9	25.5	25	19.6	29.8	24	17	21	38	Type 1
3	5	25	25	23	26	29.8	10.7	1.8	41.7	Rare inversion
4	7	30	14.5	21	33.9	74	7.4	0	18.5	n.d.
5	8	10.6	19	19	51	17	17	22	43.5	Type 1
6	37	32	17	21	32	12.5	12.5	25	50	Type 2
7	300	32.8	20.4	19.8	26.9	22.6	19.3	30.4	27.6	Type 1
8	280	16.2	34.2	17	32.4	3.4	13.7	31	51.7	n.d.
9	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
Mean		23	22.5	20.1	32.9	26.6	15.1	18.9	38	
SD		7.5	6.2	1.8	7.8	21.1	5	11.2	11.2	
Group 2										
10	1	27.8	12.8	11	27.8	27.4	39.6	17.8	14.8	Type 2
11	5	26	24.6	24.6	24.6	55	22	11	11	n.d.
12	150	30	7	28.9	33.3	24	20	28	28	n.d.
Mean		27.9	14.8	21.5	28.5	35.5	27.2	18.9	17.9	
SD		2	8.9	9.3	4.4	17	10.8	6.9	8.9	
Mean, $^{n=11}$		25.3	20.4	20.5	31.8	29.0	18.4	20.1	32.6	
SD		6.8	7.5	4.5	7.1	19.7	8.5	9.1	13.8	

n.d. - not determined

5.3. Inhibition of FVIII:C activity by the patient's antibodies, the putative mechanism (II)

After the characterization of the specificity of FVIII antibodies during the ITT of patient TK, we attempted to explore the mechanism of FVIII antibodies in the inhibition of FVIII:C activity in relation to epitope specificity. Therefore, we studied whether patient TK's antibodies interfered with the FVIII interaction with VWF and/or the binding of FVIII to PS, since these interactions are most commonly impaired by the FVIII-light-chain-specific antibodies. No influence on the FVIII interaction with VWF or the FVIII binding to PS was detected when FVIII was pre-incubated with the patient's IgG before adding it to either VWF- or PS-coated microplates, as observed in the respective ELISA assays. Monoclonal antibody BO2C11, which interfered with both of these interactions in a concentration dependent manner, was used as a control in each assay.

Since the FVIII antibodies of patient TK were not able to interfere with the FVIII-PS interaction, we assessed whether the antibodies impair the FVIII interaction with FIXa and/or FX and hinder the formation of the FXase complex. We used a factor Xa generation assay, which was dependent on the presence of phospholipids, FVIII as well as Ca^{2+} and if one of these was not added to the reaction mixture, FXa generation was abolished.

Preliminary experiments showed that in the presence of the light chain of FVIII, FXa generation was lower than in the presence of rFVIII. The recombinant FVIII heavy chain led to the generation of a negligible amount of FXa only. Due to a limited amount of plasma available for IgG purification, we investigated only four samples in this assay. At the beginning of treatment the patient's antibodies at the IgG concentration of 500 μg/ml completely abolished the generation of FXa induced by rFVIII or the FVIII light chain (Figure 10). The concentration-dependence of the inhibition of FXa generation by patient TK's antibodies is shown in Figure 11.

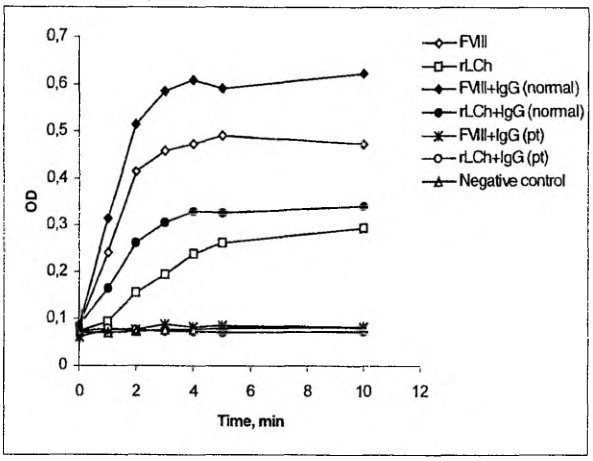

Figure 10. Inhibition of FXa generation induced by rFVIII (rFVIII) or the recombinant light chain of FVIII (rLCh) with patient TK's FVIII antibodies (0.5 mg/ml IgG) taken at the beginning of ITT. Each data point represents the mean of at least two individual experiments.

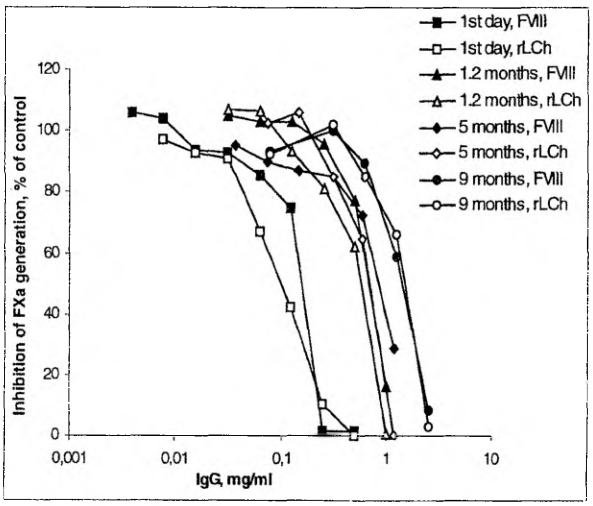

Figure 11. Inhibition of FXa generation induced by rFVIII or the recombinant light chain of FVIII (rLCh) with patient TK's FVIII antibodies. The amount of FXa was estimated after 5 min of induction. Each data point represents the mean of at least two individual experiments.

Patient TK's antibodies also inhibited the generation of FXa induced by the FVIII light chain, although a slightly lower concentration of IgG was needed for that, as compared to the rFVIII-induced FXa generation (Figure 10). Probably, FVIII light chain antibodies were mainly responsible for the inhibition of FVIII:C activity by interfering with the FVIII interaction with FIXa and FX. The IgG concentrations that caused 50% of the inhibition of FXa generation induced by rFVIII or the light chain of FVIII are shown in Figure 8B.

Since the tested FVIII antibodies hindered the formation of the FXase complex, we assessed whether or not the interaction of FVIII with FX takes place in the presence of patient TK's antibodies. The microplates coated with phosphatidylserine were used to ensure the specific orientation of FVIII on the surface allowing of an efficient binding of FX. This strategy for FVIII immobilization was chosen, because the tested antibodies did not interfere with the binding of FVIII to phosphatidylserine, and the orientation of FVIII was known to be highly important for the interaction with FX. The amount of FX bound to FVIII was estimated by a subsequent activation of FX with Russell's viper venom and the ability of the formed FXa to cleave a specific substrate. All four tested IgG samples characterized by the FXa generation assay, were able to inhibit the FX binding to FVIII immobilized to PS (Figure 12). The progressively higher concentrations of IgG obtained from the subsequent plasma samples during treatment were needed for the inhibition of the FX binding to FVIII analogously to the FXa generation assay. Since the light chain of FVIII has been reported to participate in the FX binding to FVIII (Nogami *et al.*, 1999), we assessed the ability of the FVIII light chain to interact with FX in a respective experimental set-up. The binding of FX to the light chain of FVIII was also inhibited in the presence of patient TK's antibodies. IgG fractionated from the plasma of healthy donors did not impair the interaction of FX with FVIII or its light chain and was therefore used as a control.

FX itself could also bind to PS in a FVIII-independent manner and give rise to high background values. Consequently, in parallel with an oriented immobilization of FVIII onto a phosphatidylserine layer, we also attempted the FVIII binding to surface-attached antibodies. The antibodies which are known to be targeted at the regions of FVIII, which do not participate in the interaction with FX (e.g. BO2C11, ESH4) bound efficiently to FVIII. Unfortunately, the ability of FVIII to interact with FX was completely lost upon binding to these monoclonal antibodies (data not shown).

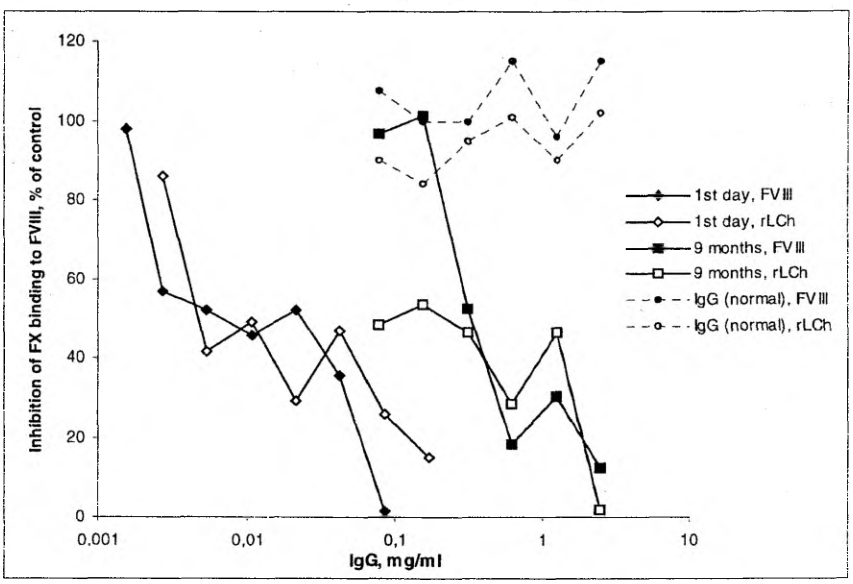

Figure 12. Interaction of FX with rFVIII and its recombinant light chain bound to phosphatidylserine in the presence of patient TK's antibodies. Each data point represents the mean of at least two individual experiments.

5.4. Characterization of VWF in FVIII preparations (IV)

We characterized the composition and the amount of VWF in various FVIII preparations registered in Estonia. Due to different protocols for isolating the FVIII-VWF complex from plasma dissimilar FVIII preparations are produced in which VWF serves as a biological carrier and stabilizer of FVIII (Vlot *et al.*, 1998). However, the degradation of VWF by protelytic cleavage can occur during different manufacturing procedures resulting in the cleavage of VWF high-molecular-weight multimers. We used various assays to characterize the VWF content in FVIII preparations. Compared to other investigated preparations, Haemoctin SDH showed a higher ratio between the results of VWF antigen and collagen binding, and high-molecular-weight multimers were not detected, which facts both indicate that some proteolytic cleavage of VWF had occurred (Table 7). The highest VWF content was in product Koate-HP, showing a good correlation between the results of FVIII:C activity and VWF:Ag ($r=0.94$, $p<0.05$) and also between the results of VWF antigen and collagen binding assays ($r=0.96$, $p<0.05$). Factor VIII preparation Haemoctin SDH (Biotest) showed correlation only between the results of VWF:Ag and

RCof activity (r=0.94, p<0.05). Factor VIII preparation Haemate had the ratio VWF:Ag/CBA 1.1, while for the lyophilized cryoprecipitate (Kryo-AHG) the ratio 1.4 ± 0.4 was found, which shows the presence of functional VWF in both products.

Table 6. Properties of the von Willebrand factor in different FVIII preparations. Multimerization is shown as the total number of multimers.

FVIII preparation	**FVIII:C IU/ml**	**VWF:Ag IU/ml**	**VWF:RCo IU/ml**	**VWF:CB IU/ml**	**Ratio VWF:Ag/ VWF:CB**	**Multi-meri-zation**
Haemoctin SDH *(Bio-test)* n=6	58.4 ± 3.5	31.8 ± 4.6	13.3 ± 3.2	8.5 ± 1.9	4.0 ± 1.6	14
Koate–HP *(Bayer)* n=6	114.4 ± 39.2	251.4 ± 79.5	118.8 ± 1.7	96.5 ± 42.0	2.4 ± 0.4	16
Kryo-AHG *(North Estonian Blood Centre)* n=6	8.5 ± 2.2	12.2 ± 3.1	8.4 ± 1.7	10.2 ± 3.1	1.4 ± 0.4	16
Haemate *(Aventis)* n=1	47.6	89.1	85.5	81.8	1.1	17

6. DISCUSSION

6.1. Inactivation of FVIII by antibodies, the protective effect of VWF

The results of our study indicate that the protective effect of the von Willebrand factor on the inhibition of the FVIII:C activity by FVIII antibodies depends primarily on the concentration of antibodies specific of the light chain of FVIII. All 12 investigated samples contained antibodies targeted at both the light chain and the heavy chain of FVIII. We found that the samples with a higher amount of the FVIII-light-chain-specific antibodies caused a significantly lower inhibition of FVIII:C activity in the presence than in the absence of VWF. Similar inhibition pattern of FVIII:C by FVIII antibodies developed in haemophilia A patients using FVIII preparations of different purity has been observed in *in vitro* experiments also by several other investigators (Amano *et al.*, 1995; Berntorp *et al.*, 1996; Gensana *et al.*, 2001; Littlewood *et al.*, 1991). The results of our study are well in line with the report by Suzuki *et al.* who has shown a lower inhibition of FVIII complexed with VWF by antibody samples that contained only C2-domain antibodies compared to the antibody sample containing C2- and A2-domain antibodies (Suzuki *et al.*, 1996).

The VWF binding sites are located within the a3 and C2 domains of FVIII (Saenko and Scandella, 1997). The proper conformation of these regions and an intact thrombin cleavage site at the amino-acid position 1689–1690 of FVIII contribute to a high affinity binding to VWF (Saenko *et al.*, 1999c). Recently it has been reported that the recombinant C1-C2 fragment binds to VWF at least 2 orders of magnitude more strongly than the C2 domain alone (Liu and Thomson, 2000). The C1-domain-specific antibodies are reported to interfere with the FVIII binding to VWF (Jacquemin *et al.*, 2000). Several regions of the FVIII light chain participate in the interaction of FVIII with VWF, therefore we used the recombinant FVIII light chain in order to characterize a panel of FVIII antibodies, which could influence the binding of FVIII to VWF. On the other hand, VWF could hinder the binding of antibodies to FVIII by binding to their epitopes, by steric hindrance or by inducing such conformational changes as a result of which the respective epitopes are buried or are not recognized by antibodies any more.

Whether the presence of VWF in FVIII preparations is necessary or not has been under discussion since immuno-affinity chromatography was applied for the production of plasma-derived FVIII preparations of very high purity. Haemophilia A patients have a normal level of VWF and half of the administered FVIII could bind to VWF in 10 seconds (Vlot *et al.*, 1996). In order to mimic the *in vivo* situation, plasma samples containing FVIII antibodies were used in neutralization experiments allowing the complex formation between

rFVIII and VWF of the patient's plasma. Still, the FVIII:C activity of the rFVIII preparation was more strongly inhibited by the patient's antibodies than the activity of the FVIII-VWF concentrate. This could be caused by a looser complexing of VWF with rFVIII or easier dissociation of the formed complex compared to FVIII-VWF complexes present in respective concentrates. On the other hand, FVIII antibodies could have a higher affinity to FVIII than to VWF or may have complexed with FVIII faster than with VWF.

Although a small number of plasma samples was investigated, two samples out of 12 contained almost an equal amount of FVIII light chain and heavy chain antibodies and one plasma sample containing predominantly FVIII heavy chain antibodies. Our data are in good agreement with the results by Scandella and the co-workers who found that 2 out of the 10 investigated plasma samples had almost an equal amount of antibodies recognizing the A2 domain and the light chain of FVIII as judged by immuno-precipitation (Scandella *et al.*, 1995b). Apparently, the development of antibodies with an almost equal relative contribution to binding activity and neutralization activity towards both the light and the heavy chains of FVIII is not typical.

Comparison of the neutralization activity of FVIII antibodies towards the FVIII or FVIII-VWF complex and the estimation of the amount of FVIII antibodies targeted at the light chain of FVIII might be useful in order to select the most suitable FVIII preparation for the treatment of the patient and thereby contribute to the lowering of treatment costs. Characterization of the antibody response to the FVIII-VWF concentrate administered for the immune tolerance treatment was another aim of this study.

6.2. Characterization of the patient's FVIII antibodies during immune tolerance treatment with FVIII preparation containing VWF

6.2.1. Epitope specificity of FVIII antibodies

Immune tolerance induction with the FVIII-VWF concentrate into a high responder patient TK (the Bethesda titer >5 BU/ml) resulted in a persistent production of antibodies targeted mainly at the light chain of FVIII analogously to the situation observed at the beginning of treatment. The concentration of inhibitory and non-inhibitory antibodies decreased significantly and *in vivo* recovery normalized at the end of treatment. The FVIII-light-chain-specific antibodies recognised mainly the C1 domain, but also the C2 domain, and a negligible amount of antibodies was targeted at the a3 domain. A significantly lower amount of antibodies recognized the FVIII heavy chain. Antibodies were

targeted at the C-terminal parts of the A1 domain and A2 domain (Figure 13). The heterogeneous antibody response like the one observed in the tested patient is generally characteristic of haemophilia A patients, while in the case of acquired haemophilia the antibodies are targeted at a smaller number of epitopes (Prescott *et al.*, 1997). Antibody epitopes often localise within the C2 domain of the light chain or the A2 domain of the heavy chain of FVIII, which are believed to be the most immunogenic regions on FVIII (Fulcher *et al.*, 1985; Scandella *et al.*, 1989; Scandella *et al.*, 2001).

To our knowledge, this is the first time when clinically significant C1-domain specific antibodies have been detected in a severe haemophilia A patient. Rare presence of antibodies of such specificity has been demonstrated by using synthetic peptide arrays (Palmer *et al.*, 1997). Recently a monoclonal C1-domain-specific antibody 2E9, which interferes with the FVIII binding to VWF, has been obtained by the immortalization of B-lymphocytes of a patient with mild haemophilia A (Jacquemin *et al.*, 2000). In order to compare the epitope specificity of the C1 domain antibodies developed in our patient with mAb 2E9, we used a competitive binding assay. The tested antibodies were not able to compete with mAb 2E9 for binding to FVIII, hence these antibodies probably possess a different specificity. On the other hand, mAb 2E9 was capable of inhibiting the interaction of FVIII with VWF, but patient TK's anti-FVIII antibodies did not interfere with FVIII-VWF interaction. This corroborates the suggestion that the epitopes of these antibodies were not overlapping.

Epitope mapping of FVIII C2-domain antibodies showed that patient TK's antibodies were targeted at both the N-terminus and C-terminus of the C2 domain, since the patient's antibodies were able to compete with monoclonal antibodies F29B10 (aa 2170–2195) and ESH4 (aa 2303–2332) for their binding sites on FVIII respectively. The binding of ESH4 (aa 2303–2332) to FVIII was abolished by the tested antibodies. Several reasons allow us to assume, that the C1-domain-specific antibodies could also inhibit the ESH4 binding to FVIII. The disulphide bonds in the C2 domain (Cys2174-Cys2326) and in C1 domain (Cys2021-Cys2169) connect the N-terminal and C-terminal ends of domains in close proximity (Pratt *et al.*, 1999; Stoilova-McPie *et al.*, 2002) (Figure 13). Therefore, antibodies which were able to interfere with the mAb F29B10 binding to FVIII could in principle also interfere with the binding of ESH4 to FVIII. Factor VIII fragment 2303–2332, the putative binding site of ESH4, contains β strands 18 and 19 of the C2 domain, which localize opposite the C1 domain. This part of the C2 domain participates in the FVIII binding to PL, being one of the four different loops of the C2 domain (aa 2222–2227, 2196–2201, 2313–2315, and 2249–2255) that are shown to be involved in interaction with the negatively charged phospholipids' head groups (Barrow *et al.*, 2001; Spiegel *et al.*, 2001). Unfortunately, the epitope of ESH4 is not precisely mapped. Probably ESH4 could induce conformational changes of the C2 domain and abolish further interactions of FVIII with VWF or PL. The patient's

antibodies, particularly the C1-domain-specific antibodies, however, interfered with the FVIII binding to ESH4 probably by steric hindrance or by inducing conformational changes, but had no inhibitory effect on FVIII-VWF and FVIII-PS interactions.

Although we could detect antibodies recognizing the C-terminus of the C2 domain and a3 domain, the tested antibodies were neither able to interfere with the FVIII binding to VWF nor to compete with monoclonal antibodies BO2C11 and 2E9, which are both able to interfere with this interaction (Jacquemin *et al.*, 2000; Spiegel *et al.*, 2001). This suggests that VWF present in the FVIII preparation used for the immune tolerance treatment could bury the respective immunogenic regions on the FVIII surface and thereby prevent the development of antibodies towards those epitopes (Figure 14). This result is in accordance with the observation reported by Prescott, who characterized the differences in antibody epitope specificity in patients treated with pdFVIII or rFVIII preparations (Prescott *et al.*, 1997). Antibodies against the a3-A3-C1 fragment were three-fold more common in the group of patients treated with pdFVIII than the patients treated with the rFVIII preparation, while the development of antibodies recognizing the A2 domain and/or the C2 domain was a characteristic response to the rFVIII treatment (Prescott *et al.*, 1997).

Some of patient TK's antibodies were targeted at the A1 and A2 domains of the FVIII heavy chain. Monoclonal antibody mAb15 (aa 720–740) binding site on FVIII is often a target of A2 domain antibodies developed in haemophilia A patients (Gilles *et al.*, 1993; Gilles, *et al.*, 1996). The A1-domain-specific antibodies are rare, although by using synthetic peptide arrays a small fraction of antibodies recognizing region 20–349 has also been found in haemophilia A patients (Palmer *et al.*, 1997). Patient TK antibodies were able to interfere with the binding of mAb F7B4 (aa 356–360) to FVIII. Region 337–372 is exposed on the surface of both the FVIII activated and inactivated form (Amano *et al.*, 1998a; Pemberton *et al.*, 1997; Stoylova *et al.*, 1999), which could explain why this region was also a target for antibodies developed in the tested patient.

It has been shown earlier that during the immune tolerance treatment the epitope specificity of anti-FVIII antibodies is changing. Immunoblotting of FVIII and its thrombin-cleaved fragments that was used for the characterization of the specificity of FVIII antibodies demonstrated the changes in the epitope specificity in five haemophilia A patients out of 13 (Fulcher *et al.*, 1988). The changes in the concentration of antibodies recognizing the A2 domain of FVIII were observed in most of these patients. In our study we used recombinant FVIII fragments, which enabled us a more exact characterization of the changes in antibody specificity. In our patient antibody epitope specificity remained rather unchanged throughout the immune tolerance treatment, except an increase in C2-domain-specific antibodies caused by the interruption of treatment. However, the concentration of inhibitory and non-inhibitory FVIII antibodies decreased significantly. This result is in agreement with a previous study by Gilles and co-workers, where no changes in the profile of antibody

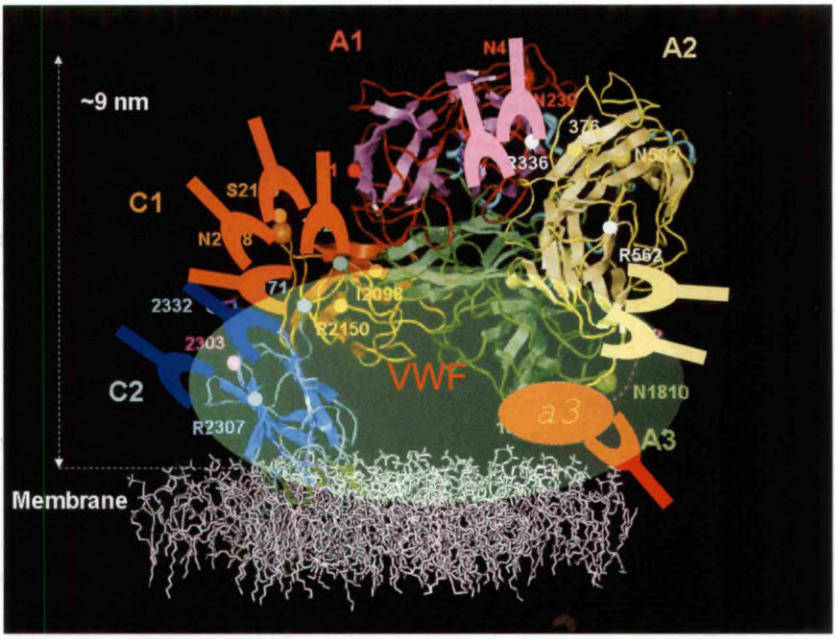

Figure 13. Model of membrane-bound FVIII and putative binding sites and the relative amount of patient TK's FVIII antibodies. Antibodies targeted at different domains of FVIII are coloured respectively. Putative location of the *a3* domain and VWF bound to FVIII are indicated. Adapted from Stoilova *et al.*, 2001, courtesy of Geoffrey Kemball-Cook.

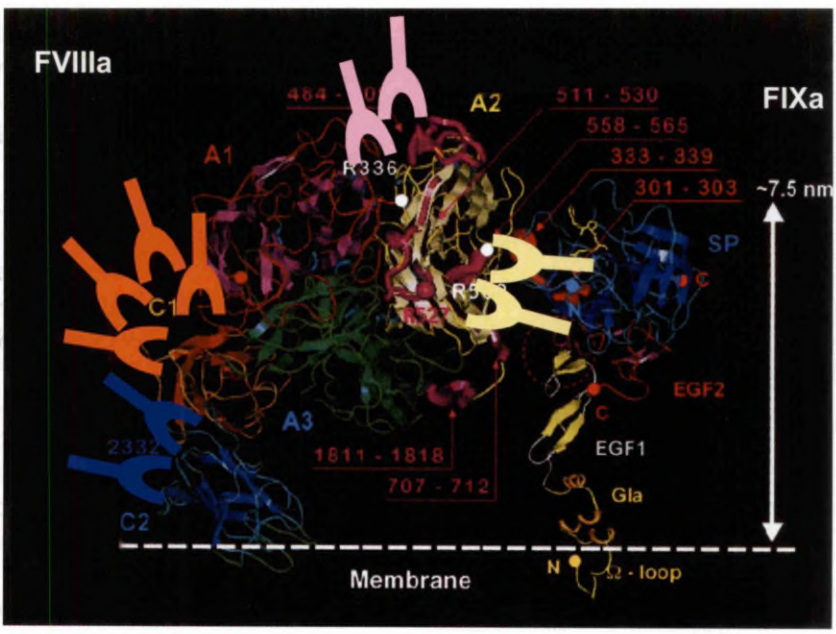

Figure 14. Model for the membrane-bound FVIII-FIXa complex and the putative location of binding sites of patient TK's FVIII antibodies. Adapted from Stoilova-McPhie *et al.*, 2002, courtesy of Geoffrey Kemball-Cook.

specificity were observed in the course of the immune tolerance treatment in 2 patients, except the changes in FVIII antibody concentration (Gilles *et al.*, 1996). They observed that successful desensitization led to the production of anti-idiotypic antibodies in both patients. A fair relationship was reported between FVIII administration and the following increase in the concentration of anti-idiotypic antibodies (Gilles *et al.*, 1996). We observed a significant decrease in the Bethesda titer after 2 weeks of treatment, while no change in anti-FVIII antibody concentration was detected, which could also be due to the development of anti-idiotypic antibodies. Unfortunately, we could not study the presence of anti-idiotypic antibodies in our patient because of the limited volume of the investigated plasma samples collected for our investigation.

To our knowledge, this is the first detailed characterization of the antibody response during the immune tolerance treatment although the antibody response has been described only in a single patient treated according to an uncommon treatment protocol. Usually, the amount of antibodies is determined by the Bethesda assay and in some studies the samples taken during treatment have also been characterized by immunoblotting with FVIII (Fulcher *et al.*, 1985; Fulcher *et al.*, 1987) or by the immunoprecipitation assay with recombinant FVIII fragments (Klinge *et al.*, 2001; Scandella *et al.*, 1988; Scandella *et al.*, 2000). Interruption of the treatment and a subsequent increase and decrease in FVIII antibody production allowed us to compare the situation with the one at the beginning of treatment. Although the titer of inhibitory and non-inhibitory antibodies increased after the second continuous infusion, no significant changes in epitope specificity were observed, except a slightly elevated level of the C2-domain-specific antibodies and antibodies that were able to compete with mAb ESH4 for binding to FVIII. However, the FVIII binding to VWF or PL was not interfered by these FVIII antibodies. An increase in the C2 domain antibody level suggests that the patient had a tendency to produce antibodies of such specificity, but their production was suppressed by the administration of the FVIII-VWF concentrate. The sample taken several months before the immune tolerance treatment from the same patient contained predominantly the FVIII light chain antibodies. Their inhibitory activity towards FVIII complexed to VWF was 14.5-fold lower than towards FVIII itself (Table 5, Patient 2). It is generally believed, that C2-domain antibodies capable of interfering with the FVIII-VWF binding are responsible for a lower neutralization ability in the presence of VWF (Shima *et al.*, 1995b; Suzuki *et al.*, 1996). We were unable to characterize antibodies in a this particular sample in a more detailed manner, but apparently the concentration of the C2-domain-specific antibodies was significantly higher and the epitope specificity differed from FVIII antibodies produced during the immune tolerance treatment. Therefore, minor changes in antibody epitope specificity during treatment were probably caused by the administration of this particular FVIII-VWF concentrate.

Our patient has an inversion in intron 22 of the FVIII gene, which is the most common gene defect in a severe form of haemophilia A. This gene defect

abolishing the synthesis of FVIII protein, is usually accompanied by a higher prevalence of the FVIII antibody development (Schwaab et al., 1995b; Tuddenham and McVey, 1998; Antonarakis *et al.*, 1995; Tuddenham *et al.*, 1991). Characterization of antibodies developed in these patients has shown a wide variety of epitopes with no prevalence of any binding site either on the heavy or the light chain of F VIII (Scandella *et al.*, 2001).

6.2.2. The IgG subclass distribution of FVIII antibodies

Inhibitory and non-inhibitory FVIII antibodies of the IgG4 subclass dominated during the whole immune tolerance treatment of patient TK, but the FVIII light chain antibodies of all IgG subclasses were expressed at an almost equal level, except the IgG2 subclass. The subclass distribution of FVIII light-chain antibodies during ITT differed from that of the patients with persistent inhibitory antibodies possessing mainly the IgG4 subclass FVIII-light-chain-specific antibodies (Table 6). However, the results of our study are in good agreement with the data published earlier, which report that the on-demand treatment of haemophilia A patients causes the development of the IgG1 or IgG4 subclass FVIII antibodies mainly (Algiman *et al.*, 1992; Fulcher *et al.*, 1987).

We detected the elevated IgG1 subclass levels of FVIII antibodies at two different time points during treatment. Both increased levels of IgG1 subclass antibodies were observed after the administration of a higher dosage of the FVIII preparation, indicating the presence of a novel clone that produces FVIII antibodies with a different epitope specificity or affinity. The level of IgG1 subclass antibodies rises quickly after antigen administration, while the production of the IgG4 subclass probably needs more support on the part of cytokines produced by Th2 cells (Mosmann *et al.*, 1986).

This observation was supported by the results of the epitope mapping of FVIII antibodies. The first rise in the IgG1 subclass FVIII antibodies was accompanied by elevated levels of the C1 domain antibodies and antibodies that were able to compete with mAb F29B10 for their binding sites on the C2 domain. The second rise in the IgG1 subclass level of FVIII antibodies was obviously caused by the interruption and resumption of treatment and reflected probably the increased concentration of the A2-domain-specific antibodies, which were able to compete with mAb 15 for the binding site on FVIII. Although an increase in the C2-domain-specific antibody level was also observed at the same time point, we found no significant increase in FVIII light chain antibodies of the IgG1 subclass. It might be that the C2-domain-specific antibodies belonged to the IgG4 subclass. Unfortunately, we were unable to detect the IgG distribution of C2-domain-specific antibodies due to a limited plasma volume.

The results of our study are in agreement with the results obtained in the mouse haemophilia A models, where the IgG1 subclass (corresponds to human IgG4) FVIII antibodies dominate (Rossi *et al.*, 2001; Sasgary *et al.*, 2002; Wu *et al.*, 2001). Rossi with the co-workers found that the distribution of FVIII antibodies into IgG subclasses correlated well with the results of cytokine assays indicating that the antibody response is very complex and might involve both Th1 and Th2 type cells and is especially dependent on the production of IL-10 (Rossi *et al.*, 2001). A recent report by Sasgary *et al* suggested that the IFN-γ producing cells were most prominent, but decreased along with the increase in number of IL-10 producing FVIII–specific T cells (Sasgary *et al.*, 2002). They also observed the presence of two types of IL-10-positive cells; the first produced only IL-10 and the second produced, in addition to IL-10 also IFN-γ (Sasgary *et al.*, 2002). IFN-γ is known to support the production of the IgG2a subclass and inhibit the production of IgG1 subclass antibodies in mice. Based on that we assume that the antibody response in the tested haemophilia A patient was determined by the dominance of IFN-γ producing cells or IL-10 producing cells at different time points during the immune tolerance treatment, which was also reflected in the changes in IgG subclass levels.

A very recent study reported that the concentration of FVIII antibodies in haemophilia A patients may poorly correlate with a proliferative response to FVIII of their CD4+ blood lymphocytes (Reding *et al.*, 2002). A response to FVIII by CD4+ lymphocytes of healthy donors was somewhat lower and transient as compared to haemophilia A patients with and without FVIII antibodies. But CD4+ lymphocytes of the patient who was tolerated to FVIII did not respond to FVIII at all (Reding *et al.*, 2002). The normal level of FVIII in healthy persons may induce anergy or apoptosis of FVIII-specific CD4+ cells, when FVIII epitopes are presented without co-stimulatory signals (Johnson and Jenkins, 1994; Schwartz, 1996). We were unable to study the proliferation of FVIII-specific CD4+ cells of our patient during treatment, although we could suppose that the activated FVIII-specific CD4+cells persisted rather long, especially when the level of FVIII in plasma was low and transient and not sufficient to sustain the persistent toleration of CD4+ cells. Probably, in haemophilia A patients such FVIII-specific CD4+ cells have not been eliminated during ontogenesis of the immune system due to the absence of FVIII and, therefore, a higher level of FVIII is needed for successful toleration. However, information about the role of different cytokines producing CD4+ cells in a FVIII antibody response and in the toleration of haemophilia A patients is rather limited and further studies are needed.

6.3. Inhibition of FVIII:C activity by the patient's antibodies, the putative mechanism

The antibodies of patient TK recognized mainly the FVIII light chain and were unable to interfere with the FVIII binding to PS or to VWF, but on the other hand they inhibited FXa generation and the FX binding to FVIII. These results are rather different from a well-characterized inhibitory effect of FVIII antibodies developed in most patients, since FVIII-light-chain-specific antibodies in general are able to block the binding of FVIII to VWF or to PS (Saenko and Scandella, 1995; Shima *et al.*, 1995b; Shima *et al.*, 1993).

Patient TK's antibodies inhibited FXa generation induced by FVIII or by its light chain. The generation of FXa involves successive processes starting with the FVIII binding to PS and the FIXa binding to FVIII, followed by the interaction of FX with the formed complex. FIXa binding to FVIII takes place at three sites: two within the A2 domain (aa 558–565 and aa 698–710) that have been found to be responsible for the binding to the FIXa heavy chain (Fay *et al.*, 1994; Lenting, *et al.*, 1998) and the third region within the A3 domain (aa 1811–1818) that binds to the FIXa light chain (Lenting *et al.*, 1996). The tested antibodies recognised the C-terminus of the A2 domain, which obviously contributes to the FVIII binding to FIXa. Unfortunately, we were not able to test the effect of the patient's antibodies on the FIXa binding to FVIII because of our limited sample volume. The concentration of A2-domain-specific antibodies as well as the inhibitory activity of FVIII heavy chain antibodies were significantly lower than that of the FVIII light chain antibodies, therefore the hindrance of the FIXa binding to FVIII was probably not the main mechanism of the inhibition of FXa generation. In addition, FX generation induced by the FVIII light chain was also inhibited by the patient's antibodies indicating that FVIII light chain antibodies were mainly responsible for interfering with FXa formation, pointing to a novel, yet unknown mechanism of the inhibition of FVIII:C activity by FVIII light chain antibodies specific of the C1 domain.

The patient's antibodies were capable of interfering with the FX interaction with FVIII on the phosphatidylserine layer. FVIII-light-chain-specific antibodies were responsible for that effect as the FX binding to the FVIII light chain on PS was also impaired by the patient's antibodies. We found that C2-domain-specific antibodies ESH4 and BO2C11 were also able to inhibit the FVIII interaction with FX. However, since both of these antibodies interfere with the FVIII binding to PS, we used another strategy, coupling first FVIII to an antibody on the microplates, but unfortunately, FX was unable to bind to the formed complex. Obviously, ESH4 is able to induce such a conformation change in the FVIII C2 domain that FVIII was not able any more to bind to VWF or to PS and the interaction with FX was also abolished. Surprisingly, we encountered an analogous inhibition of the FVIII binding to FX by using mAb BO2C11, which is shown to interact with the PS binding site of the C2 domain

(aa 2251–2252, 2199–2200). The C2-domain-specific antibodies (ESH4, BO2C11) and C1-domain-specific antibodies of the tested patient were able to interfere with the FVIII binding to FX, confirming that the conformation of the FVIII light chain as well as the distance between the binding sites of FVIII and FX are extremely important for efficient interaction. Nogami with the co-workers has studied the role of the FVIII C2 domain in the FVIII interaction with FXa (Nogami, 2002; Nogami, *et al.*, 1999). They showed that monoclonal antibody ESH8 (aa 2248–2285, non-competitive with ESH4 used in our experiments) abolished proteolytic cleavage at Arg1689 and Arg1721 of the light chain of FVIII by FXa and partially inhibited cleavage at Arg372 of the heavy chain of FVIII both in the presence and absence of PL. Another mAb NMC-VIII/5 (aa 2170–2337) had no protective effect and on the contrary, even tended to promote cleavage by FXa. VWF was also able to protect FVIII against proteolytic activation by FXa. It was demonstrated with synthetic peptides that amino acids 2253–2270 within the C2 domain are essential for FXa binding. Since ESH8 did not interfere with the FVIII binding to PL, they suggested that the FXa binding site should be within the N-terminal of the C2 domain (Nogami, 2002; Nogami *et al.*, 1999). Probably the FX and FXa binding sites on FVIII are not completely overlapping. The tested patient's antibodies were targeted at the N-terminus of the C2 domain and were able to hinder the FVIII binding to FX but were not interfering with the FVIII binding to PL. Therefore the patient's antibodies could also interfere with the FXa binding to FVIII. Since the tested antibodies were a mixture of C2 domain antibodies and antibodies against other epitopes within the light chain and heavy chain of FVIII, the mechanism of inhibiting FVIII coagulation activity by every antibody in this mixture has in principle to be investigated by using monoclonal antibodies against the same epitope as the patient's antibodies. However, the properties of FVIII antibodies in haemophilia A patients vary in the course of therapy in their affinity, concentration, epitope specificity, neutralization mechanism, etc. and often only the major traits can be characterized.

So far little is known about the function of the C1 domain in FVIII. Liu and Thompson have reported that compared to the C2 domain alone, C1-C2 fragment has a higher binding affinity to VWF, FX/FXai and FIX/IXai (Liu and Thomson, 2000). They also reported that the VWF binding site was different from and not overlapping with the binding sites for FX/Xai and FIX/IXai, however, FX and FIX were competing for the binding site within C1-C2 fragment (Liu and Thomson, 2000). The patient's antibodies were unable to interfere with the FVIII binding to a C1-domain-specific monoclonal antibody 2E9, which interferes with the FVIII binding to VWF. However, the patient's antibodies were capable of impairing the FVIII binding to FX. These findings are in good accordance with the results obtained by Liu and Thompson, showing that different regions of the C1 domain are involved in FVIII-VWF and FVIII-FX interactions.

The patient's antibodies recognized the A1 domain and were able to compete for the binding site on FVIII with mAb F7B4 (aa 356–360), therefore the A1 domain antibodies were obviously also able to interfere with the FX binding to FVIII. It has been shown that, human antibodies directed to the epitope of amino-acids 338–362 (Ware *et al.*, 1988) or to the epitope of amino-acids 351–361 within the A1 domain interfere with the FVIII binding to FX (Foster *et al.*, 1990a). On the other hand, amino-acids 337–372 of the a1 domain are suggested to participate in the interaction of FVIII with FX (Lapan and Fay, 1997). Therefore, A1-domain-specific antibodies of the tested patient could inhibit the interaction FVIII-FX by covering FX binding site on the a1 domains. The IgG concentrations needed for the inhibition of the FVIII binding to FX or the FVIII light chain binding to FX were slightly different, thus indicating that A1-domain-specific antibodies were also involved in the inhibition of this interaction. However, the inhibitory effect of FVIII light chain antibodies dominated.

Two types of antibodies can be distinguished by the inhibitory character of FVIII:C activity in haemophilia A patients (Biggs *et al.*, 1972a; Biggs *et al.*, 1972b; Gawryl and Hoyer, 1982; Ling *et al.*, 2001). Patient TK's antibodies inhibited FVIII:C activity incompletely even at very high antibody concentrations, and we observed a non-linear relationship between FVIII:C activity and antibody concentration. Analogous inactivation pattern was observed when the patient's antibodies were pre-incubated with the recombinant FVIII light chain and the ability of the remaining antibodies to neutralize FVIII:C activity was measured by the chromogenic method (Figure 5) and also by the FXa generation assay. Therefore, we can conclude that FVIII light chain antibodies were mainly responsible for Type 2 inhibition pattern of FVIII:C, which has also been reported by other investigators (Peerlinck *et al.*, 1997). However, FVIII-light-chain inhibitory antibodies with the Type 2 inactivation pattern are usually targeted at the C2 domain and interfere with the FVIII binding to VWF and to PS (Peerlinck *et al.*, 1997; Sawamoto *et al.*, 1998).

6.4. Characterization of the von Willebrand factor content in FVIII preparations

The amount and the functional activity of VWF was characterized in various FVIII preparations including Haemoctin SDH, which was used in *in vitro* neutralization experiments as a source of the FVIII–VWF complex and also for the immune tolerance treatment. Haemoctin SDH had a higher ratio between the results of the VWF antigen and collagen binding compared to other investigated preparations, and high-molecular-weight multimers were absent in Haemoctin SDH. This indicates that the proteolytic degradation of VWF occurred in the

course of the manufacturing process. Our data are in good agreement with the results obtained by Kotitchke and his co-workers: VWF collagen binding of about 12 IU/ml and the ratio of VWF:Ag to VWF:CB being 4.1 (Kotitschke *et al.*, 1999).

Despite the lack of high- molecular-weight multimers and the increased level of intermediate- and low-molecular weight multimers, VWF in this particular preparation was able to protect FVIII:C inactivation caused by FVIII antibodies in *in vitro* experiments. Presumably, the multimerization of VWF is necessary for an efficient binding of VWF to the platelet Gp Ib-IX-V receptor complex and integrin $\alpha_{IIb}\beta_3$ for the induction of platelet adhesion and aggregation, respectively. On the other hand, the multimerization of VWF is not obligatory for the complexing with FVIII, its protection and stabilization, since each VWF monomer has a FVIII binding site and can therefore bind to FVIII (Vlot *et al.*, 1996). By using the platelet function analyzer, PFA-100, platelet adhesion to the collagen surface was studied, and the authors concluded that FVIII preparations of high purity and intermediate purity lacking high-molecular-weight multimers were also able to promote platelet adhesion in a dose-dependent manner upon the flow at high shear stress (Siekmann *et al.*, 2002). These results can explain why these concentrates are effective in treating haemorrhages in VWD Type 3 patients, although without correcting the prolonged bleeding time (Rodeghiero *et al.*, 1992).

We investigated the VWF binding to collagen by an ELISA assay, which measures the ability of VWF to bind to collagen in static conditions. As reported by us and other investigators, the source and the concentration of collagen, and some more parameters are critical for an effective VWF binding to collagen (Favaloro 2000; Favaloro *et al.*, 2000; Siekmann *et al.*, 1998). The measuring of ristocetin cofactor activity of VWF (VWF:RCo) enables researchers the characterization of VWF-mediated platelet-platelet interaction and quantifies the level of intermediate- and high-molecular-weight multimers (Favaloro, 1999; Favaloro, 2001). An optimised VWF collagen binding (VWF:CB) assay estimates the amount of the largest VWF multimers, which have the highest functional activity. Although the VWF:CB assay is recommended for use by European Pharmacopoeia (European Pharmacopoeia, 2001), a potential official European Pharmacopoeia assay for the characterization of VWF in factor VIII preparations is still under development (Neugebauer *et al.*, 2001). Therefore, we designed a respective ELISA for the characterization of VWF composition in FVIII preparations. The optimal collagen III concentration (10 µg/ml) in the assay enabled us to lower of the variation coefficient (4.8% for normal values), to discriminate well between different types of VWD and fairly correlate between the values of VWF:CB and VWF multimers in the VWD patients' plasmas as well as in FVIII preparations. Ramasamy with the co-workers showed the applicability of the VWF:CB assay for the characterization of VWF in the course of a small-scale manufacturing process of the preparation. They used collagen Type I in the assay and the assay had the coefficient of variance

12.5% for normal values (Ramasamy *et al.*, 1998). Results of the multi-laboratory characterization of VWF in different FVIII preparations including Haemate P (Aventis Behring, USA), showed results similar to our findings (Favaloro *et al.*, 2002).

Analogously to other investigators, we used a local plasma standard, which was calibrated against the VWF:RCo activity of the WHO plasma standard, because the VWF:CB values were not available at that time and the VWF:RCo activity was the only well-characterized functional activity of VWF (Casonato *et al.*, 2001; Favaloro *et al.*, 2000; Siekmann *et al.*, 1998). Recently the National Institute of Biological Standards and Controls (NIBSC, UK) has completed an international study on the measuring of VWF:CB in one of its international plasma standards and in the proposed WHO 1st International Standard for FVIII preparations containing VWF (Montgomery, 2001). Both these standards will be available in the near future and improve the standardization of the VWF:CB assay.

In principle, the FVIII preparation containing VWF, is of a lower price as compared to the FVIII preparations of a very high purity obtained by immuno-affinity chromatography. The cost of treatment is always considered to be important, especially that of the immune tolerance treatment. The cost of FVIII preparations accounts for 80–90% of the total ITT costs (Colowick *et al.*, 2000). After a change of the FVIII preparation of a very high purity to the FVIII-VWF concentrate used for achieving tolerance in haemophilia A patients with FVIII antibodies, resulted in successful immune tolerance in several patients as reported by Kreuz and co-authors (Kreuz *et al.*, 1995b). According to the results obtained in our study, the screening of FVIII antibodies for their neutralization activity against FVIII and FVIII-VWF, the estimation of the amount of the FVIII light chain antibodies and the information about the functional activity of VWF in a particular preparation helps with the in optimization of treatment and especially with choosing the most appropriate FVIII preparation in order to achieve a better outcome.

7. CONCLUSIONS

The results of the present study allow us to conclude the following:

1. The von Willebrand factor complexed with FVIII is able to counteract the inhibition of the FVIII coagulation activity by antibodies that are targeted at the light chain of FVIII. The concentration of the FVIII-light-chain-specific antibodies determines to which extent the inhibition of the FVIII coagulation activity by these antibodies is reduced in the presence of VWF (FVIII-VWF complex) compared to FVIII itself.

2. Proteolytic degradation of VWF high-molecular-weight multimers in the FVIII preparation does not affect the protective effect on the FVIII:C activity by masking the epitopes on FVIII antibodies.

3. The administration of the FVIII-VWF concentrate for immune tolerance treatment led to the development of a unique pattern of FVIII antibody specificity: antibodies were targeted mainly at epitopes exposed on the surface of the FVIII-VWF complex. Factor VIII light chain antibodies (C1-domain-, C2-domain-specific) dominated during the whole treatment period, and these antibodies were unable to interfere with the FVIII binding to VWF.

4. The immune tolerance treatment caused no significant changes in epitope specificity, although the concentration of inhibitory and non-inhibitory antibodies decreased significantly in the course of treatment. The interruption of treatment resulted in an increased FVIII antibody production with different epitope specificity.

5. FVIII-specific IgG antibodies with inhibitory and non-inhibitory activity were mainly of the IgG4 subclass. A change in the IgG subclass distribution of FVIII antibodies, particularly an increase in the IgG1 subclass antibody levels was accompanied by a change in the production of FVIII antibodies with different epitope specificity or affinity.

6. The patient's antibodies that were targeted mainly at the light chain of FVIII were unable to interfere with the FVIII binding to PL or to VWF. However, these antibodies interfered with FXa generation by inhibiting the FX binding to FVIII, which is a novel so far undescribed mechanism of FVIII antibodies in inhibiting the FVIII:C activity.

7. The binding sites of FVIII for VWF and for FX within the C1 domain of the light chain are different, hence the patient's antibodies targeted mainly at the C1 domain of FVIII were able to interfere with the FVIII binding to FX, but unable to inhibit the FVIII binding to VWF. Therefore, the patient's antibodies were unable to compete for binding to FVIII with the C1-domain-specific monoclonal antibody 2E9 (University of Leuven, Belgium), which inhibits the FVIII binding to VWF.

8. The well-characterized monoclonal C2-domain-specific antibodies ESH4 (American Diagnostica, USA) and BO2C11 (University of Leuven, Belgium) in addition to their ability to interfere with the FVIII binding to PL and VWF, can also block the binding of FX to FVIII. This suggests that the FVIII-light-chain-specific antibodies can inhibit the FVIII coagulation activity by different mechanisms, and that the light chain of FVIII participates in several different interactions of FVIII with other components of the coagulation cascade.

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VIII HÜÜBIMISFAKTORI VASTASED ANTIKEHAD HEMOFIILIA A HAIGETEL

Kokkuvõte

Hemofiilia A on pärilik kaasasündinud vere hüübimishäire, mis on tingitud VIII hüübimisfaktori geeni mutatsioonist, põhjustades mittefunktsionaalse hüübimisfaktori sünteesi või täieliku puudumise. VIII hüübimisfaktori geen asub X kromosoomi pikas õlas ning seetõttu on haigus peamiselt meestel sagedusega 1:5000 (Larsson, 1984). Sõltuvalt VIII hüübimisfaktori koagulantsest aktiivsusest ja kliinilistest sümptomitest eristatakse rasket (FVIII koagulantne aktiivsus, FVIII:C 1 IU/dl), keskmist (FVIII:C 1–5 IU/dl) või kerget (5–40 IU/dl) hemofiilia A vormi. 35% raske hemofiilia A juhtumitest on põhjustatud inversioonist FVIII geeni 22 intronis, mis on kõige sagedasem geenidefekt (Antonarakis *et al.*, 1995). Spontaansete ja traumajärgsete veristuste ravi seisneb puuduva VIII hüübimisfaktori asendamises eksogeensega. Tänapäeval on kasutada nii plasmast fraktsioneeritud kui rekombinantse DNA meetodil valmistatud FVIII kontsentraadid, mille õigeaegne manustamine peatab verejooksu ja parandab tunduvalt haige elu kvaliteeti. Siiani on raskeimaks komplikatsiooniks VIII hüübimisfaktori manustamisel tema aktiivsust inhibeerivate antikehade teke ligi 25% hemofiilia A haigetest (Scharrer and Neutzling, 1993). Vastavate antikehade tekke põhjused on siiani teadmata, kuid nende olemasolul on veritsuse peatamine komplitseeritud. Antikehad VIII hüübimisfaktori vastu tekivad sagedamini just raske hemofiilia A korral (kuni 60%-l haigetest (Kemball-Cook and Tuddenham, 1997)), tavaliselt esimese 5 eluaasta jooksul ning 9–15 manustamispäeva järel (Scharrer and Neutzling, 1993). Faktor VIII antikehade tekke sageduse ja asendusteraapias kasutatava preparaadi puhtusastme vahel seni seost leitud ei ole (Scharrer and Neutzling, 1993), välja arvatud kahe kontsentraadi korral, mille tootmisprotsessis kasutati pastöriseerimist viiruste inaktiveerimiseks, mis seetõttu põhjustasid FVIII antikehade tekke ka eelnevalt ravitud haigetel (Laub, *et al.*, 1999; Peerlinck, *et al.*, 1997; Peerlinck *et al.*, 1993b). Tootmisprotsess võib mõjutada FVIII omadusi ja aktiivsust ning seetõttu on tähelepanu pööratud FVIII füsioloogilisele stabiliseerijale, von Willebrandi faktorile (VWF). Vereplasmas moodustab von Willebrandi faktor kompleksi VIII hüübimisfaktoriga, kaitstes teda enneaegse aktivatsiooni ja sellele järgneva inaktivatsiooni eest. VWF juuresolekul ei seondu FVIII membraani fosfolipiididega (veresoone vigastuskohal), samuti on inhibeeritud interaktsioon aktiveeritud IX hüübimisfaktoriga ning aktiveeritud X faktor ei saa lõhustada ega aktiveerida VIII hüübimisfaktorit. Samal ajal VWF aga ei takista FVIII aktiveerimist trombiiniga, millele järgnevad FVIII molekuli konformatsioonilised muutused võimaldavad mitmesuguseid interaktsioone, mis omakorda kiirendavad trombiini teket ja stabiilse fibriinivõrgustiku moodustumist vigastuskohale. Faktor VIII juuresolekul on FX generatsioon 500 korda

efektiivsem kui selle puudumisel (Mertens *et al.*, 1985). Aminohapete homoloogia põhjal saab VIII hüübimisfaktoris eristada järgmisi domeene: A1-a1-A2-a2-B-a3-A3-C1-C2. FVIII antikehad tekivad kõige sagedamini A2 domeeni vastu raskes ahelas (A1-A2-B) ja C2 domeeni vastu kerges ahelas (A3-C1-C2) (Scandella, *et al.*, 2001). Antikehi, mis seonduvad FVIII molekuli A1 domeeni või A3-C1 domeeniga on kirjeldatud harva (Palmer *et al.*, 1997; Prescott *et al.*, 1997; Shibata *et al.*, 2000). Von Willebrandi faktori seondumiskoht asub FVIII kerges ahelas, C2 domeenis ja a3 domeenis (Saenko and Scandella, 1997; Saenko *et al.*, 1994). Seetõttu inhibeerivad vastavad antikehad FVIII:C aktiivsust, takistades FVIII seondumist VWF-ga ja/või fosfolipiididega (Saenko and Scandella, 1995; Shima *et al.*, 1993). Võiks arvata, et FVIII kontsentraadis olev VWF on võimeline neutraliseerima FVIII antikehade inhibeerivat toimet ning madala antikehade tiitri korral oleks hea kasutada veritsuse peatamiseks haigel eelkõige FVIII-VWF kompleksi sisaldavaid kontsentraate. Immuuntolerantsuse kujundamisel on selliseid kontsentraate edukalt rakendatud (Scharrer *et al.*, 1999), samal ajal on olnud tulemuslik ka rekombinantsete preparaatide kasutamine, mis ei sisalda VWF (Batlle *et al.*, 1999; Rothschild *et al.*, 1998). On oluline märkida, et seni ebaefektiivse immuuntolerantsuse ravi jätkamine neljal hemofiilia A haigel VWF-i sisaldava kontsentraadiga andis hea tulemuse (Kreuz *et al.*, 1995b). Sellest võib järeldada, et ravi efektiivsus sõltub oluliselt kasutatavast FVIII preparaadist ja sellest, millise FVIII piirkonna vastu on haige antikehad tekkinud.

Uurimuse eesmärgid

Lähtuvalt eespool toodust olid uurimuse esmärgid järgmised.

- Uurida, kas VWF on võimeline kaitsma VIII hüübimisfaktori aktiivsust antikehade inhibeeriva toime eest *in vitro*.
- Iseloomustada VIII hüübimisfaktori vastaste antikehade epitoobispetsiifilisust ja IgG subklasse ning muutusi immuuntolerantsuse ravi käigus hemofiilia A haigel, keda raviti FVIII-VWF kontsentraaadiga.
- Uurida FVIII antikehade toimemehhanismi FVIII koagulantse aktiivsuse inhibeerimisel.
- Iseloomustada VWF funktsionaalset aktiivsust FVIII kontsentraatides, mida kasutati *in vitro* ja *in vivo* uurimustes.

Uuritavad ja meetodid

FVIII antikehade inhibeerivat toimet VWF juuresolekul uuriti hemofiilia A haigete plasmaproovides (n=12), mis sisaldasid FVIII kerge ja raske ahela vastaseid antikehi. Antikehade epitoobispetsiifilisust ja selle võimalikke

muutusi uuriti plasmaproovides, mis olid võetud hemofiilia A haigelt immuuntolerantsuse ravi jooksul. FVIII antikehade sisalduse ja omaduste iseloomustamiseks plasmaproovides kasutati Bethesda meetodit ja immunoloogilisi meetodeid. Antikehade epitoobispetsiifilisuse määramiseks tarvitati rekombinantseid FVIII raske ja kerge ahela fragmente ning GST-liitvalke FVIII erinevate domeenidega (A1, A2, a1, a3, A3, C1, C2) ja meetodina konkureerivat seondumist monoklonaalsete FVIII antikehadega, mille seondumiskoht FVIII molekulis oli teada. Antikehade toimemehhanismi uurimisel rakendati ELISA meetodit (FVIII seondumine VWF-le või fosfolipiididele) ja kromogeenset meetodit FXa hulga määramiseks. Von Willebrandi faktori iseloomustamiseks FVIII kontsentraatides määrati von Willebrandi faktori antigeeni sisaldus (ELISA), kollageeniga seondumise võime (ELISA), multimeersuse aste (*Western blot*) ja võime indutseerida trombotsüütide agregatsiooni antibiootikum ristotsetiini juuresolekul (agregomeetriline meetod).

Uurimuse tulemused

Uurimuse tulemusel ilmnes, et VWF juuresolekul inhibeerisid antikehad FVIII:C aktiivsust vähem kui VWF puudumisel. Eriti märgatav erinevus ilmnes siis, kui plasmaproovis olid peamiselt antikehad, mis seondusid FVIII kerge ahelaga. Kui plasmaproov sisaldas aga peamiselt FVIII raske ahelaga seonduvaid antikehi, siis oli erinevus minimaalne või puudus. Seega võis *in vitro* tulemuste põhjal eeldada, et haigel, kellel on dominantsed FVIII kerge ahela spetsiifilised antikehad, saab kasutada veritsuse peatamiseks FVIII-VWF kompleksi sisaldavat kontsentraati.

In vitro katses kasutatud FVIII kontsentraadis VWF funktsionaalse aktiivsuse iseloomustamisel ilmnes, et selles puudusid VWF kõrge molekulmassiga multimeerid ning VWF antigeeni sisaldus oli tunduvalt suurem kui von Willebrandi faktori funktsionaalne aktiivsus. Samal ajal olid ka keskmise ja madala molekulmassiga VWF-i multimeerid võimelised stabiliseerima VIII hüübimisfaktorit ja vähendama antikehade inhibeerivat toimet FVIII koagulantsele aktiivsusele.

Sama preparaadi manustamisel hemofiilia A haigele immuuntolerantsuse kujundamiseks ilmnes, et kogu ravi jooksul domineerisid vastupidiselt oodatule FVIII kerge ahela spetsiifilised antikehad. Põhimõtteliselt oleks VWF pidanud katma ja kaitsma epitoobid FVIII kerges ahelas ning seega ka reguleerima FVIII kerge ahela vastaste antikehade produktsiooni B-rakkude poolt, kuna need epitoobid ei ole FVIII-VWF kompleksis eksponeeritud. Täpsemal uurimisel selgus, et FVIII antikehad olid C1, C2, A2, A1 domeenide spetsiifilised, kusjuures C1-spetsiifiliste antikehade kontsentratsioon oli tunduvalt kõrgem kui teiste domeenide vastastel antikehadel. Ravi käigus vähenes nii FVIII:C aktiivsust inhibeerivate kui ka mitteinhibeerivate omadustega FVIII antikehade kontsentratsioon, kuigi ei ilmnenud olulisi muutusi epitoobispetsiifilisuses. Ravi

katkestamisel ja uuesti jätkamisel aga suurenes FVIII antikehade produktsioon, sealhulgas ka C2-domeeni spetsiifiliste antikehade kontsentratsioon, kuid vaatamata sellele jäi C1-domeeni spetsiifiliste antikehade kontsentratsioon kõrgemaks. Kuigi haige antikehad olid suunatud peamiselt FVIII kerge ahela vastu, ei olnud ühegi antikeha seondumiskoht niisuguses piirkonnas, mis oleks takistanud VWF seondumist FVIII-ga, või oli selliste antikehade kontsentratsioon märgatava mõju avaldamiseks liiga madal. Seega olid epitoobid peamiselt FVIII-VWF kompleksi pinnal. Uurides FVIII antikehade IgG jaotust subklasside järgi, selgus, et enamus nii FVIII kui ka FVIII kerge ahela spetsiifilistest antikehadest oli IgG4 klassi. Ravi jooksul detekteeriti IgG1 klassi antikehade kontsentratsiooni suurenemine kahel ajahetkel ning samades proovides ilmes ka FVIII antikehade produktsiooni muutus.

Immuuntolererantsuse ravi käigus tekkinud antikehade inhibeeriva toime uurimisel selgus, et kuigi enamus antikehi olid FVIII kerge ahela spetsiifilised, ei blokeerinud need FVIII seondumist von Willebrandi faktoriga ega fosfolipiididega. Samal ajal olid haige antikehad võimelised inhibeerima FXa generatsiooni, takistades FX seondumist FVIII-ga. Sellist antikehade toimemehhanismi FVIII:C aktiivsuse inhibeerimisel ei ole eelnevalt kirjeldatud.

Antud mehhanismi edasisel uurimisel leidsime, et FVIII C2-domeenispetsiifilised monoklonaalsed antikehad BO2C11 ja ESH4 on samuti võimelised seonduma FVIII-ga nii, et selle interaktsioon FX-ga on välistatud. Mõlemad ülalnimetatud monoklonaalsed antikehad inhibeerivad ka FVIII interaktsiooni VWF-ga ja fosfolipiididega (Scandella *et al.*, 1995a; Spiegel *et al.*, 2001).

C1-domeeni tähtsusest FVIII aktiivsusele on praeguseks vähe teada. Antud uurimuse tulemused näitavad, et FX seondumiskoht C1-domeenis erineb VWF seondumiskohast, sest haige C1-spetsiifilised antikehad ei konkureerinud monoklonaalse C1-domeeni-spetsiifilise antikehaga, mis on võimeline blokeerima FVIII seondumist VWF-ga (Jacquemin *et al.*, 2000).

Kokkuvõttes võib järeldada, et FVIII kerge ahela antikehad on võimelised inhibeerima FVIII koagulantset aktiivsust erinevalt, kusjuures von Willebrandi faktoriga seondumise takistamine on mitmetest võimalustest ainult üks. FVIII antikehade inhibeeriva toime erinevuse määramine FVIII ja FVIII-VWF kompleksi suhtes ning FVIII kerge ahela vastaste antikehade kontsentratsiooni hindamine võimaldab valida sobivaima ja efektiivseima FVIII kontsentraadi haige veritsuste raviks või immuuntolerantsuse kujundamiseks, võimaldades suurendada ravi efektiivsust ja vähendada ravi kulusid.

ACKNOWLEDGEMENTS

The present study was carried out at the Department of Pharmacy, Institute of Cell and Molecular Biology of the University of Tartu and at the Children's Hospital of the Tartu University Clinicum. The work was supported by the Estonian Science Foundation (Grants No 0413 and 2137).

I am greatly indebted to so many people, who have contributed in one way or another to this thesis.

- My warmest gratitude goes to my teachers Prof. Hele Everaus and Ass. Prof. Toivo Hinrikus for their support and advice during all these years.
- I wish to express my deepest gratitude to Dr. Margus Pooga for his excellent supervision, encouragement, inspiration, and for being beside me whenever his advice was needed.
- My special thanks are expressed to Tiit Talpsep for a wonderful opportunity to work with him, for his support and endless discussions including those about research.
- I wish to express my sincerest thanks to Dr. Erik Berntorp for an opportunity to improve my knowledge and experience in coagulation and for encouraging my research and allowing me to work at the Coagulation Laboratory and with the research projects in Malmö.
- I am also thankful to Dr. Stephan Lethagen for introducing me the interesting world of the von Willebrand factor, to Dr. Jan Astermark for valuable discussions, and to Maj Ekman and Margoth Gunnarsson and all the staff at the Coagulation Laboratory and at the Wallenberg Laboratory for their hospitality and friendship during my stay in Malmö.
- I sincerely thank Dr. Rolf Ljung and Elsy Sjörin for performing the DNA analyses of the haemophilia A patients.
- My deepest gratitude belongs to Dr. Jean-Marie Saint-Remy for his guidance, enjoyable discussions full of grandiose ideas, for providing us with monoclonal FVIII antibodies, and for an opportunity to work at the Center of Molecular and Vascular Biology at Leuven.
- I also thank Dr. Marc Jacquemin, Abdellah Benhida and Renaud Lavenhomme for their invaluable collaboration, hospitality and friendly attitude during my stay in Leuven.
- I am grateful to Prof. Raivo Uibo for revision and constructive comments on manuscript.
- I express my gratitude to all my colleagues at the Department of Pharmacy for their patience and support.

- I am grateful to Prof. Peep Veski, head of the department, for enjoyable discussions and assistance, whenever financial support for research was needed.
- I am thankful to Sulev, Arvi, Viljar, Ilvi and all others at the Institute of Cell and Molecular Biology for a friendly and inspiring research atmosphere, and Prof. Toivo Maimets for an opportunity to work at his laboratory.
- My sincere thanks go to all the patients, without whose participation the research could not have been carried out.
- I thank the staff of the Children's Hospital of the Tartu University Clinicum, especially Anu Saag, Siiri Telling, Dr. Ene Luik for taking care of the patients and Dr. Ain Kaare for on opportunity to work at his department and Dr. Sam Schulman for his enthusiasm to share his experience in coagulation with the colleagues in Tartu in the framework of collaboration between haemophilia centers of the Children's Hospital of the Tartu University Clinicum and Karolinska Hospital in Sweden.
- I thank Dr. Mirella Ezban for providing the recombinant light chain and heavy chain of FVIII, and Peeter Toomik for providing the chromogenic substrates which enabled us to carry out numerous fantastic experiments.
- I sincerely thank Ele Kaldjärve, who skilfully revised the English of the thesis.
- I also thank companies Baxter (USA) and Biotest (Germany) for providing FVIII preparations for *in vitro* analyses.
- My special thanks go to my family, my mother and my brother for their care and support.
- Finally, I would like to thank my financial supporters for their scholarships, namely

  The University of Tartu
  The Baxter Grant for Coagulation Research in the Nordic countries
  Liisa Kolumbus Foundation.

PUBLICATIONS

I

Kallas A, Talpsep T.
Von Willebrand factor in factor VIII concentrates protects against neutralization by factor VIII antibodies of haemophilia A patients.
Haemophilia 2001; 7 (4): 375–380

Haemophilia (2001), 7, 375–380

von Willebrand factor in factor VIII concentrates protects against neutralization by factor VIII antibodies of haemophilia A patients

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Summary. We investigated the neutralization activity of factor VIII (FVIII) antibodies of 12 haemophilia A patients, acquired during treatment with plasma-derived FVIII concentrates. All plasma samples, drawn in a clinically stable situation before any immunotolerance treatment, contained anti-A2 domain and anti-light-chain FVIII antibodies. In nine patients' plasmas, containing relatively high amounts of FVIII light-chain antibodies (53–96%), a higher neutralization activity was found against recombinant FVIII concentrate (Recombinate) than against plasma-derived von Willebrand factor (vWF)-containing concentrate (Haemoctin SDH). No difference in neutralization of the two concentrates was found in two patients' plasmas with almost equal content of FVIII light- and heavy-chain antibodies, or one plasma with predominantly heavy-chain antibodies. These results suggest that haemophilia A patients with relatively high amounts of FVIII light-chain antibodies in plasma might benefit by infusion of FVIII concentrates containing vWF because vWF appears to have some protective effect on FVIII. This hypothesis should be tested by a clinical study.

Keywords: factor VIII antibody, factor VIII, haemophilia A, von Willebrand factor.

Introduction

Factor VIII (FVIII) circulates in plasma as a heterodimer composed of a heavy chain (the domains A1–A2–B) and a light chain (domains A3–C1–C2) [1]. Non-covalent complexing with vWF protects FVIII from dissociation and from interaction with phospholipids and thrombin-activated platelets [2]. The half-life of infused FVIII depends on the circulating level of vWF [3]. Haemophilia A patients with blood group O have lower vWF antigen and activity levels and a shorter half-life of administered FVIII [4]. Plasma-derived FVIII concentrates, which are used for replacement therapy of haemophilia A patients, differ in purity and vWF content. Development of FVIII antibodies (inhibitors) in 5–25% of haemophilia A patients is the most serious consequence of replacement therapy [5]. Patients treated with plasma-derived FVIII concentrates (pdFVIII) are more prone to develop antibodies against the heavy-chain A2 domain and the light chain domains, A3, C1 and C2, while patients treated with recombinant FVIII are more likely to produce antibodies against the A2 and C2 domains [6]. The vWF binding site is located within the light chain of FVIII [1] therefore vWF may protect FVIII from neutralization by antibodies recognizing the epitopes in the light chain. The aim of our study was to investigate the protective effect of vWF on FVIII binding and neutralization by the plasmas of 12 haemophilia A patients with inhibitors, in relation to the distribution of antibodies against the most common epitopes of FVIII.

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Accepted 22 March 2001

Materials and methods

Investigated FVIII antibodies

Plasma samples from 12 haemophilia A patients with FVIII antibodies were included to the study. Anti-FVIII antibodies had developed in all the patients in response to on-demand treatment with plasma-derived FVIII concentrates. Plasma samples were

drawn in a clinically stable situation before any immunotolerance treatment. The study protocol was approved by the Ethics Committee of the University of Tartu, Estonia. Plasma samples contained 1–300 Bethesda units (BU) mL^{-1} of FVIII antibodies. All investigated FVIII antibodies reacted with the A2 domain of the FVIII heavy chain cleaved by thrombin (Sigma Chemical Co., St Louis, MO, USA) and the FVIII light chain, detected by Western blotting using plasma-derived FVIII concentrate (Haemoctin SDH; Biotest, Dreieich, Germany) and recombinant FVIII fragments (FVIII light chain, 6318 IU mL^{-1}, and heavy chain, 7366 IU mL^{-1}; kindly provided by Dr Mirella Ezban from Novo Nordisk, Bagsuaerd, Denmark). All plasma samples contained antibodies preventing FVIII interactions with vWF and phosphatidylserine as measured by ELISA assays previously described by Shima and coworkers [7]. FVIII antibodies of all plasma samples competed with murine monoclonal antibody ESH4 (American Diagnostica, Greenwich, CT, USA) for the phospholipid binding site of the C-terminus of the light chain of FVIII, as detected by ELISA.

Neutralization assay in the presence and absence of vWF

Recombinant FVIII concentrate (Recombinate; Baxter, USA; specific activity > 4000 IU mg^{-1} of protein) and plasma-derived concentrate (Haemoctin; specific activity 100 IU mL^{-1} of protein) were used in the neutralization experiments. Haemoctin SDH vials with labelled potency of 500 IU contained 58.3 ± 3.9 IU mL^{-1} of FVIII:C, 31.8 ± 4.6 IU mL^{-1} of vWF:Ag, 12.9 ± 2.9 IU mL^{-1} of RCof, 8.5 ± 1.9 IU mL^{-1} of CBA, after dissolving.

Both concentrates were diluted to 1 IU mL^{-1} of FVIII:C with Tris-buffered saline (TBS; 20 $mmoL^{-1}$ Tris-HCl, 150 $mmoL^{-1}$ NaCl; pH 7.2) containing 1% bovine serum albumin (BSA) and incubated with an equal volume of serially diluted (1 : 2 to 1 : 1000 in TBS containing 1% BSA) FVIII antibody plasma sample for 1 h at 37 °C. The maximum neutralization of FVIII:C activity was achieved after 1 h. Residual FVIII:C was measured by a chromogenic test according to the manufacturer's instructions (Coatest FVIII; Chromogenix Instrumentation Laboratory, SpA, Milan, Italy) on a 96-well microplate (Micro-Well™; Nunc, Roskilde, Denmark). Percentage neutralization was calculated relative to a control. The control had the test plasma sample replaced by plasma from a CRM-negative haemophilia A patient without FVIII antibodies. The test plasma sample dilution that gave 50% neutralization of FVIII:C activity was found using both FVIII concentrates. The results were used to calculate a ratio of 50% neutralization of FVIII:C with rFVIII to that with FVIII-vWF.

Distribution of FVIII light-chain and heavy-chain neutralizing antibodies measured by an ELISA assay

Microplates (PolySorp™; Nunc) were coated with rFVIII (Recombinate) diluted to 8 IU mL^{-1} in carbonate buffer (50 $mmoL^{-1}$ sodium carbonate–bicarbonate buffer; pH 9.6) and incubated overnight at 2–8 °C. Prior to using, the wells of microplate were blocked with TBS containing 3% BSA for 1 h at room temperature and washed with TBS–Tween (0.05% Tween 20). Plasma samples with FVIII antibodies were diluted to 4 BU mL^{-1}, except plasma samples no. 9 and 10, which were used undiluted. The samples were incubated with an equal volume of serially diluted recombinant FVIII fragments, from 0.2 to 12.6 IU mL^{-1} according to labelled activity, for 1 h at 37 °C. After incubation, aliquots were removed and added to the wells of the microplate and incubated for 2 h at 37 °C. Bound FVIII antibodies were detected using rabbit antihuman IgG conjugated with alkaline phosphatase (AP) diluted 1 : 1000 (Dako, Glostrup, Denmark). The reaction was visualized with the AP substrate *p*-nitrophenyl phosphate (pNPP; Sigma) and the colour change was read at 405 nm. Two controls were included with each test: one mixture that had the antibody plasma sample replaced by buffer and a second mixture that used buffer instead of the recombinant FVIII fragments. The percentage neutralization was calculated as follows:

$$100 - [(\text{binding with fragment} - \text{minimum binding}) / (\text{maximum binding} - \text{minimum binding})] \times 100.$$

The maximum binding measurement was obtained from the incubation in the microplate of the antibody sample and buffer without recombinant fragments. Minimum binding was measured from the incubation of buffer alone. The plateau was defined as the point beyond which increasing the FVIII fragment concentrations did not lead to a greater neutralization of FVIII antibodies. When FVIII antibodies were partially neutralized by recombinant fragments, all values within the plateau region were averaged.

Distribution of FVIII light-chain and heavy-chain neutralizing antibodies measured by a chromogenic method

Factor VIII antibody plasma samples were diluted to a neutralization activity of 4 BU mL^{-1}, except

plasma samples no. 9 and 10, which were used undiluted, and incubated with an equal volume of serially diluted recombinant FVIII fragments as described in the previous assay. An aliquot was removed for measuring residual FVIII antibodies using an ELISA assay. To the remaining mixture, an equal volume of rFVIII (Recombinate), diluted to 2 IU mL^{-1} was added and incubated for 1 h at 37 °C. Residual FVIII:C activity was measured by the chromogenic method. Percentage neutralization was calculated using the above-mentioned formula, but binding capacity was substituted with FVIII:C activity. Maximum FVIII:C activity was measured from the mixture of recombinant fragments and rFVIII without antibody sample, incubated in the same conditions. If the tested plasma contained an FVIII antibody level < 10 BU mL^{-1}, the plasma sample from the CRM-negative haemophilia A patient without FVIII antibodies was used in the mixture to obtain the maximum FVIII:C activity. Minimum FVIII:C activity was found for each test sample incubated with rFVIII without any competitive FVIII fragments. The plateau value was similarly measured as in the previous assay.

Statistical analysis

Calculation of correlation was based on the Spearman rank order correlation coefficient, with $P < 0.05$ considered significant.

Results

Two groups were identified with respect to the relative amount of FVIII light-chain antibodies among the heterogeneous mixture of antibodies in the patients' plasmas, and to the neutralization of FVIII in the presence and the absence of vWF. The first group, consisting of nine plasma samples (samples no. 1–9 in Table 1), had significantly lower neutralization activity when exposed to FVIII containing vWF (FVIII-vWF; Fig. 1A). The ratios of 50% neutralization with rFVIII over that with FVIII-vWF ranged from 1.5 to 18.3 (mean 6.8). The group 1 samples contained higher amounts of recombinant FVIII light-chain neutralizing antibodies (53–96%; Fig. 2A). Three samples (nos. 10–12) belonged to the second group with no difference in neutralization of FVIII:C exposed to FVIII or FVIII-vWF (mean ratio 1.0; Fig. 1B). Two plasma samples contained equal amounts of antibodies neutralized by rLCh and rHCh (Fig. 2B) and one sample (no. 12) contained a higher amount of recombinant FVIII heavy-chain neutralizing antibodies. Figure 3 illustrates the

Table 1. Characterization of investigated FVIII antibodies.

Plasma sample	FVIII antibodies		Neutralization of rFVIII and FVIII-vWF			Distribution of antibodies against recombinant light chain (rLCh) and heavy chain (rHCh)					
	Activity (BU mL^{-1})	Epitope specificity	rFVIII ID 50%	FVIII-vWF ID 50%	Ratio rFVIII/ FVIII-vWF	ELISA			FVIII:C		
						rLCh (%)	rHCh (%)	Ratio rLCh/rHCh	rLCh (%)	rHCh (%)	Ratio rLCh/rHCh
Group 1											
1	140	A2, LCh	750	150	5	96	7	13.7	53	8	6.6
2	9	A2, LCh	9	2	4.5	100	7	14.3	78	30	2.6
3	5	A2, LCh	27.5	1.5	18.3	97	25	3.9	82	42	1.9
4	7	A2, LCh	25	1.5	16.7	92	20	4.6	93	3.5	26.6
5	8	A2, LCh	5	2	2.5	62	20	3.1	75	27	2.8
6	37	A2, LCh	30	8	3.7	93	58	1.6	82	21	3.9
7	300	A2, LCh	265	110	2.5	90	7	12.8	95	7	13.6
8	280	A2, LCh	350	50	7	95	6	15.8	96	6	16
9	4	A2, LCh	3	2	1.5	90	3	30	83	15	5.5
Group 2											
10	1	A2, LCh	0.7	0.7	1	n.d.	n.d.	n.d.	21	21	1
11	5	A2, LCh	2	2	1	70	74	0.9	64	65	1
12	150	A2, LCh	28	23	1.2	18	94	0.2	26	74	0.4

n.d., not determined; ID50%, dilution of plasma sample neutralizing 50% of FVIII:C activity.

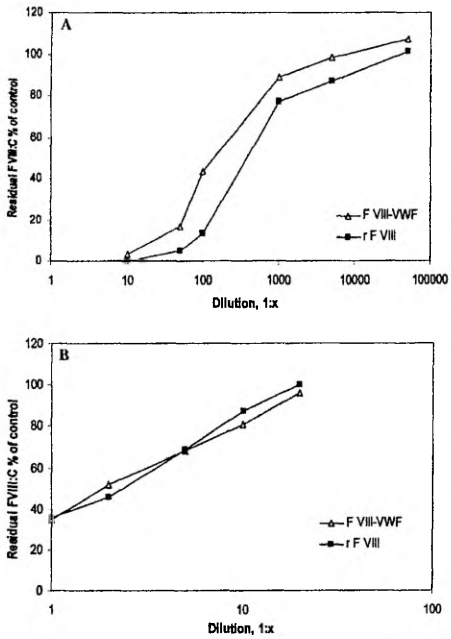

Fig. 1. Neutralization of FVIII:C activity by FVIII antibodies from plasma sample no. 1 (A) and plasma sample 11 (B) in the presence and absence of vWF. Plasma dilutions were incubated with recombinant FVIII concentrate (rFVIII; Recombinate, Baxter) and FVIII concentrate containing vWF (FVIII-vWF; Haemoctin SDH, Biotest). The residual FVIII:C activity was measured by chromogenic method. Each datapoint represents the mean of two individual experiments.

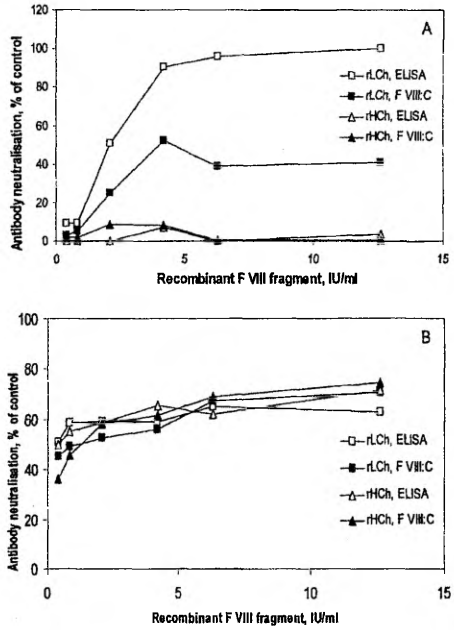

Fig. 2. Neutralization of FVIII antibodies from plasma sample no. 1 (A) and plasma sample 11 (B) by recombinant FVIII light chain (rLCh) and heavy chain (rHCh). Plasma sample was diluted to 4 BU mL^{-1} and incubated with increasing concentrations of rLCh and rHCh. The neutralization of antibodies was measured by an ELISA assay (ELISA) and a chromogenic test (FVIII:C) as described in 'Materials and methods'. Each datapoint represents the mean of at least two individual experiments.

correlation between the distribution of rLCh-neutralizing antibodies relative to rHCh antibodies measured by the chromogenic method (ratio rLCh to rHCh) and FVIII neutralization in the absence or presence of vWF (ratio rFVIII to FVIII-vWF) for all investigated samples (Spearman analysis $r = 0.59$; $P = 0.04$).

Discussion

We studied the effect of vWF on neutralization of FVIII by plasma antibodies. All studied plasma samples, collected from haemophilia A patients, contained FVIII antibodies with reactivity against the light chain and the A2 heavy chain domain of FVIII. Plasma-derived FVIII concentrate with vWF was significantly less neutralized by most plasma samples than recombinant FVIII concentrate. The extent of neutralization correlated with the relative amount of FVIII light-chain antibodies in a patient's plasma. A lower neutralization of FVIII coagulation activity in the presence of vWF was associated with a higher amount of FVIII light-chain antibodies compared to FVIII heavy-chain antibodies (Fig. 3).

The interaction between FVIII and vWF has been studied extensively. vWF binds to different domains of the FVIII molecule. Antibodies reacting with epitopes within the C1 [8] and C2 [7] domains, or the acidic region A3 in the light chain of FVIII, inhibit the binding of FVIII to vWF. As only the intact light chain has the maximum vWF binding affinity [2], we used the recombinant full-length FVIII light chain in neutralization experiments to cover the whole panel of plasma antibodies interfering with FVIII–vWF interaction.

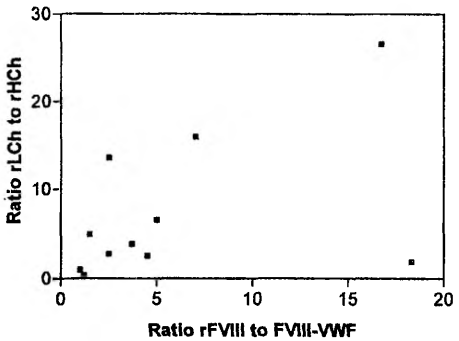

Fig. 3. The relationship between neutralization activity of FVIII antibodies in the absence and in the presence of vWF (ratio rFVIII to FVIII-vWF) and the distribution of recombinant FVIII light-chain (rLCh) neutralizing antibodies compared to recombinant heavy-chain (rHCh) neutralizing antibodies measured by a chromogenic test (ratio rLCh to rHCh).

The different reactivity of FVIII antibodies against a panel of FVIII concentrates with varying levels of purity has been described [9,10]. Plasma antibodies with FVIII C2 domain specificity from haemophilia A patients possess a lower ability to neutralize FVIII, if it is in complex with vWF [11]. FVIII antibodies investigated in our study developed as a response to treatment with pdFVIII concentrates, varying in their content of functional vWF. Compared to rFVIII and recombinant fragments, factor VIII, when complexed with vWF, expresses a significantly different number of epitopes, especially conformational epitopes. The epitope specificity of FVIII antibodies was detected in reduced and unreduced conditions by Western blotting. The same antibody-binding pattern was observed using plasma-derived FVIII concentrate or recombinant light- and heavy-chain fragments. Antibody binding was not altered because of the linear structure of epitopes. As epitopes are presented in pdFVIII and in recombinant fragments, von Willebrand factor, having occupied the binding sites in the light chain of FVIII, can block epitopes on the light chain of FVIII or interfere with antibody binding by steric hindrance.

The limitation of our study is the small number of investigated plasma samples. Only two samples out of 12 contained an almost equal amount of FVIII antibodies against the light and heavy chains and one plasma sample contained predominantly FVIII heavy-chain antibodies. However, these results agree with those reported by Scandella and coworkers, who found that two out of 10 plasma samples investigated by an immunoprecipitation method [12] had an almost equal amount of antibodies neutralizing the A2 heavy chain domain and the light chain of FVIII. Apparently, antibodies with almost equal relative contribution to binding activity and neutralization activity are uncommon.

The data presented here represent a situation at one timepoint. However, the composition of antibodies can vary over time and the epitope specificity of antibodies can change due to treatment or independently [13].

The results of our *in vitro* study suggest that a screening of the neutralizing activity of plasma antibodies before treatment might be useful in determining the selection of FVIII concentrate for treatment. These findings might have implications for the prediction of a better response of FVIII–vWF concentrate and thus contribute to lower treatment costs. Further experiments and clinical studies should establish the correlation between *in vitro* and *in vivo* observations.

Acknowledgements

We wish to thank the haemophilia centres in Malmö (Sweden) and in Tartu (Estonia) for providing plasma samples of haemophilia A patients. We also gratefully acknowledge Professor Rainer Seitz for critical reading of the manuscript. The study was in part supported by a Nordic coagulation grant.

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Kallas A, Pooga M, Benhida A, Jacquemin M, Saint-Remy J-M.
Epitope specificity of anti-FVIII antibodies during
immune tolerance therapy with FVIII preparation containing
von Willebrand factor.
Submitted for publication

EPITOPE SPECIFICITY OF ANTI-FVIII ANTIBODIES DURING IMMUNE TOLERANCE THERAPY WITH FVIII PREPARATION CONTAINING VON WILLEBRAND FACTOR

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Summary

The study aimed at characterizing the putative changes in the epitope specificity of anti-FVIII antibodies during a successful immune tolerance treatment of the haemophilia A patient with the factor VIII (FVIII) preparation containing the von Willebrand factor (VWF). At the beginning of treatment, anti-FVIII inhibitory antibodies recognizing predominantly the light chain of FVIII were prevalent and persisted throughout treatment. More detailed characterization of the FVIII antibody epitope specificity by using GST-fusion proteins corresponding to different FVIII domains revealed the prevalence of C1-domain-specific antibodies, while a remarkably lower amount of antibodies were targeted at the C2 and the a3 domains of the FVIII light chain and towards the A2 and the A1 domain of the FVIII heavy chain. The epitope specificity of antibodies remained rather unchanged throughout treatment except the elevated level of C2-domain-specific FVIII antibodies after a temporary interruption of treatment. The patient's antibodies were unable to interfere with the FVIII binding to VWF or to phospholipids, but inhibited FXa generation and the binding of FX to FVIII on the phospholipid monolayer. Thus, a unique pattern of the epitope specificity of FVIII antibodies and the mechanism to inhibit FVIII:C activity by FVIII-light-chain-specific antibodies were characterized. The uncommon epitope distribution of FVIII antibodies of this patient was probably caused by the administration of the FVIII preparation containing VWF.

Keywords: Factor VIII antibody, Factor VIII, haemophilia A, immune tolerance

Introduction

Defects or malproduction of factor VIII (FVIII) in haemophilia A patients causes bleedings, which are treated by the administration of functional FVIII. The most common serious complication of substitution therapy is the development of antibodies against exogenous FVIII in up to 20% of haemophilia A patients (Hoyer, 1995). It is generally accepted that patients with a certain defect in the FVIII gene have a higher risk of the FVIII antibody response to occur (Schwaab *et al.*, 1995). According to the updated database, up to 60% of haemophilia A patients carrying an inversion in intron 22 and 38% of patients with large deletions in the FVIII gene have an elevated FVIII antibody response (Kemball-Cook *et al.*, 1998). Different point mutations usually yield a moderate or mild form of the disease and are accompanied by a lower incidence of FVIII antibodies.

A large number of studies have been focused on the FVIII antibody development in patients treated with FVIII preparations of different purity. However, a higher purity of plasma-derived preparations and the application of the recombinant DNA technology for FVIII production have not been accompanied by any significant change in the incidence of FVIII antibody formation (Scharrer *et al.*, 1999). Two preparations produced by using treatment with a solvent-detergent and heat pasteurization for virus inactivation have been exceptions causing a remarkably higher antibody response, however, neither of these is in use any more (Laub *et al.*, 1999; Peerlinck *et al.*, 1997).

The FVIII molecule consists of a series of domains A1-a1-A2-a2-B-a3-A3-C1-C2 serving as a basis for internal sequence homology (Lenting *et al.*, 1998). Normal processing yields the FVIII heterodimer, comprising the heavy chain (A1-a1-A2-a2-B) and the light chain (a3-A3-C1-C2), circulating as a non-covalent complex with the von Willebrand factor (VWF). FVIII survival depends largely on the presence of VWF. The von Willebrand factor protects FVIII from premature activation and also from inactivation by proteolytic degradation. The VWF binding sites are located within the a3 domain and the C2 domain of the light chain of FVIII (Saenko and Scandella, 1997). Up to 68% of FVIII antibodies developed in the patients treated with plasma-derived FVIII preparations recognize epitopes within the A2 domain and the light chain of FVIII (Prescott *et al.*, 1997; Scandella *et al.*, 2001). The FVIII-light-chain-specific antibodies possess a lower inhibitory activity towards FVIII in the complex with VWF than towards FVIII itself as shown by *in vitro* experiments (Gensana *et al.*, 2001; Kallas and Talpsep, 2001; Suzuki *et al.*, 1996). The change of the FVIII preparation to the one containing VWF has resulted in a beneficial suppression of FVIII antibodies in the immune tolerance treatment (ITT) of the patients (Kreuz *et al.*, 1995). Among various immune tolerance therapy protocols the administration of high doses of FVIII preparations is one of the strategies to reduce the FVIII antibody level (Berntorp *et al.*, 1996). Here we present a case, where the FVIII preparation containing VWF was used for

the treatment of a patient with high titer antibodies targeted at the FVIII light chain and the heavy chain. The FVIII light chain antibodies in the studied patient were mainly C1-domain-specific. The patient's antibodies were unable to interfere with the FVIII binding to VWF or with the FVIII binding to phosphatidylserine (PS), but they were able to inhibit FXa generation by interfering the FX binding to FVIII.

Materials and methods

Reagents

Recombinant FVIII (rFVIII, Recombinate™ and albumin-free rFVIII, provided by Baxter, Glendale, CA, for laboratory use only) was used throughout the study. Recombinant FVIII fragments (the recombinant heavy chain, rHCh, and the light chain, rLCh) were kindly provided by Dr. Mirella Ezban (Novo Nordisk, Denmark). Bovine serum albumin (BSA), casein, L-(-phosphatidyl)-l-serine, p-nitrophenylphosphate (pNPP), and avidin-peroxidase were purchased from Sigma (Sigma Chemicals, St. Louis, MO, USA), biotin derivate (EZ Link sulfo-NHS-LC-biotin) from Pierce company (Rockford, IL, USA), and substrate o-phenyldiaminedihydrochloride (OPD) from Dako (Glostrup, Denmark). The test kit for measuring FVIII:C activity (Coatest FVIII:C), purified bovine FX, factor IXa with factor X (FIXa+FX reagent), phospholipid emulsion, Russel's viper venom and substrates S-2288 and S-2337 were purchased from the Chromogenix Instrumentation Laboratory SpA (Milano, Italy). The *in vitro* assays were carried out in Tris-buffered saline (TBS, 0.02 M Tris, 0,15 M NaCl, pH 7.2), phosphate-buffered saline (PBS, 0.08 M, pH 7.2), carbonate buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$, pH 9.6) or glycine-buffered saline (GBS, 0.02 M glycine, 0.034 M NaCl, pH 9.2). All chemicals were of an analytical grade.

Patient description

A 3-year-old severe haemophilia A patient carrying intron 22 inversion in the FVIII gene (Type 1) had developed FVIII inhibitors under therapy with various plasma-derived (pd) FVIII preparations, reaching a maximal historical titer of 26 BU/ml. The immune tolerance (IT) therapy was started after 23 exposure days to FVIII (Bethesda titer 17,5 BU/ml), using the FVIII preparation Haemoctin SDH (Biotest, Dreieich, Germany). The immune tolerance protocol comprised continuous infusion (40 IU/kg/h for 8 days), followed by bolus injections of 100 IU/kg/day for 1 month, after which treatment was continued with a dose of 100 IU/kg twice a week for 10 months (Figure 1 E). After 2

months of therapy, the antibody titer was reduced to 0.7 BU/ml (Figure 1 D). During treatment with 100 IU/kg twice a week, difficulties in venous access were encountered, causing the interruption of treatment. The antibody titer increased then to 1.2 BU/ml. The second continuous infusion was therefore administered for 8 days with a dose of 150 IU/kg/h. The antibody titer peaked to 3.5 BU/ml and then dropped again to 0.8 BU/ml after 8 months of ITT. The three last plasma samples taken at the end of treatment showed the Bethesda titers of 0.8 BU/ml. The *in vivo* recovery had normalized by month 13, and prophylactic treatment (dose of 100 IU/kg twice a week) was started on the patient. No bleeding episodes occurred during immune tolerance treatment.

Functional activity of FVIII antibodies

The FVIII antibody levels were measured by the Bethesda method, as described by Kasper *et al.* (Kasper and Pool, 1975). The local plasma pool (made from 22 plasma samples of healthy donors) was used as a source of FVIII. The residual FVIII:C was determined by the chromogenic method according to the manufacturer's instructions (Coatest Factor VIII, Chromogenix AB, Italy) in a 96-well microplate (Micro-Well™, Nunc, Denmark). FVIII:C activity was calculated relative to a control in which the test sample was replaced by a buffer solution (TBS containing 1% of BSA).

Purification of IgG from plasma samples

IgG was purified from plasma samples by using affinity chromatography on Protein-G Sepharose (HiTrap™ Protein G, Pharmacia, Uppsala, Sweden) according to the manufacturer's instructions and IgG-containing fractions were dialysed against PBS at 4°C overnight. The concentration of total IgG was measured by a sandwich-type ELISA using the rabbit anti-human IgG antibody (Bio Rad, Hercules, CA). A human IgG reference sample (Bio Rad, Hercules, CA) was used for constructing a standard curve. Antibodies specific of FVIII were detected by a direct-binding ELISA using rFVIII-coated (Recombinate™) microplates.

Biotin labelling of recombinant FVIII

Recombinant carrier-free FVIII (specific activity >4000 IU/mg of protein) was labelled with biotin by using EZ Link sulfo-NHS-LC-biotin under mild conditions according to the manufacturer's instructions. Briefly, rFVIII (100 µg/ml) was dialysed against the HEPES buffer (0.01 M HEPES, 0.15 M

NaCl, 0.01 M $CaCl_2$, pH 8.5). The biotin derivative was dissolved in water to a final concentration of 0.01 mg/ml. Nine volumes of rFVIII and 1 volume of biotin solution were reacted for 2 h at RT. The free residual biotin derivative was removed by dialysis against the HEPES buffer (HEPES 0.01 M, NaCl 0.15 M, $CaCl_2$ 0.01 M, pH 7.2). Biotinylation did not alter FVIII:C activity, the binding to phosphatidylserine or the binding to VWF as analysed by the respective assays.

Inhibition of FVIII binding to phospholipids

The wells of polystyrene microplates (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with L-(-phosphatidyl)-l-serine (PS, 100 µl of 5 µg/ml solution in methanol) and were allowed to dry up at room temperature (RT). The wells were blocked with 3% of BSA in TBS for 1 h at RT. The wells were rinsed with washing buffer (TBS containing 0.1% of Tween 20) and a mixture containing an equal volume of biotin-labelled rFVIII 2 µg/ml in TBS-BSA (1% of BSA in TBS buffer), as well as the serially diluted (1:2 to 1:1000) purified IgG of test plasma samples were added and further incubated for 2 h at RT. The amount of FVIII-biotin bound to the wells was determined with avidin-peroxidase (1 µg/ml in PBS with 1% of BSA) by using OPD as a substrate. For each series of tests, a control was included in which buffer was used instead of the test plasma sample dilution.

Inhibition of FVIII binding to the von Willebrand factor

Microplate wells (MaxiSorp™, Nunc, Roskilde, Denmark) were coated with 100 µl of the mouse anti-human VWF polyclonal antibody (2 µg/ml, Dako, Denmark) in GBS buffer and incubated overnight at 4°C. The wells were blocked with 0.5% of casein in TBS for 30 minutes. A normal plasma pool at a dilution of 1:20 in TBS-BSA was used to saturate the wells of the microplate with VWF (plasma pool of 22 plasmas of healthy persons) for 1 h at RT. Factor VIII was dissociated from VWF by incubation with 0.4 M $CaCl_2$ for 30 min at RT. A mixture containing a tested IgG sample dilution and an equal volume of the rFVIII-biotin conjugate diluted at 2 µg/ml in TBS-casein were preincubated for 30 min at 37°C before adding it to the microplates. The amount of the bound rFVIII was determined as described in the previous assay after a further 2h of incubation at RT.

Factor Xa generation, the chromogenic assay

Recombinant FVIII (Recombinate™, 2 IU/ml in TBS-BSA) was preincubated with various concentrations (4 to 2500 μg/ml) of the tested IgG for 30 min at 37°C. The resulting FVIII-antibody complex mixture was reacted with purified bovine FIXa+FX (final concentration 2.4 IU/ml according to labelled activity) in the presence of phospolipids for 10 min at 37°C. Factor Xa generation was initiated by adding the $CaCl_2$ solution to the mixture (final concentration 6.25 mM). Aliquots were removed at appropriate times to assess the initial rates of product formation and the reaction was stopped with the EDTA solution (final concentration 11 mM). The amount of generated FXa was determined by the activity of cleaving chromogenic substrate S-2288 (final concentration 1.1 mg/ml) reading absorbance at 405 nm. Each assay included 2 controls, namely, in the first mixture the tested IgG was replaced by the IgG prepared from the plasmas of healthy donors (a positive control), and in the other, the tested IgG and FVIII were substituted by buffer TBS-BSA (a negative contol). The amount of generated FXa in the sample was expressed as a percentage of FXa generated in the positive control. The concentration of the tested IgG yielding 50% inhibition of FXa generation was calculated for each tested IgG sample. In an analogous assay, rFVIII was substituted by the recombinant FVIII light chain diluted to 12 IU/ml in TBS-BSA in order to estimate the effect of the patient's antibodies on FXa generation induced by the FVIII light chain.

Inhibition of FX binding to FVIII

The microplates (PolySorp™) were coated with PS (5 μg/ml in methanol) and air-dried. The wells were blocked with 5% of BSA in TBS for 1 h at RT. An equal amount of rFVIII (2 IU/ml in buffer A, 0.05 M Tris, 0.015 M NaCl, 0.015 M $CaCl_2$, BSA 1%) and the tested IgG at indicated concentrations (7 to 2500 μg/ml) were preincubated for 30 min at 37°C and transferred to PS-coated wells of the microplate. After 2h of incubation, the non-bound material was removed by washing the wells with buffer B (0.05 M Tris, 0.015 M NaCl, 0.05 M $CaCl_2$, Tween-20 0.1%, pH 7.2). Purified bovine FX diluted to 0.1 U/ml in buffer A (according to the labelled activity) was added to the wells and incubated for 15 min at RT. The wells were rapidly washed with 3 changes of buffer B. Subsequently, substrate S-2337 (final concentration 0.6 mg/m) was added to the wells and FX was activated by the FX-activating enzyme from Russel's viper venom (RVV, 21.5 μg/ml) in the presence of $CaCl_2$ (0.025 M). The inhibition of the FX binding was calculated relative to the control, which contained the IgG preparation from heathly donors instead of the tested IgG

sample (100% of binding). Control experiments confirmed that the RVV enzyme by itself did not hydrolyse the chromogenic substrate.

Assays for studying the epitope specificity of FVIII antibodies

FVIII heavy and light chain specificity of FVIII antibodies, ELISA assay

Microplates (PolySorp™, Nunc, Denmark) were coated with 8 IU/ml FVIII (Recombinate™) in a carbonate buffer overnight at 2–8°C. Non-specific binding sites were blocked with 3% of BSA in TBS for 1 hour at RT, and BSA was washed off with washing buffer. Plasma samples with FVIII antibodies were diluted to 4 BU/ml or used undiluted if the antibody titer was ≤4 BU/ml. Plasma samples were incubated for 1 h at 37°C with an equal volume of recombinant FVIII fragments (the recombinant heavy chain or the light chain of FVIII) by using concentrations ranging from 0.2 to 12.6 IU/ml according to the labelled activity. Aliquots were then transferred to rFVIII-coated microplates for an incubation of 2 h at 37°C. The amount of bound FVIII antibodies was determined by using the rabbit anti-human IgG conjugated with alkaline phosphatase (AP, Dako, Glostrup, Denmark) diluted to 1:1000 and visualized with AP-substrate pNPP (p-nitrophenyl phosphate). Two controls were included in each test: one mixture in which the plasma sample was replaced by buffer (minimal binding) and the other where buffer instead of recombinant FVIII fragments was used (maximal binding). The percentage of neutralization was calculated as follows: 100 – [(binding after incubation with fragment – minimal binding) / (maximal binding – minimal binding)] x 100. The plateau concentration was defined as a minimal concentration of the rFVIII fragment, which yielded a maximum optical density (OD), and all OD values within the plateau region were averaged.

FVIII heavy- and-light-chain specificity of FVIII antibodies, chromogenic method

Plasma sample dilutions were prepared as above. An aliquot was taken for measuring the concentration of FVIII antibodies by the ELISA assay. To the remaining solution an equal volume of rFVIII (Recombinate™) 2 IU/ml in TBS containing 2% of BSA was added and incubated for 1 h at 37°C. Residual FVIII coagulation activity (FVIII:C) was measured by the chromogenic method (Coatest Factor VIII, Chromogenix AB, Italy). The percentage of neutralization was calculated by using the above-mentioned formula using FVIII:C activity instead of binding capacity. Maximum FVIII:C activity was measured in the mixture of a particular recombinant fragment and rFVIII in the absence of an

antibody. When the tested plasma contained an FVIII antibody <10 BU/ml, a plasma sample from a haemophilia A patient (CRM-negative and without FVIII antibodies) instead of buffer was used in the mixture in order to obtain the maximal FVIII:C activity. Minimal FVIII:C activity was measured for each test sample incubated with rFVIII without any competitive FVIII fragments. The plateau value was calculated as described above.

Recombinant GST-fused FVIII fragments

The FVIII fragments for cloning were generated by PCR using primers with the added restriction cloning sites. The PCR products were cloned in frame into the pGEX4T-2 expression vector and controlled by sequencing in both directions with the T7 sequencing kit (Pharmacia, Uppsala, Sweden). The respective glutathione S-transferase (GST) fusion proteins were expressed in the DH5 α *Escherichia coli* strain. Fusion protein aggregates were dissolved by using 1.5% Sarkosyl (Sigma, USA) and 2% Triton X-100 and purified by using a glutathione column.

Binding of FVIII antibodies to recombinant FVIII fragments

MaxiSorp™ microplates were coated with the respective recombinant FVIII fragment (Figure 2) diluted to 2 µg/ml in GBS buffer for 2 h at RT. The wells were blocked with TBS-casein for 30 min. IgG fractions obtained from the patient's plasma samples were serially diluted from 15 µg/ml to 500 µg/ml in TBS-casein before addition to the wells of the microplate for a further incubation for 2 h at RT. The bound FVIII antibodies were detected by using the peroxidase-conjugated rabbit anti-human IgG and OPD. As a positive control, monoclonal antibodies specific of particular recombinant FVIII fragments were included in each experiment. Results were expressed as OD values corrected by subtracting background values (non-specific binding to GST-fused sham protein).

Competitive binding between FVIII-specific monoclonal and the patient's antibodies

Microplates were coated with different human or mouse monoclonal antibodies against human FVIII (hFVIII). The following FVIII-specific antibodies were used: human anti-hFVIII antibodies BO2C11 and 2E9; and mouse anti-hFVIII antibodies F7B4, F29B10, mAb 15, and ESH4. All these antibodies had been produced in our laboratory (CMVB, Leuven), except ESH4 (American Diag-

nostica, Greenwich, CT, USA) and were used at the dilution of 2 µg/ml in GBS buffer for 2 h at RT to coat the microplates. The wells of the microplate were then washed and blocked with TBS-casein for 30 min at RT. Mixtures containing an equal volume of the test IgG sample (15 µg/ml to 100 µg/ml) and the rFVIII-biotin (2 µg/ml) were then applied and incubated for 2 h at RT. The bound rFVIII-biotin was detected by using avidin-peroxidase as described above. The assay was validated by using the same monoclonal antibody than the one used to coat microplates instead of the tested antibody. Background values (no antigen in the coating solution) were subtracted from OD values. The degree of competition was calculated relative to the maximal binding obtained when no competitor (buffer instead of the test IgG sample) was used.

Statistical analysis

All the calculations were carried out by using the GraphPad Prizm Program Package. The Spearman rank order correlation test was used. The coefficient of p 0.05 was considered to be significant. The student paired t-test was used to confirm the difference in the concentration of antibodies bound to different FVIII fragments.

Results

Inhibition of FVIII:C activity by the patient's FVIII antibodies

The Bethesda assay was used to estimate the FVIII antibody content in samples taken during the immune tolerance treatment of the patient. The treatment resulted in two increases in the Bethesda titer. The first one was observed directly after the change from continuous infusion to bolus injection with an increased FVIII dosage (from 3.5 to 16.5 BU/ml, Figure 1D). The other increase in the Bethesda titer was detected as a response to the second continuous infusion that was applied after a temporal interruption of treatment (from 1.2 to 3.5 BU/ml).

The relationship between the inhibition of FVIII:C activity and FVIII antibody concentration was not linear and reflected complex kinetics known as Type 2 kinetics according to Biggs *et al.* (Biggs *et al.*, 1972a; Biggs *et al.*, 1972b). This non-linear neutralization pattern was observed for all plasma samples containing more than 4 BU/ml of FVIII antibodies.

Binding of the patient's antibodies to recombinant FVIII fragments

We characterized the distribution of inhibitory and non-inhibitory FVIII antibodies directed towards the light chain or the heavy chain of FVIII in plasma samples taken during treatment. At the beginning of treatment, the majority of the patient's FVIII antibodies recognized epitopes within the light chain of FVIII and only about 10% of antibodies recognized epitopes within the heavy chain of FVIII as assayed by ELISA (Figure 3). Moreover, FVIII-light-chain-specific antibodies possessed higher inhibitory activity than FVIII-heavy-chain-specific antibodies as estimated by the chromogenic method. During treatment, the antibody specificity persisted; most of the antibodies with inhibitory and non-inhibitory activity were directed against the FVIII light chain (Figure 1C). However, at the beginning of treatment, the proportion of FVIII-heavy-chain antibodies rose and the maximum was detected in 1.5 months (30%). The amount of antibodies directed towards the FVIII heavy chain decreased below detection limit in month 10.

To characterize the epitope specificity of antibodies more precisely, we studied the antibody binding to the following recombinant FVIII fragments, namely the A1, a1, A2, a3, A3, C1, C2 domains in an ELISA. The respective fragments were expressed as the fusion proteins with glutathione S-transferase (GST) in *E. coli* and were immobilized to the microplates. The relative amount of the bound antibodies was expressed as an OD value with the background value obtained with GST fusion with an irrelevant protein being subtracted. The patient's antibody binding to recombinant fragments corresponding to the FVIII light chain (a3, A3, C1, C2) is shown in Figure 1A and binding to fragments of the FVIII heavy chain (A1, A1, A2) in Figure 1B. At the beginning of treatment, the concentration of antibodies recognizing the C1 domain significantly exceeded the concentration of the C2-domain-bound antibodies ($p<0.01$). No antibody binding to the A3 domain was detected, but a negligible amount of antibodies bound to the a3 domain. On the second day of treatment, the amount of antibodies bound to recombinant fragments decreased significantly. This was accompanied by a decrease in the Bethesda titer, which can probably be explained by the neutralization of FVIII antibodies by the administered FVIII. The decrease in the concentration of C1 domain antibodies and a3 domain antibodies during treatment was linear (linear regression coefficient r=0,87, r=0.61, respectively). The binding of the patient's antibodies to the recombinant C1 domain immobilized to the polystyrene surface is shown in Figure 4. The binding of the patient's antibodies to the C1 domain was linearly dependent on antibody concentration.

During the following treatment period, the amount of antibodies recognizing the FVIII light chain fragments remained rather unchanged, except the significantly elevated level of the C2-domain-specific antibodies ($p=0.02$, t-test) observed in samples taken in the 5th month of treatment.

The amount of antibodies recognizing the recombinant FVIII heavy chain fragments was lower. We could detect antibodies, which bound to epitopes within the A2 domain or the A1 domain. No binding to the a1 domain was detected. During the treatment the concentration of the A1 and A2 domain specific antibodies decreased linearly ($r=0.56$, $r=0.81$, $P<0.05$ respectively).

Competitive binding assay using monoclonal FVIII antibodies

Further we characterized whether the tested FVIII antibodies were capable of competing with the monoclonal FVIII antibodies for the known binding sites in FVIII. In the competitive binding assay we used a mouse monoclonal FVIII antibody F29B10, which binds to the epitope within amino-acids 2170–2195, because both recombinant FVIII fragments, the C1 and C2 domain, contained this epitope (common region of amino-acids 2125–2222). Antibody F29B10 bound to the recombinant C2 domain only, while no binding to the C1 domain was detected. At the beginning of treatment the patient's antibodies inhibited the mAb F29B10 binding to FVIII by 12% (Figure 5A), and during the immune tolerance treatment we also found a small amount of antibodies able to interfere with the FVIII binding to mAb F29B10. No correlation was found between the extent of the inhibition of the mAb F29B10 interaction with FVIII by the patient's antibodies and the concentration of C2-domain-specific FVIII antibodies.

Another monoclonal antibody, F7B4, whose binding site locates within amino-acids 356–360 of the a1 domain, was used in the competitive binding assay. The result that antibody F7B4 bound with a similar affinity to the recombinant fragments a1 and A2 was expected, since the epitope of this antibody was present in both these recombinant FVIII fragments. At the beginning of treatment, the patient's antibodies inhibited the mAb F7B4 binding to FVIII by 25% (Figure 5A), but the amount of such antibodies decreased concomitantly during treatment. The inhibition of FVIII interaction with this mAb correlated with the concentration of antibodies bound to the recombinant A2 domain (Spearman rank order correlation coefficient $r=0.881$, $p<0.01$). Contrary to mAb F7B4, the tested patient's antibodies were unable to bind to the a1 recombinant fragment, thus the epitopes of the patient's FVIII-heavy-chain-specific antibodies located obviously within the A2 domain or A1 the domain and interfered with the mAb F7B4 binding to FVIII indirectly.

The proper conformation of the A2 domain of FVIII is critical for the interaction with FIXa (Fay *et al.*, 1994; Fay *et al.*, 1999). This interaction is probably abolished by the binding of mAb 15 to a conformational epitope in the C-terminus of the A2 domain of the FVIII heavy chain resulting in a complete inhibition of the FVIII coagulation activity (Gilles *et al.*, 1996; Gilles *et al.*, 1997). The FVIII binding to mAb 15 was interfered with by some of the tested

antibody samples, showing that the patient's antibodies were targeted at this region of the C-terminus of the A2 domain (Figure 5A).

Interaction of FVIII with VWF is abolished by human monoclonal antibodies 2E9 and BO2C11, which bind to the specific epitopes of the C1 (Jacquemin *et al.*, 2000) and the C2 domain of the FVIII light chain (Jacquemin *et al.*, 1998; Spiegel *et al.*, 2001), respectively. Whether the patient's FVIII-light-chain-specific antibodies recognized these specific epitopes was next assessed by the competitive binding assay. None of the investigated antibodies was able to interfere with the FVIII binding to mAb BO2C11 or the FVIII binding to mAb 2E9, neither when added directly to the wells of the microplate nor later, after 30 min preincubation with the FVIII-biotin conjugate. The C1 domain was the main target for the FVIII antibodies of the patient, but the patient's antibodies recognized different epitopes of the C1 domain of the FVIII light chain than the monoclonal antibody 2E9 binding site.

Surprisingly, the patient's antibodies were able to compete with mAb ESH4 for its binding site (2303–2332) within the C2 domain. At the beginning of treatment the binding of ESH4 to FVIII was abolished by the patient's antibodies (Figure 5A). During treatment the concentration of antibodies able to compete with ESH4 for the binding site on FVIII decreased.

Inhibition of the FVIII binding to VWF and to PS by FVIII antibodies

In order to unravel the mechanism of the action of FVIII antibodies in the inhibiting FVIII:C activity, we studied whether the patient's antibodies interfered with the FVIII interaction with VWF and/or the binding of FVIII to PS, which are the most common interactions hindered by FVIII-light-chain-specific antibodies. No interference with FVIII interaction with VWF or the FVIII binding to PS was detected when FVIII was preincubated with the patient's IgG before adding to either VWF- or PS-coated microplates as observed in the respective ELISA assays. Monoclonal antibody BO2C11, which interfered with both of these interactions in a concentration-dependent manner, was used as a control in each assay.

Inhibition of FXa generation

Since FVIII antibodies were not able to interfere with the FVIII-PS interaction, we assessed whether the antibodies impair the FVIII interaction with FIXa and/or FX and abolish the formation of the FXase complex. We used a factor Xa generation assay, which was dependent on the presence of phospholipids, FVIII as well as Ca^{2+}; when one of these was not added to the reaction mixture, the generation of FXa was abolished. Preliminary experiments showed that in the presence of the light chain of FVIII, FXa generation was lower than in the

presence of rFVIII. Use of the recombinant FVIII heavy chain instead of rFVIII, led to the generation of a negligible amount of FXa only. Due to the limited amount of plasma available for IgG fractionation, we could investigate only four samples in this assay. At the beginning of treatment the patient's antibodies completely abolished the generation of FXa at the IgG concentration 500 µg/ml in the presence of rFVIII or the FVIII light chain (Figure 6). The concentration-dependent inhibition of FXa generation by the patient's antibodies is shown in Figure 7. The antibodies of the patient also inhibited the generation of FXa induced by the FVIII light chain, although a slightly higher concentration of IgG was needed than in the presence of rFVIII in an analogous assay (Figure 7). Obviously, the FVIII-light-chain-specific antibodies were responsible for the inhibition of FVIII:C activity by interfering with FVIII interaction with FIXa and FX. The IgG concentrations that caused 50% inhibition of FXa generation induced by rFVIII or the light chain of FVIII are shown in Figure 5B.

Inhibition of the FX binding to FVIII

The tested FVIII antibodies hindered the formation of the FXase complex, therefore we assessed whether or not the interaction of FVIII with FX takes place in the presence of the patient's antibodies. The microplates coated with phosphatidylserine were used to ensure a proper orientation of FVIII to the surface enabling an efficient binding of FX. This strategy for FVIII immobilisation was chosen because the tested antibodies did not interfere with the binding of FVIII to phosphatidylserine, and the orientation of FVIII was known to be highly important for interaction with FX. The amount of FX bound to FVIII was estimated by a subsequent activation of FX with Russell's viper venom and the ability to cleave a specific substrate. All four tested IgG samples, which were characterized by the FXa generation assay, were able to inhibit the FX binding to FVIII immobilized to PS (Figure 8). Since the light chain of FVIII has been reported to participate in the FX binding to FVIII (Liu and Thomson, 2000), we assessed the ability of the FVIII light chain to interact with FX in the respective experimental set-up. The binding of FX to the light chain of FVIII was also inhibited by the patient's antibodies. IgG fractionated from plasmas of healthy donors did not impair the interaction of FX with FVIII or its light chain and was therefore used as a control.

FX itself could also bind to PS in an FVIII-independent manner and give rise to high background values. Therefore in parallel with the oriented immobilization of FVIII onto the phosphatidylserine layer, we also attempted the immobilization of FVIII via surface-attached antibodies. The antibodies which are known to be targeted at the regions of FVIII participating in the interaction with PS (e.g. BO2C11, ESH4) bound efficiently to FVIII. Unfortunately, the ability of FVIII to interact with FX was completely lost upon binding to these monoclonal antibodies (data not shown).

Discussion

A high responder haemophilia A patient having predominantly FVIII-light–chain-specific antibodies was tolerated to FVIII by the administration of the FVIII preparation containing VWF. During treatment the concentration of C1-domain–specific antibodies was significantly higher than the concentration of antibodies targeted at the C2 domain, a3 domain of the light chain and the A1 domain and A2 domain of the heavy chain of FVIII. The patient's antibodies were not able to interfere with the FVIII binding to phospholipids and to VWF. We found that the antibodies of the tested patient were able to inhibit FXa generation and the binding of FX to FVIII.

The epitope specificity pattern of the patient's antibodies was rather unique. Usually FVIII antibodies develop towards the C2 domain and A2 domain in both previously untreated patients (Scandella *et al.*, 2001) as well as previously treated patients (Laub *et al.*, 1999; Sawamoto *et al.*, 1998). However, the results of our study are in agreement with the results described by Prescott *et al* (Prescott *et al.*, 1997). They reported a higher prevalence of FVIII light chain antibodies targeted at the A3-C1 fragment in patients who were treated with the pdFVIII preparation, while A2– and C2-domain-specific antibodies were predominant in a group of patients treated with the rFVIII preparation (Prescott *et al.*, 1997).

We were unable to detect any significant changes in the antibody epitope specificity during immune tolerance treatment. However, the interruption of treatment caused a significant increase in antibody production and a rise in the level of C2-domain-specific antibodies. Still, the patient's antibodies were unable to interfere with the FVIII binding to VWF or to PS. Obviously, the concentration of these antibodies was not sufficiently high to interfere with these bindings or the antibodies were targeted at another region of the C2 domain. This indicates that the development of C2-domain-specific antibodies is a common response to the administration of FVIII, but the production of antibodies with such epitope specificity was probably suppressed, since VWF containing the FVIII preparation was used for treatment. In principle, VWF could have masked the respective epitopes on the C2 domain and lowered the response to the C2 domain.

The epitope specificity of the patient's antibodies was characterized by using recombinant FVIII fragments and by the competitive binding assay with monoclonal antibodies whose binding sites on FVIII were defined. The latter strategy was chosen because the recombinant fragments may possess a different conformation as compared to these domains in the native FVIII. Therefore, a higher number of antibody binding sites could be detected on recombinant fragments. This could also explain why the amount of antibodies bound to the A1 domain and A2 domain exceeded the amount of antibodies neutralising the FVIII heavy chain. This suggests that antibody binding sites on the recombinant A1 domain and the A2 domain were more easily accessible than in the native

FVIII. On the other hand, the recombinant FVIII heavy chain and light chain, which were expressed in CHO cells could have possessed a different glycosylation pattern compared to the recombinant fragments produced by *E. coli*. Using synthetic peptide arrays the FVIII antibody binding to a high number of epitopes has been shown (Palmer *et al.*, 1997). However, the binding of antibodies to certain epitopes could impair the FVIII coagulation activity. Therefore, the monoclonal antibodies were chosen for the competitive binding assay according to the relevance of the binding site for the functional activity of FVIII focusing mainly on the interaction of FVIII with VWF.

We demonstrated that the antibodies of the patient were targeted at the epitopes that were exposed on the surface of the FVIII-VWF complex, except the a3 domain. One of the VWF binding sites locates within the a3 domain (aa 1675–1684 (Shima *et al.*, 1992), however, the antibodies of the patient were not able to hinder the FVIII binding to VWF. Presumably, the concentration of the a3-domain-specific antibodies was low or the binding sites of the patient's antibodies were not overlapping with the VWF binding sites. The antibodies of the patient did not recognize the recombinant A3 domain, which shares a common region with the a3 domain (aa 1692–1700) and the C1 domain (aa 1981–2043). This again could be explained by the presence of VWF in the administered FVIII preparation, which could have covered the respective immunogenic regions of FVIII or, on the other hand, by the different structure of the A3 domain in the recombinant fusion protein that was used in an ELISA. It could be expected that the A3 domain is also one of the targets of the antibodies of the patient, since several studies have shown the presence of antibodies against the A3-C1 fragment (Palmer *et al.*, 1997; Prescott *et al.*, 1997; Scandella *et al.*, 1995).

The antibodies of the patient were targeted at the C2 domain and were able to compete with monoclonal antibodies ESH4 and F29B10 for their binding sites on the C-terminus and the N-terminus of the C2 domain, respectively. No correlation between the amount of C2 domain antibodies and antibodies that compete with mAb ESH4 or mAb F29B10 was found. In spite of a low concentration of C2 domain antibodies, the binding of FVIII to ESH4 was abolished. Presumably, the antibodies of the patient were targeted at regions close to the ESH4 binding site on the FVIII light chain. Antibodies targeted at the N-terminus of the C2 domain as well as C1-domain-specific antibodies could both be responsible for that, since the disulphide bonds position the C-terminus and N-terminus of the C1 domain and the C2 domain into close proximity as shown in the 3D model of FVIII (Liu *et al.*, 2000; Stoilova-McPhie, 2002).

Studying the putative mechanism of the patient's antibodies we discovered that these antibodies were able to inhibit FXa generation by interfering with the FX binding to FVIII. The formation of the FXa complex necessitates several subsequent interactions, namely the binding of FVIII to the PS monolayer, association of FIXa with the formed complex in the presence of Ca^{2+} and

finally, the incorporation of FX into the complex. The FVIII binding to PS was not interfered by the tested patient's antibodies. We were unable to study the interaction of FVIII with FIXa in the presence of the tested antibodies, since the volume of plasma samples available for the experiments was limited. The FIXa binding sites locate within the A2 domain (aa 558–565, 698–710, Fay *et al.*, 1994; Jorquera *et al.*, 1992) and the A3 domain (aa 1811–1818, Lenting *et al.*, 1996) of FVIII and the tested antibodies recognized the A2 domain and interfered with the mAb 15 binding to FVIII, therefore we assume that the antibodies of the patient could inhibit the FIXa binding to FVIII.

The FX binding site locates within the A1 domain (aa 337–372, Lapan and Fay, 1997) of FVIII, and a small amount of the patient's antibodies was also able to interfere with the binding of mAb F7B4 (aa 356–360) to FVIII. These A1-domain-specific antibodies were obviously able to inhibit the FX binding to FVIII, because in the presence of rFVIII a slightly lower amount of the patient's antibodies was needed to abolish this interaction than in the presence of the FVIII light chain. Recently the FXa binding site on C2 domain amino acids 2253–2270 has been mapped (Nogami *et al.*, 2000; Nogami *et al.*, 1999). On the other hand, the presence of the FX and FXai binding sites within the C1-C2 fragment has been reported (Liu and Thomson, 2000). However, these binding sites were different from that of VWF within the same fragment (Liu and Thomson, 2000). Most of the tested patient's antibodies were targeted at the C1 domain, but the antibody binding sites were different from the epitopes of monoclonal antibody 2E9, which was able to interfere with the FVIII interaction with VWF. Our results are in agreement with the report by Liu *et al.* confirming that the binding site for FX is obviously within the C1 domain and differs from the binding site for VWF. Surprisingly, we discovered that C2-domain-specific antibodies ESH4 and BO2C11, which were able to interfere with the FVIII binding to PS abolished the binding of FVIII to FX. This stresses the significance of a proper conformation of the C1-C2 domains in FVIII for binding to FX.

The antibody response was characterized in a single patient, who was treated according to an uncommon treatment protocol. The interruption of treatment caused the prolongation of the treatment period as described also in other studies (Kreuz *et al.*, 1995; Oldenburg *et al.*, 1999). Regardless of the elevated FVIII antibody level, the tolerance of the patient was achieved and the *in vivo* recovery normalized at the end of treatment. Although the Bethesda titer decreased to 0.8 BU/ml, we could still confirm the presence of FVIII antibodies by an ELISA at the end of treatment. A similar case proving that it was impossible to detect antibodies by the Bethesda assay during the immune tolerance treatment has been described in another study (Klinge *et al.*, 2001). However, in that study an immuno-precipitation assay was used for detecting the respective antibodies (Klinge *et al.*, 2001).

To our knowledge, this is the first detailed characterization of the FVIII antibody response during the immune tolerance treatment. Further studies are

definitely needed in order to clarify the effect of VWF in the FVIII preparations on FVIII antibody production and epitope specificity. The exact determination of the C1 domain antibody binding sites of antibodies of the tested patient could lead to a better understanding of the structure as well as function of the C1 domain and could be of benefit to the development of FVIII pharmaceutical preparations in the future.

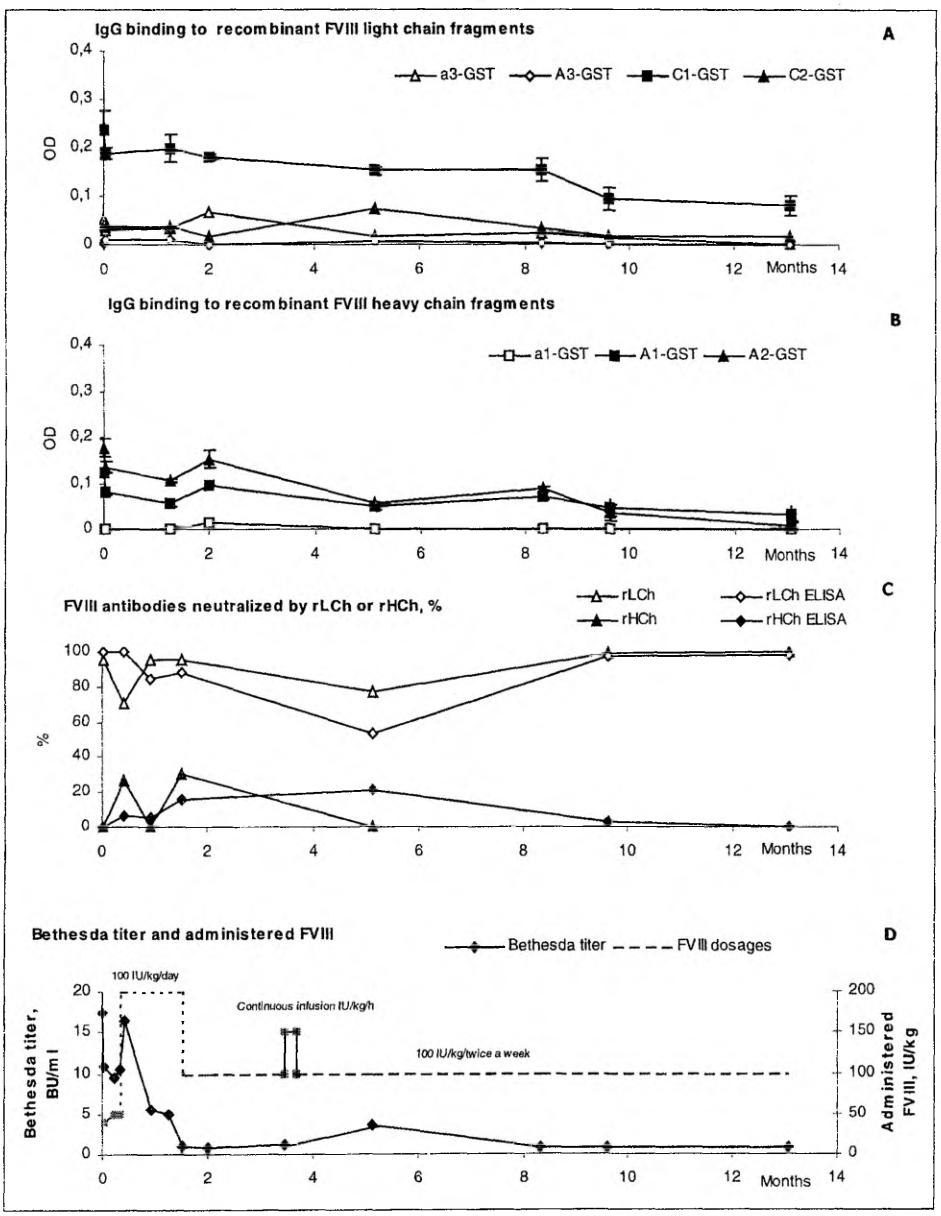

Figure 1. Characterization of properties of the patient's FVIII antibodies during ITT. The binding of the patient's antibodies to the recombinant fragments of the FVIII light chain (**A**) and the recombinant fragments of the FVIII heavy chain (**B**) at 100 µg/ml IgG concentration (mean ± SD). Neutralization of the patient's anti-FVIII antibodies by the recombinant FVIII light chain and the heavy chain as estimated by using the chromogenic method (LCh, HCh) or an ELISA assay (LCh ELISA, HCh ELISA) (**C**). The Bethesda titer and the administration scheme of FVIII preparation Haemoctin SDH (Biotest, Germany) (**D**).

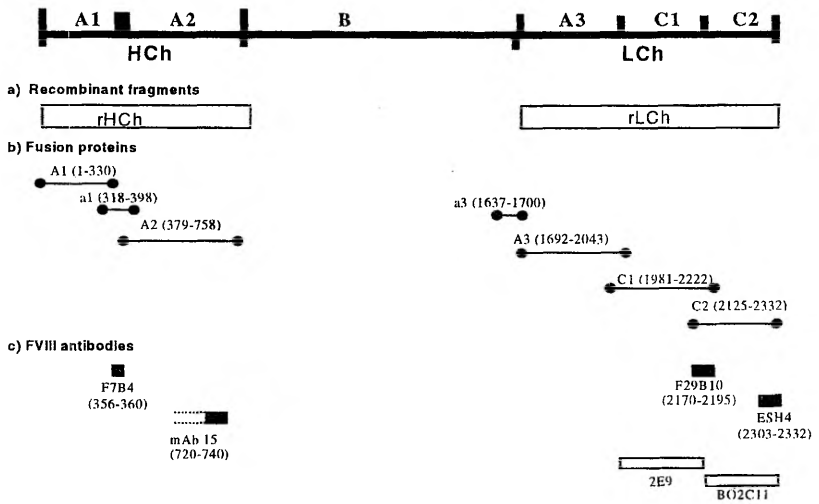

Figure 2. FVIII recombinant fragments used in the neutralization experiments (**a**) and GST-fusion proteins used for the characterization of the epitope specificity in ELISA assays (**b**). The binding sites of monoclonal FVIII antibodies that were used in the competition assay (**c**).

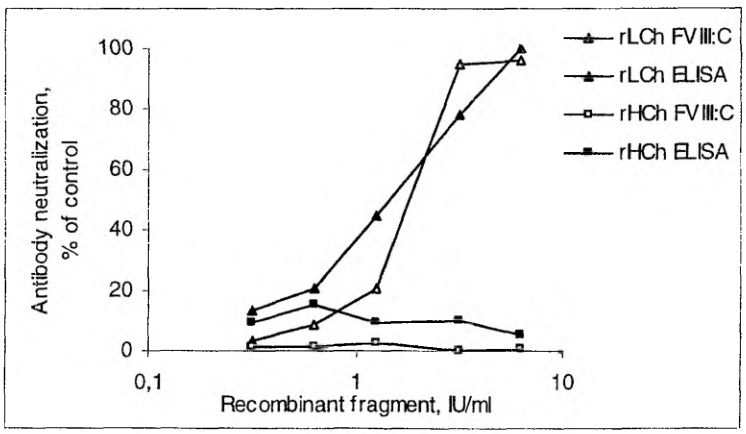

Figure 3. Neutralization of the patient's anti-FVIII antibodies by the recombinant light chain and the heavy chain of FVIII as measured by the chromogenic method (rLCh FVIII:C, rHCh FVIII:C) or an ELISA assay (rLCh ELISA, rHCh ELISA). Tested plasma sample was taken at the beginning of ITT.

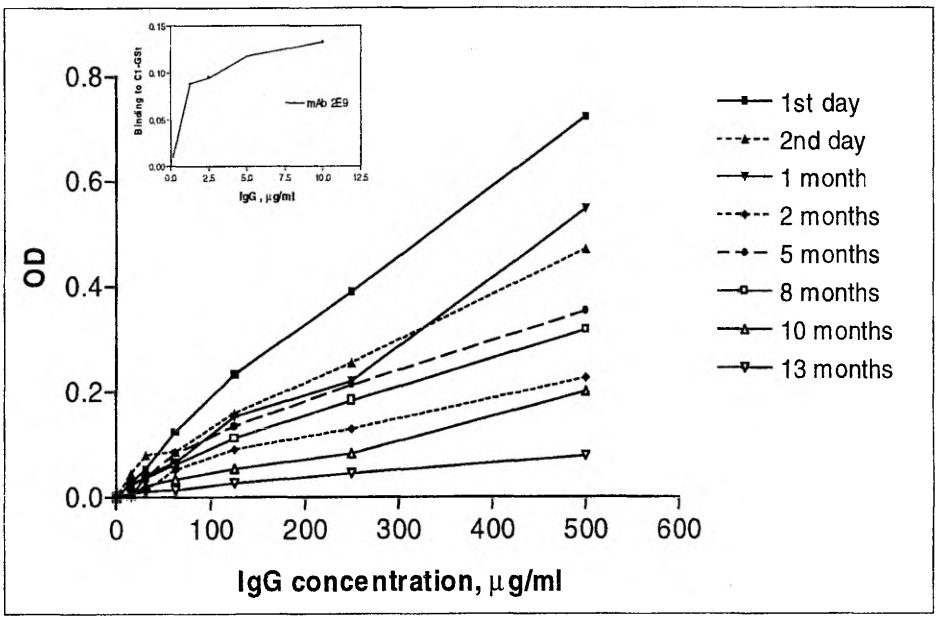

Figure 4. The binding of the patient's antibodies to the recombinant C1 domain in an ELISA. Monoclonal antibody 2E9 specific to the C1 domain was used as a control (insert)

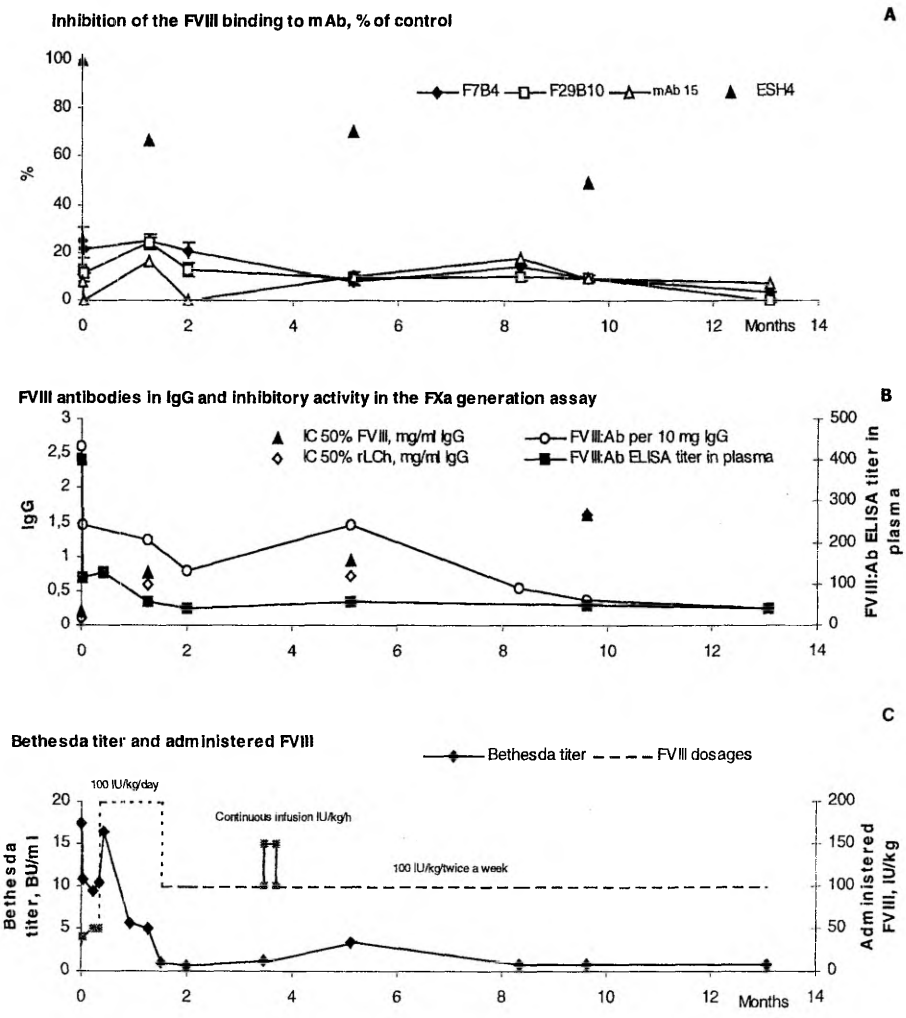

Figure 5. Inhibition of the FVIII binding to different monoclonal FVIII antibodies by the patient's IgG (mean ± SD) (**A**). Concentration of FVIII antibodies in purified IgG (μg per 10 mg of IgG) and the ELISA titer estimated in plasma. The inhibitory activity of FVIII antibodies in the FXa generation assay is shown as IgG concentration yielding 50% of inhibition of FXa generation induced by rFVIII (IC 50% FVIII) or the recombinant light chain of FVIII (IC 50% rLCh) (**B**). The Bethesda titer and the administration scheme of FVIII preparation Haemoctin SDH (Biotest, Germany) (**C**).

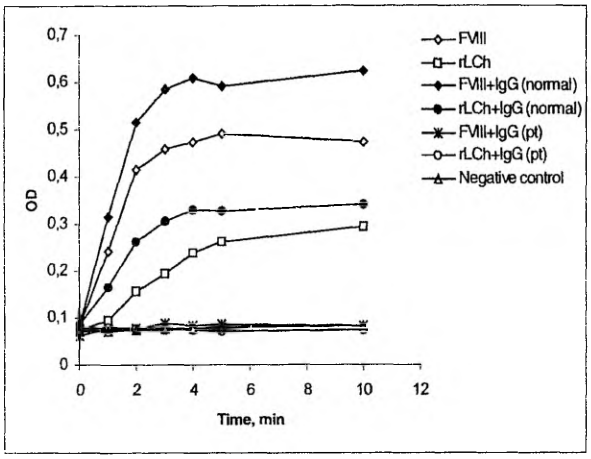

Figure 6. Inhibition of FXa generation induced by rFVIII (rFVIII) or the recombinant light chain of FVIII (rLCh) with the patient's FVIII antibodies (0.5 mg/ml IgG) at the beginning of ITT. Each data point represents the mean of at least two individual experiments.

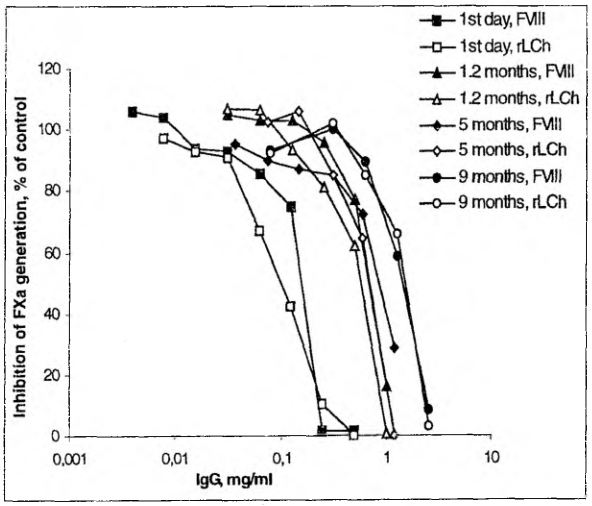

Figure 7. Inhibition of FXa generation induced by rFVIII (rFVIII) or the recombinant light chain of FVIII (rLCh) with the patient's FVIII antibodies at different time point of ITT. The amount of FXa was estimated after 5 min of induction. Each data point represents the mean of at least two individual experiments.

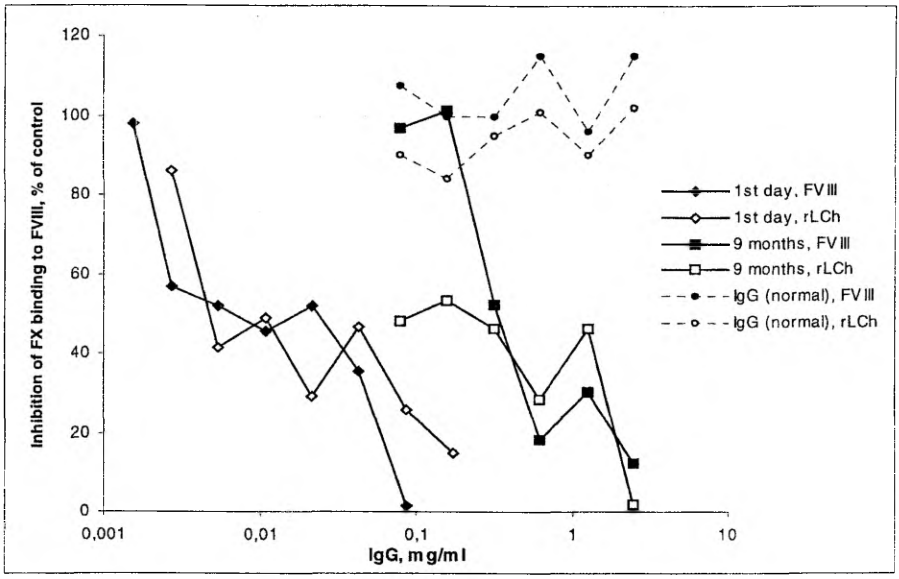

Figure 8. Interaction of FX with FVIII or its recombinant light chain bound to phosphatidylserine in the presence of the patient's FVIII antibodies. Each data point represents the mean of at least two individual experiments.

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III

Kallas A, Talpsep T, Everaus H.
Changes in epitope specificity and in distribution of IgG subtypes
of FVIII antibodies during immune tolerance therapy (ITT)
in hemophilia A patients with FVIII antibodies – a case report.
In Scharrer I., Schramm W. (eds.) *31st Hemophilia Symposium.*
Springer Verlag Berlin Heidelberg, 2001, 23–40

Changes in Epitope Specificity and in Distribution of IgG Subtypes of FVIII Antibodies during Immune Tolerance Therapy (ITT) in Hemophilia A Patients with FVIII Antibodies – a Case Report

A. KALLAS, T. TALPSEP and H. EVERAUS

Introduction

Replacement therapy of patients with inherited coagulation factor VIII deficiency (hemophilia A) comprises administration of either plasma derived factor VIII concentrates or recombinant factor VIII. Up to 25% of patients may develop antibodies against FVIII (FVIII:Ab). Antibodies arise more often in patients with severe form of hemophilia A caused by inversion, large deletion or stop codon in FVIII gene rather than in patients with mild or moderate forms of the disease (point mutations, small deletion in FVIII gene) [1, 2]. It is not known why only some patients develop FVIII antibodies whereas others do not in spite of extensive replacement therapy.

Factor VIII circulates in plasma as a heterodimer composed of the heavy chain (domains A1-A2-B) and the light chain (domains A3-C1-C2), and forms a complex with von Willebrand factor (vWF) via the light chain [3] (Fig. 1). Von Willebrand factor stabilizes FVIII and can interfere with antibody binding to FVIII either by

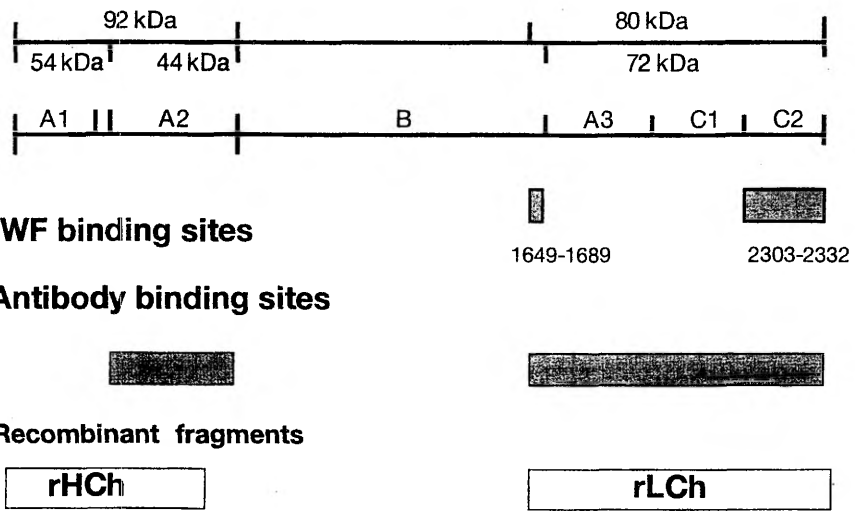

Fig. 1. The structure of FVIII molecule. VWF binding sites and antibody binding sites are indicated

I. Scharrer/W. Schramm (Ed.)
31st Hemophilia Symposium Hamburg 2000

direct blocking of epitopes on FVIII light chain or by steric hindrance. Most inhibitory antibodies to human factor VIII developed in hemophilia A patients bind to epitopes within A2 domain, C2 domain, and A3-C1 domains [4]. Antibodies reacting with C2 domain of the FVIII light chain possess a lower ability to neutralize FVIII coagulation (FVIII:C) activity, when it is in a complex with vWF (FVIII-vWF) [5]. In a previous study we have shown that the decreased neutralization of FVIII in the presence of vWF by FVIII antibodies was in good correlation with the level of antibodies against FVIII light chain [6]. These in vitro findings suggest that detecting the epitope specificity and the concentration of antibodies can be used for selecting FVIII concentrate for replacement therapy and for immune tolerance induction. Different protocols of immune tolerance therapy (ITT) like the Bonn protocol, the Malmö method, and their modifications demonstrate the similar efficacy in suppressing the production of antibodies to a non-detectable level [7]. While using FVIII concentrate alone for IT therapy [8, 9], the success rate may depend upon the purity of FVIII concentrate used [10].

The effect of different FVIII concentrates on the immune system of hemophilia A patients has been intensively studied both in vitro and in vivo. Lymphocytes respond to FVIII with enhanced cytokine production in vitro [11], but in vivo the situation is more complicated and depends also on the availability of cytokine receptors, which determine the magnitude of immune response to FVIII concentrates. First exposure to FVIII concentrate may cause production of IgM type FVIII antibodies, while isotype switch occurs soon in order to produce IgG isotype antibodies. Distribution of different IgG antibody subtypes depends on whether the Th1 or Th2 phenotype is dominant in a particular patient [12, 13].

We investigated the changes of epitope specificity and distribution of IgG subtypes of FVIII antibodies in hemophilia A patient during ITT with FVIII-vWF concentrate. The IgG subtype distribution of FVIII antibodies was estimated using sensitive ELISA assay and results were compared with those obtained from the samples of hemophilia A patients with persistent FVIII antibody titer, and from the samples taken from the patient with low titer antibodies during on-demand treatment with FVIII concentrates of different purity.

Patients

Patient 1 Undergoing ITT

A 3-year-old severe hemophilia A patient with intron 22 inversion in FVIII gene (type 1) had FVIII antibody titer of 26 BU/ml. Immune tolerance therapy with FVIII concentrate, Haemoctin SDH (Biotest, Germany) was begun when he had had 23 exposure days to FVIII. Haemoctin SDH was the only available FVIII concentrate at that time in Estonia. The specific activity of Haemoctin SDH was 100 IU of FVIII per mg of protein. The immune tolerance therapy comprised continuous infusion (CI, 40 IU/kg/h for 8 days + 8 days via vascular line), followed by bolus injections 100 IU/kg/day for 1 month and thereafter treatment was continued with the dose 100 IU/kg twice a week for 10 months (Fig. 2). After 2 months of ITT, the antibody

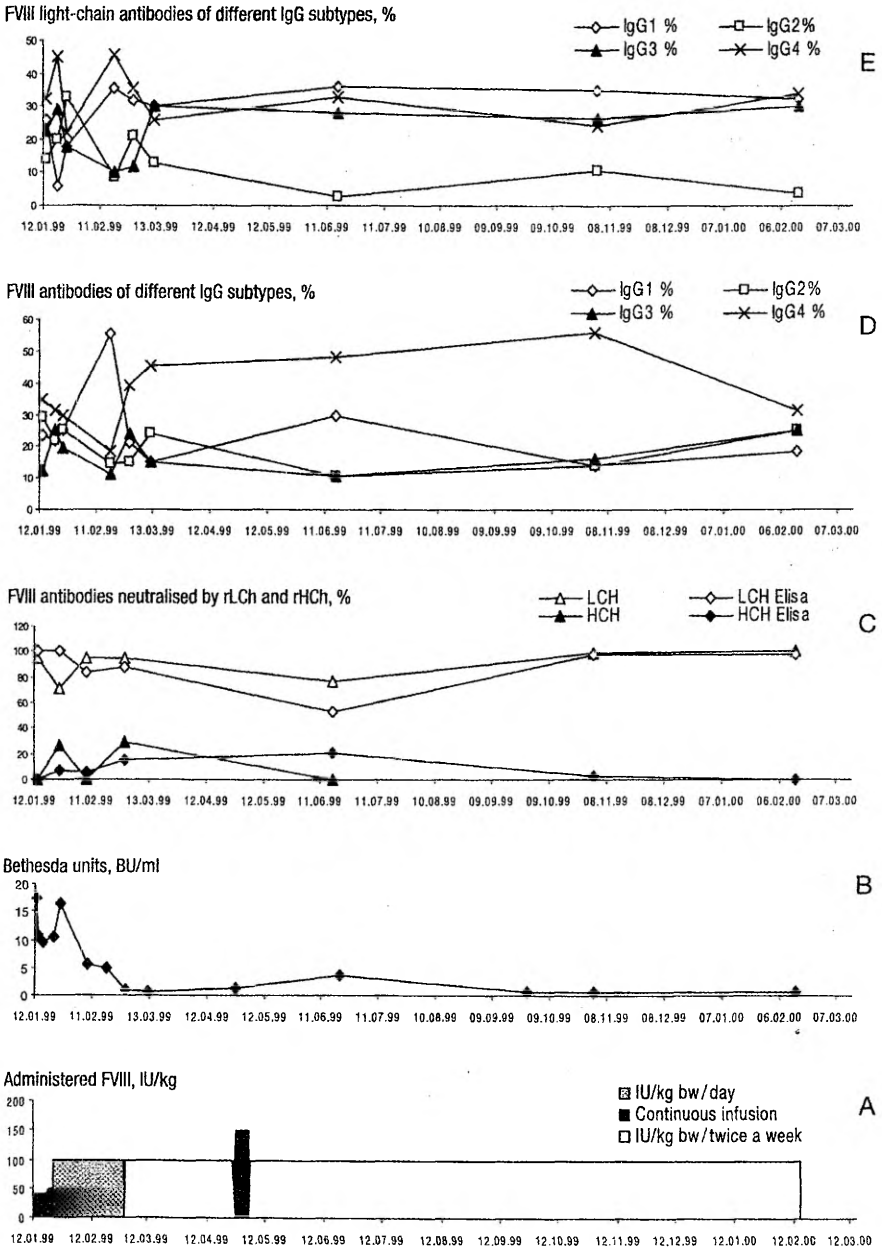

Fig. 2a–e. Immune tolerance therapy in patient 1 with high titer FVIII antibodies. *a* The course of the treatment with FVIII concentrate Haemoctin SDH (Biotest); *b* Changes in FVIII antibody titer measured by Bethesda assay; *c* Relative distribution of FVIII light chain (rLCh) and FVIII heavy chain (rHCh) antibodies measured by an ELISA assay (ELISA) and by a chromogenic method; *d* IgG subtype distribution of FVIII antibodies; **e** IgG subtype distribution of FVIII light chain antibodies

titer had reduced from 17.5 BU/ml to 0.7 BU/ml. During the course of the treatment with a dose of 100 IU/kg twice a week, problems in venous access appeared, which were accompanied by an increase in antibody titer to 1.2 BU/ml. Therefore, the second continuous infusion for 8 days with a dose of 150 IU/kg/h was given. In response to this treatment, the antibody titer peaked to 3.5 BU/ml, and then dropped to 0.8 BU/ml after 9 months of IT therapy. The last three samples contained only antibodies with non-inhibitory activity (Bethesda titer 0.8 BU/ml). In vivo recovery normalized and the patient started prophylactic treatment (dose of 100 IU/kg twice a week). During ITT the patient had no bleeding episodes.

Patient 2 Treated with FVIII Concentrates with Different Purity (On-demand Treatment)

A severe hemophilia A patient with intron 22 inversion in FVIII gene (type 2) developed FVIII antibodies (1.1 BU/ml) at the age of 6 years. Patient's HLA genotype was DR4 (DRB1*04), DR8 (DRB1*08), DQ4 (DQB1*04), and DQ7 (DQB1*0301) as detected by PCR method. He has had 8 exposure days to FVIII concentrates Hemofil-M (Baxter, USA), 8 exposure days to cryoprecipitate and 4 to plasma. The amount of FVIII concentrates used and the characterization of FVIII antibodies in plasma samples taken at different time points are shown in Fig. 3. Treatment of the patient with FVIII concentrates Koate-HP (Bayer, USA) and Kryobulin Tim 3 (Baxter), both of which contain vWF, caused a decrease in FVIII antibodies from 1.0

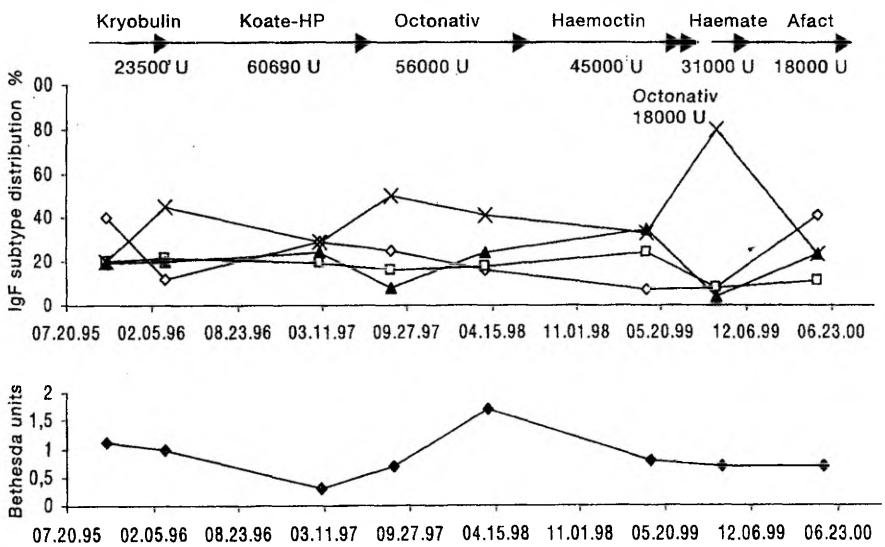

Fig. 3. Changes in IgG subtype distribution of FVIII antibodies of hemophilia A patient 2 during on-demand therapy with FVIII concentrates with different purity. FVIII antibodies of different IgG subtypes are shown as follows: IgG1 (◇), IgG2 (□), IgG3 (▲), IgG4 (×), Bethesda units (Bu/ml) ◆)

to 0.3 BU/ml. However, changing to FVIII concentrate, Octonativ-M (Pharmacia & UpJohn), which was purified by monoclonal antibody, resulted in an increase in FVIII antibodies up to 1.7 BU/ml. The respective antibodies reacted with FVIII light chain and A2 domain. In vivo FVIII recovery was 32.4% after administration of 25 IU/kg of body weight of Octonativ-M. After 15 months of treatment with Octonativ-M, FVIII concentrate was replaced by Haemoctin SDH (Biotest). In September 1998, in vivo recovery was 64.8% (the dosage 25 IU/kg of body weight). Antibody titer had slightly decreased (0.8 BU/ml), and antibodies had reactivity against the light chain and A2 domain of FVIII. Haemoctin SDH was used for on-demand treatment for the next 11 months. The titer of antibodies had decreased to 0.7 BU/ml and antibody reactivity against the light chain and the A2 domain persisted. In vivo recovery with Haemoctin-SDH was 80.5%. Thereafter treatment was switched to FVIII concentrate Octonativ-M for 3 months, followed by treatment with Haemate (Aventis Behring, USA) for 5 months, and then monoclonal antibody purified FVIII concentrate Aafact (CLB, the Netherlands).

Hemophilia A Patients with Persistent FVIII Antibodies

Plasma samples from 12 hemophilia A patients having FVIII antibodies were studied. The study protocol was approved by the Ethics Committee of the University of Tartu, Estonia. Anti-FVIII antibodies had developed in all the patients in response to the on-demand treatment with plasma-derived FVIII concentrates. Plasma samples were drawn in a clinical stable situation before any immune tolerance induction. Plasma samples contained 1 to 300 BU/ml of FVIII antibodies. All investigated FVIII antibodies reacted with A2 domain and the light chain of FVIII as detected by Western blotting using plasma derived FVIII concentrate Haemoctin SDH (Biotest, Germany). Moreover, all plasma samples contained antibodies interfering FVIII interactions with vWF and also interaction with phosphatidylserine measured by ELISA assays, as described by Shima and coworkers [14]. FVIII antibodies competed with murine monoclonal antibody ESH4 (American Diagnostica, USA) for phospholipid binding site at light chain C-terminus of FVIII as detected by an ELISA.

Materials

Recombinant FVIII (rFVIII, Recombinate, Baxter) was used throughout the study. Recombinant FVIII fragments (recombinant heavy chain, rHCh, and light chain, rLCh) were kindly provided by Dr. Mirella Ezban. Bovine serum albumin (BSA) and pNPP (p-nitrophenylphosphate) were purchased from Sigma. Other chemicals were of analytical grade. Majority of in vitro assays was carried through either in TBS (0.02 M Tris buffered saline, pH 7.2) or carbonate buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6).

Methods

FVIII antibodies in plasma samples taken during IT therapy were measured by Bethesda assay and by an ELISA method. Recombinant FVIII (Recombinate) was used as a coating agent for microplates.

Neutralization of FVIII:C Activity in the Presence and Absence of vWF

Plasma-derived FVIII-vWF concentrate (Haemoctin SDH) and recombinant FVIII (Recombinate) were diluted to 1 IU/ml of FVIII:C with TBS buffer containing 1% BSA and incubated with an equal volume of serially diluted (1:2 to 1:1000 in TBS-BSA) FVIII antibody plasma sample for 1 h at 37°C. Residual FVIII:C was determined by chromogenic method according to manufacturer's instructions (Coatest, Chromogenix AB, Italy) on a 96-well microplate. Percentage neutralization was calculated relative to a control. The control had the test plasma sample replaced by buffer or plasma from CRM negative hemophilia A patient without FVIII antibodies. The test plasma sample dilution giving 50% of neutralization of FVIII:C activity was found using both concentrates. The results were used to calculate a ratio of 50% neutralization of FVIII:C with rFVIII to that with FVIII-vWF.

Distribution of FVIII Light Chain and Heavy Chain Neutralizing Antibodies Measured by an ELISA Assay

Microplates (PolySorp, Nunc, Denmark) were coated with rFVIII (Recombinate) diluted to 8 IU/ml in carbonate buffer and incubated overnight at 2–8°C. The sites of nonspecific binding in the wells of the microplate were blocked with TBS containing 3% BSA for 1 h at room temperature and BSA was washed off with TBS-T (washing buffer, TBS with 0.1% Tween 20). Plasma samples with FVIII antibodies were diluted to 4 BU/ml, except test plasma samples with FVIII antibody titer $\leq$ 4 BU/ml, which were used undiluted. To neutralize FVIII antibodies, plasma samples were incubated with an equal volume of recombinant FVIII fragments diluted from 0.2 to 12.6 IU/ml according to labelled activity, for 1 h at 37°C. After incubation, aliquots of incubation mixture were transferred to antigen in the wells of the microplate and incubated for 2 h at 37°C. Bound FVIII antibodies were detected using rabbit anti-human IgG conjugated with alkaline phosphatase (AP) diluted 1:1000 (Dako, Denmark) and visualized with AP-substrate pNPP (p-nitrophenyl phosphate) and the color change was read at 405 nm. Two controls were included with each test: one mixture that had the antibody plasma sample replaced by buffer (minimal binding), and the second mixture that used buffer instead of recombinant FVIII fragments (maximum binding). The percentage neutralization was calculated as follows: 100 – ([binding with fragment – minimum binding]/[maximum binding – minimum binding]) × 100. The plateau was defined as the minimal concentration of FVIII fragment, which yielded the maximal (plateau) optical density (OD). When FVIII antibodies were partially neutralized by recombinant fragments, all values within the plateau region were averaged.

Distribution Antibodies Neutralized by FVIII Light Chain and Heavy Chain Measured by Chromogenic Method

The plasma samples were diluted to neutralization activity of 4 BU/ml, except plasma samples, which contained ≤ 4 BU/ml of FVIII antibodies and were used undiluted, and incubated with an equal volume of serially diluted recombinant FVIII fragments as described in the previous assay. An aliquot was removed for measuring FVIII antibodies by an ELISA assay. To the remaining mixture an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml was added and incubated for 1 h at 37 °C. Residual FVIII coagulation activity (FVIII:C) was measured by the chromogenic method. Percentage of neutralization was calculated using the above-mentioned formula but binding capacity was substituted with FVIII:C activity. Maximum FVIII:C activity was measured from the mixture of recombinant fragments and rFVIII without an antibody sample, incubated in the same conditions. If the tested plasma contained an FVIII antibody level <10 BU/ml, the plasma sample from the hemophilia A patient (CRM-negative and without FVIII antibodies) was used in the mixture to obtain the maximum FVIII:C activity. Minimum FVIII:C activity was found for each test sample incubated with rFVIII without any competitive FVIII fragments. The plateau value was similarly measured as in the previous assay.

ELISA Assay for Measuring FVIII Antibody IgG1–4 Subtypes

Immunoplates (PolySorp) were coated with 8 IU/ml recombinant FVIII (Recombinate) in carbonate buffer and incubated overnight at 2–8 C. After washing with washing buffer, the wells were blocked with TBS containing 5% BSA and incubated for 1 h at room temperature (RT). Plasma samples were diluted from 1:10 to 1:5000 in TBS, transferred to the wells and incubated for 2 h at 37 °C. Bound FVIII antibodies were detected after washing with sheep monoclonal anti-human IgG1, IgG2, IgG3 or IgG4 antibody (CLB, The Netherlands) diluted 1:1000 in TBS by incubating for 1 h at 37 °C followed by rabbit anti-sheep IgG-biotin conjugate (diluted 1:1000), incubating for 1 h at RT. Streptavidin – AP conjugate was added to the wells at dilution (1:1000) and incubated for 30 min at RT. Between all incubation steps the wells were thoroughly washed with TBS-T. The reaction was visualized using pNPP solution (maximum absorbency was achieved in 30 min) and stopped with 1 M NaOH. Buffer and FVIII antibody free plasma from severe hemophilia A patients were used instead of investigated plasma sample as controls. Cut-off value was defined as a mean value of optical densities measured for controls plus 3 standard deviations. The ELISA titer was defined as the maximal dilution of sample yielding OD exceeding the cut-off value.

ELISA Assay for Estimation of FVIII Light Chain Antibody IgG Subtype Distribution

Immunoplates (PolySorp) were coated with recombinant FVIII light chain diluted 1:1000 in TBS and incubated overnight at 2–8 °C. Afterwards the assay was continued as described in the section above.

Immunoblotting Analysis

Human FVIII (Haemoctin SDH) or recombinant FVIII fragments were cleaved by thrombin (0.01 IU thrombin per 1 IU of FVIII) for 30 min at 37°C and fragments were separated by SDS-PAGE under reduced and unreduced conditions using 10–15% gradient gel.

Inhibition of FVIII Binding to Phospholipids Measured by an ELISA Assay

One hundred microliters of methanol dissolved L-(-phosphatidyl)-l-serine (Sigma) at a concentration of 5 μg/ml was added to each well of polystyrene microplates (PolySorp) and air-dried at room temperature (RT). The wells were blocked by adding TBS containing 5% BSA and incubated for 1 h at RT. After washing with washing buffer, a mixture containing an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml in TBS-BSA (1% of BSA in TBS buffer) and a serially diluted (1:2 to 1:1000) test plasma sample was added to the wells of the microplate and incubated for 2 h at RT with continuous agitation. Bound FVIII was detected by adding mouse anti-human monoclonal antibody ESH5 (against FVIII heavy-chain, American Diagnostica, USA) diluted 1:500 in TBS. After washing goat anti-mouse IgG conjugated with AP (1:1000, Amersham Pharmacia Biotech) was added and incubated at RT for 1 h. The reaction was visualized by adding AP substrate pNPP. The control included in each test contained a mixture in which test plasma sample dilution was substituted with buffer.

Inhibition of FVIII Binding to von Willebrand Factor Measured by an ELISA Assay

Microplate wells (MaxiSorp, Dako) were coated with 100 μl of rabbit anti-human vWF antibody (1:4000, Dako, Denmark) in carbonate buffer and incubated overnight at 2–8°C. After washing, standard plasma (from a plasma pool of 22 plasmas from healthy persons) diluted to 1:20 in TBS-BSA was added and incubated for 1 h at RT. Factor VIII was dissociated from vWF by incubating with 0.4 M $CaCl_2$ for 30 min at RT. After washing, a mixture of a test plasma sample dilution with an equal volume of rFVIII (Recombinate) diluted to 2 IU/ml in TBS-BSA and incubated previously for 30 min at 37°C was added to the wells of the microplate and incubated for 2 h at RT. Bound FVIII was detected as described in the previous assay.

Competitive Enzyme-Linked Immunosorbent Assay

Microplates were coated with rFVIII (Recombinate) diluted to 8 IU/ml in carbonate buffer and incubated overnight. After washing with washing buffer, plates were blocked with 5% BSA in TBS for 1 h at RT. Then a mixture containing an equal volume of test plasma sample dilution (dilutions 1:10 and 1:100) with either of the murine FVIII monoclonal antibodies ESH5 or ESH4 diluted to 25 μg/ml was added

to the wells coated with FVIII and incubated for 2 h at RT with continuous agitation. The bound murine FVIII antibody was detected by using goat anti-mouse IgG diluted to 1:1000 (AP conjugate) and the reaction was visualized with AP substrate pNPP. The competition was calculated relative to the control where test plasma samples were replaced by buffer (no competition of binding to FVIII).

Statistical Analysis

Student *t*-test was used to compare the level of different IgG subtypes. Correlation was calculated according to Spearman rank order correlation coefficient. *P* value of 0.05 was considered significant.

Results

Characterization of FVIII Antibodies on Patient Before ITT

Immunoblotting analysis showed antibody reactivity against thrombin cleaved and non-cleaved FVIII light chain and A2 domain of FVIII. The neutralization activity of FVIII antibodies was reduced in the presence of FVIII-vWF complex (Fig. 4). The ratio of 50% neutralization of FVIII:C with rFVIII to FVIII:vWF complex was 4.5. The extent to which each epitope contributed to FVIII inhibition was determined by neutralization assay. Antibodies directed towards the heavy chain accounted for 30% of the FVIII inhibitory antibodies, while adding a recombinant FVIII light

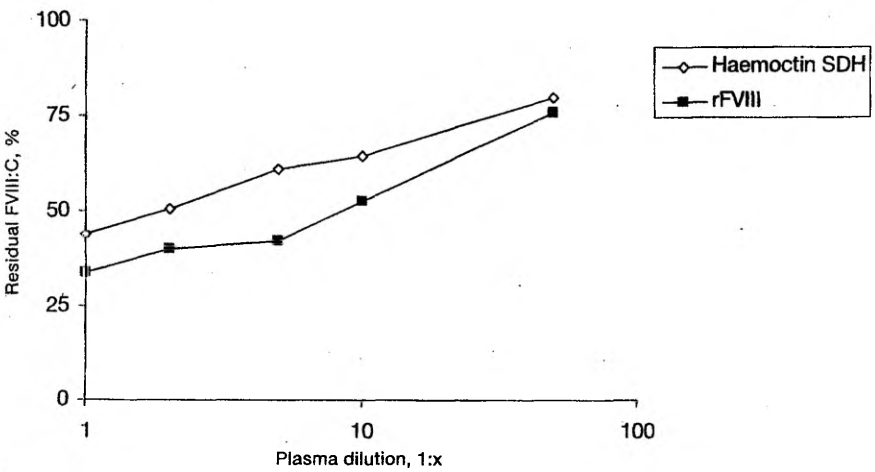

Fig. 4. Neutralization of FVIII:C activity by FVIII antibodies from the plasma sample of patient 1 taken before immune tolerance therapy. Test plasma dilutions were incubated with FVIII concentrate (rFVIII, Recombinate, Baxter) and FVIII concentrate containing vWF (FVIII-vWF, Haemoctin SDH, Biotest). The residual FVIII:C activity was measured by chromogenic method. Each data point represents the mean of two individual experiments

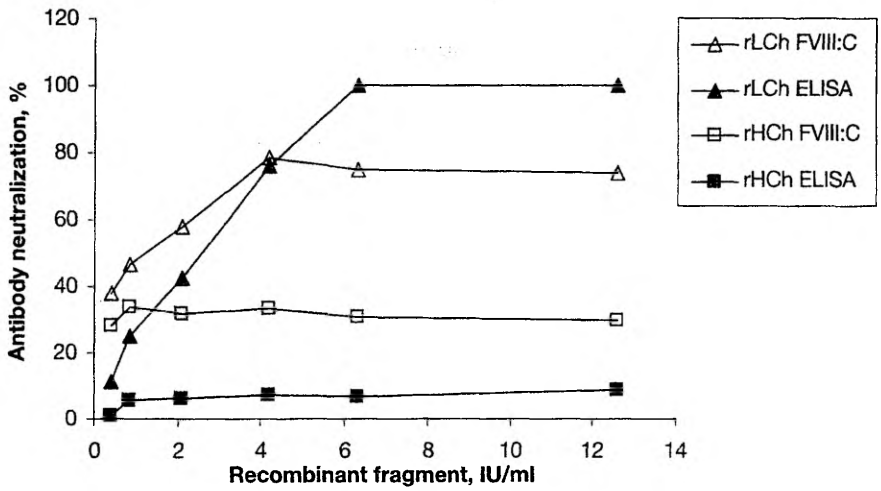

Fig. 5. Neutralization of FVIII antibodies from plasma sample from patient 1 taken before ITT. Plasma sample was diluted to 4 BU/ml and incubated with increasing concentration of recombinant FVIII light chain (rLCh) and heavy chain (rHCh). The neutralization of antibodies was measured by an ELISA assay (ELISA) and by a chromogenic method (FVIII:C) as described in Materials and Methods. Each data point represents the mean of two individual experiments

chain resulted in 78% of FVIII inhibitory antibody neutralization. In an ELISA assay 7% of antibodies were neutralized by recombinant heavy chain and 100% by recombinant light chain (Fig. 5). Antibodies inhibited FVIII interaction with phospholipids (50% of inhibition was achieved at the dilution 1:100) and FVIII interaction with vWF (50% of inhibition was achieved at the dilution 1:50) as measured by ELISA assays respectively [15]. FVIII antibodies of tested plasma samples competed with the murine monoclonal antibody ESH4 for the phospholipid binding site of the C-terminus of the light chain of FVIII as detected by ELISA.

Changes in Epitope Specificity of FVIII Antibodies During ITT

Epitope specificity of FVIII antibodies was measured in samples taken at different time points during IT therapy that contained an inhibitor titer of at least 2 BU/ml. During first continuous infusion (CI) treatment the rHCh neutralizing antibodies reached a non-detectable level measured by chromogenic method and ELISA assay (Fig. 2). However, Western blot analysis using the same recombinant fragments showed the presence of antibodies reacting with the light chain and heavy chain of FVIII in all investigated plasma samples during ITT. The epitope specificity of anti-FVIII light chain antibodies changed towards epitopes without functional activity. Factor VIII heavy chain antibodies were detected mostly by ELISA assay (6–21%) and the concentration of these antibodies reached an undetectable level at the end of the treatment. Theoretically, the total amount of FVIII light chain plus FVIII

heavy chain neutralizing antibodies should be 100%, but it was true for few plasma samples. Samples taken at the early stage of IT therapy contained in summary more than 100% of rHCh and rLCh neutralizing antibodies, while last plasma samples accounted less than 100%. Recombinant LCh or HCh concentration required for the first point of maximum neutralization was significantly different for these plasma samples. It can be explained by the presence of antibodies with different affinities to recombinant fragments. The lower slope of the neutralization curve of recombinant fragments explained this effect.

Changes in IgG Subtype Distribution During ITT

At the beginning of the ITT the patient had 29.8% of IgG4, 25.5% of IgG1, 25% of IgG2 and 19.6% of IgG3 subtype FVIII antibodies. Factor VIII light chain antibodies were present as follows: 38% of IgG4, 24% of IgG1, 21% of IgG3 and 17% of IgG2 subtypes. Results of IgG1–4 subtype distribution are shown on Fig. 2 D, E. During the first continuous infusion changes in FVIII antibody distribution were observed. Thereafter the distribution of IgG subtypes of FVIII:Ab remained unchanged concomitant with an increased level of IgG4 subtype antibodies. FVIII antibodies specific to FVIII light chain (FVIII-LCh:Ab) had a different IgG subtype distribution. During the ITT, factor VIII light chain antibodies of subtypes IgG1, IgG4, IgG3 were almost identically expressed and at a significantly higher level than IgG2 antibodies. At the beginning of treatment a fluctuation in FVIII-LCh:Ab subtype distribution was found to be analogous to that of FVIII:Ab. The amount of antibodies neutralized by recombinant FVIII light chain as measured by an ELISA assay correlated only with IgG2 subtype of FVIII:LCh:Ab (P=0.02). There were two peaks of IgG1 subtype levels. The first was observed after 1 month of treatment with the dose 100 IU/kg per day and another one after 1 month of the second continuous infusion. One of them was accompanied by an increase in the IgG1 subtype of FVIII light chain antibodies and the second one was associated with the appearance of antibodies against epitopes within the FVIII heavy chain.

FVIII Antibody Subtype Distribution in Hemophilia A Patients with Persistent FVIII Antibodies

In order to clarify whether the IgG subtype distribution of FVIII antibodies in hemophilia A patients with persistent FVIII antibodies differs from that detected during ITT, we investigated plasma samples of 12 hemophilia A patients. The characterization of patients is shown in Table 1. We used a sensitive ELISA assay for the detection of IgG subtypes directly from the plasma or serum sample. The estimated cut-off values of optical density for IgG1, IgG2, IgG3, and IgG4 FVIII antibodies were 0.113, 0.103, 0.089, and 0.089 respectively. The results are shown in Table 1. The most abundant were IgG4 type FVIII antibodies and the less abundant were IgG1 antibodies (P=0.02). IgG2 and IgG3 subtype FVIII antibodies were present in almost equal concentration, being significantly lower than IgG4 subtype

Table 1. Characterization of FVIII antibodies (*n.d.* not determined)

Plasma sample	Bethesda units	Distribution of FVIII antibodies				Distribution of FVIII light chain antibodies				Distribution of FVIII antibodies against recombinant light chain (rLCh) and heavy chain (rHCh)				Inversion in intron 22 of FVIII gene
		IgG1 %	IgG2 %	IgG3 %	IgG4 %	IgG1 %	IgG2 %	IgG3 %	IgG4 %	ELISA assay		Chromogenic method		
										rLCh %	rHCh %	rLCh %	rHCh %	
1	140	22	25.2	21	31.6	29.5	23.2	17	33.5	96	7	53	8	Type 1
2	9	25.5	25	19.6	29.8	24	17	21	38	100	7	78	30	Type 1
3	5	25	25	23	26	29.8	10.7	1.8	41.7	97	25	82	42	Rare inversion
4	7	30	14.5	21	33.9	74	7.4	0	18.5	92	20	93	3.5	n.d.
5	8	10.6	19	19	51	17	17	22	43.5	62	20	75	27	Type 1
6	37	32	17	21	32	12.5	12.5	25	50	93	58	82	21	Type 2
7	300	32.8	20.4	19.8	26.9	22.6	19.3	30.4	27.6	90	7	95	7	Type 1
8	280	16.2	34.2	17	32.4	3.4	13.7	31	51.7	95	6	96	6	n.d.
9	4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	90	3	83	15	n.d.
10	1	27.8	12.8	11	27.8	27.4	39.6	17.8	14.8	n.d.	n.d.	21	21	Type 2
11	5	26	24.6	24.6	24.6	55	22	11	11	70	74	64	65	n.d.
12	150	30	7	28.9	33.3	24	20	28	28	18	94	26	74	n.d.
Mean		25.3	20.4	20.5	31.8	29.0	18.4	20.1	32.6					
SD		6.8	7.5	4.5	7.1	19.7	8.5	9.1	13.8					

concentration ($P<0.01$ and $P<0.01$ respectively). The amount of antibodies recognizing light chain of FVIII measured by chromogenic method (Table 1) correlated well with the concentration of IgG2 subtype FVIII:Ab ($P<0.01$) in all plasma samples. The results of the Bethesda assay correlated fairly well with the ELISA titers of all four IgG subtypes of FVIII:Ab.

In another ELISA test, recombinant FVIII light chain was used to coat ELISA microplates. The relative distribution of IgG subtype FVIII light chain antibodies (FVIII-LCh:Ab) was compared with the relative subtype distribution of antibodies against a whole FVIII molecule. The estimated cut-off values for IgG1, IgG2, IgG3 and IgG4 were 0.222, 0.217, 0.167, and 0.166 respectively. The results of FVIII-LCh:Ab IgG subtypes are shown in Table 1. The higher relative amount of FVIII-LCh antibodies belonged to IgG4 subtype. The level of FVIII light chain antibodies decreased in the order IgG4>IgG1>IgG3>IgG2 and was similar to subtype distribution of antibodies against a FVIII whole molecule. The amount of FVIII light chain antibodies in all the investigated samples measured by the ELISA assay had a good correlation with IgG2 ($P=0.01$). The results of the Bethesda assay correlated with the results of all IgG subtypes ($P<0.01$).

Changes in FVIII:Ab IgG Subtype Distribution During the Treatment with Different FVIII Concentrates

One low responder hemophilia A patient (patient 2, Fig. 3) was treated with plasma derived FVIII concentrates of different purity for a long time. The samples were drawn at different time points and the subtype distribution of FVIII:Ab was studied. Factor VIII-LCh:Ab was not measured because of the limited amount of plasma. At the beginning of the treatment, IgG1 subtype FVIII antibodies were predominant; during the treatment with Kryobulin TIM 3 the level of IgG1 decreased and was replaced mainly by IgG4. In July 1997, FVIII concentrate was changed to monoclonal affinity purified FVIII concentrate Octonativ-M. A significant increase in IgG4 subtype was accompanied by a decrease in IgG1 subtype FVIII:Ab, while the levels IgG2 and IgG3 subtype remained unchanged. In Sept 1998, treatment was continued with FVIII-vWF concentrate and after 7 months of treatment all subtypes except IgG1 were expressed at almost equal levels. In June and July 1999, FVIII concentrate Octonativ-M was again administered. This caused another profound increase in IgG4 subtype level. Then the patient was treated with Haemate for 5 months and thereafter with monoclonal antibody purified FVIII concentrate (Aafact) for 6 months. As a result of this treatment the levels of FVIII antibodies of IgG1 and IgG3 subtypes increased while the levels of subtypes IgG4 and IgG2 decreased significantly.

Discussion

We investigated the changes in epitope specificity and IgG subtype distribution of FVIII antibodies influenced mainly by the administration of high purity FVIII concentrates containing vWF for immune tolerance induction. In vitro experiments

showed lower neutralization of FVIII concentrate containing vWF by FVIII antibodies of the studied patient and predicted a beneficial outcome using the same concentrate for immune tolerance therapy. The ITT effectively suppressed the production of inhibitory antibodies reacting mainly with FVIII light chain to a non-detectable level. FVIII antibodies of IgG4 subtype dominated over all other subtypes, except IgG1 subtype at two different time points, where the higher level of IgG1 was probably caused by the increased dosage of administered FVIII.

The patient was tolerated using FVIII-vWF concentrate. However, FVIII antibodies recognizing the FVIII light chain were expressed during the whole treatment, but the subtype distribution of FVIII light chain antibodies differed from that of patients with persistent inhibitor antibodies (Table 1). All IgG subtypes, except IgG2 subtype of FVIII light chain antibodies were expressed at equal levels during ITT. This could be due to the fact that the isolated FVIII light chain used in neutralization experiments and ELISA assays possesses a higher number of accessible epitopes on the surface than the form of heterodimer with FVIII heavy chain, as it circulates in plasma. The fact that the ELISA titer of antibodies bound to the FVIII light chain was higher than that bound to the whole FVIII molecule in some samples, suggests that FVIII light chain is more immunogenic than the whole FVIII molecule. Therefore after antigen processing there could be a higher number of small fragments of FVIII light chain presented by antigen presenting cells to T-lymphocytes. It has been shown that the size of epitope fragments capable of binding to the FVIII antibody is rather large (16–66 amino acids) and does not depend on whether the epitopes are linear, sequential or completely denaturated [16–18]. The antibody binding to denaturated plasma-derived FVIII and isolated recombinant FVIII light chain and heavy chain was observed in the investigated patient. The level of FVIII antibody subtypes, and the concentration and affinity of antibodies at different time points indicated the polyclonal antibody response to the treatment.

The results of our study are in agreement with previous reports, where on-demand treatment of hemophilia A patients is associated with the development of FVIII antibodies mainly of the IgG1 or IgG4 subtypes [19, 20]. The level of IgG1 subtype antibodies rises quickly after antigen administration (gene locates in 5'-end), while production of IgG2 and IgG4 subtypes probably needs more support of cytokines, produced by Th2 cells, because the genes encoding these subtypes are located close to 3'-end [12, 13]. FVIII antibodies with inhibitory and non-inhibitory antibodies were of the IgG4 subtype predominantly. During immune tolerance therapy with FVIII-vWF concentrate, the level of FVIII antibodies of IgG1 subtype increased significantly after 1 month of treatment and this rise was accompanied by the peaking of IgG1 subtype antibodies against the FVIII light chain. After the second continuous infusion the sudden rise in IgG1 subtype FVIII antibodies was observed, but the distribution of FVIII light chain antibodies remained unchanged. Surprisingly, antibodies neutralizing the FVIII heavy chain were detected in the same plasma sample. The enhanced level of the IgG1 subtype was observed as a result of the higher dosage of FVIII concentrate and could indicate the novel clone producing FVIII antibodies with different epitope specificity. One report [21] about unsuccessful ITT described an increased level of anti-A2 domain antibodies among heterogeneous antibodies in the patient and the authors suggested using the

epitope mapping as a prediction of the outcome of ITT. We found an increase in the FVIII heavy chain antibody level (IgG1 subtype), but continued ITT suppressed the production of these antibodies to undetectable level. Tolerance induction basis on the clonal selection. Therefore it is important that all FVIII antibody-producing clones have to become tolerant to FVIII administered at sufficiently high doses for a long time.

The effect of different FVIII concentrates used in replacement therapy on hemophilia A patients' immune system has been studied extensively. Results of in vitro studies showed decreased levels of IL-2, TNF, INF-γ and increased levels of IL-4, IL-10 in each different leukocyte subset after stimulation with FVIII concentrate of intermediate purity [11]. The immuno-suppressive effect of these concentrates was explained by the presence of TGF-β [11], which inhibits the proliferation of T-cells, maturation of cytolytic T-cells and the activation of macrophages. In vivo investigations in HIV-negative hemophiliacs have shown increased numbers of CD8+ cells and decreased numbers of CD4+ cells, indicating the immuno-stimulatory effect of treatment caused probably by the presence of TGF-β, which produces some Th2-like effects, like a co-stimulatory effect on CD8+ T-cells [22]. The FVIII concentrates of intermediate purity in general are more prone to decreasing the CD4+ cell count than immuno-affinity purified concentrates used for treatment of HIV-positive hemophilia A patients [23–25]. HIV-negative hemophiliacs who developed FVIII antibodies have been investigated so far only in one multicenter study [26]. This study showed no difference between FVIII-induced IFN-γ and IL-10 production by lymphocytes in hemophilia A patients who developed FVIII antibodies and those who did not.

We studied changes in FVIII antibody subtype distribution in a low responder hemophilia A patient treated with FVIII concentrates with different purities (patient 2). An ELISA assay used for estimating IgG subtypes was sensitive to detecting very low titers of FVIII antibodies. Analogously to patient 1, the higher production of IgG4 subtype FVIII antibodies was more or less pronounced in patient 2 at different time points. Long term treatment with FVIII-vWF concentrates in patient 2 resulted in a decrease of FVIII antibody titer measured in Bethesda units. The change to affinity purified FVIII concentrate Octonativ-M caused an increase in inhibitory antibodies, but isotype distribution of FVIII antibodies remained unchanged. An increase in the production of IgG1 and IgG3 subtype FVIII antibodies was observed during treatment with another immuno-affinity purified concentrate, Aafact. Unfortunately, plasma samples were taken occasionally and therefore it is not absolutely clear whether the observed changes in IgG subtype distribution were caused only by the switch to another concentrate. Apart from the purity of FVIII concentrate used for treatment, many other reasons like the dose and interval between doses could be responsible for different antibody response.

The usage of vWF in FVIII concentrates for ITT suppressed the production of FVIII light chain antibodies with inhibitory activity to non-detectable levels, while antibodies with non-inhibitory activity persisted. The beneficial outcome of using FVIII-vWF concentrates was observed also in patient 2, who had higher in vivo recovery with FVIII-vWF concentrate than with immuno-affinity purified concentrates. Probably after injection of FVIII concentrate of very high purity, both vWF and FVIII antibodies compete for binding sites on FVIII. If the antibodies possess

higher affinity than vWF, the antibody-antigen complex is formed and the FVIII molecule is removed from the circulation. It has been reported, that antibodies reacting with epitopes within the acidic region of the A3 domain (a3 region), C1 [27] and C2 domains [28], inhibit the binding of FVIII to vWF and are able to compete with vWF for binding site. As the intact light chain of FVIII has the maximum vWF binding affinity [29], we used the recombinant full-length FVIII light chain in the neutralization experiment to quantify all antibodies interfering with FVIII-vWF interaction. The vWF in the administered FVIII concentrates covers FVIII antibody binding sites on FVIII light chain and therefore delays the FVIII neutralization. Still half of the FVIII molecules dissociate from the vWF every 10 min [30]. Released FVIII is exposed to FVIII antibodies for forming FVIII-antibody complex, which is more stable than FVIII-vWF complex.

FVIII antibody response to the treatment is associated with the characteristics of the patient. The FVIII gene defect of the patient determines whether a non-functional FVIII molecule is produced or production of any protein is prevented. Hemophilia A patients with mild disease caused by point mutation in the FVIII gene may develop antibodies with higher neutralization activity against administered FVIII than against endogenous FVIII [31]. Investigated patients 1 and 2 had inversion in intron 22 in FVIII gene, which is the most common gene defect among hemophilia A patients and accompanies a higher incidence of FVIII antibody development dominantly against the FVIII light chain and the A2 domain of heavy chain [1, 32].

In addition to a gene defect causing FVIII deficiency, the antibody response to the treatment may be determined by the immune system of the particular patient. HLA genotype of MHC II alleles was characterized only in patient 2. DR8, DQ7 and DQ4 genotypes, as found in our patient, have been reported by Hay and Oldenburg, who detected these genotypes in some patients with inhibitors [33, 34]. However, no correlation between HLA allele genotypes and inhibitor incidence could be established in either of these reports.

So far no efficient treatment option has been found to prevent the development of FVIII antibodies and to achieve successful immune tolerance. However, some reports are more promising in that respect, Rossi et al. have used the blocking of CD40-CD40L interaction in mice [35]. Abolishing another important interaction, CD28-B7, caused less development of inhibitory antibodies, but unfortunately immune tolerance was not gained [36].

Thus it seems that multiple factors are involved in the development and production of FVIII antibodies in hemophilia A patients. Which factor is more relevant depends on the particular patient and further studies are needed to clarify their relative contribution to the success rate of immune tolerance therapy. Our study focused on the presence of vWF in FVIII concentrate used for ITT and we conclude that a beneficial outcome can be achieved using FVIII concentrate containing vWF in hemophilia A patients with antibodies reacting mainly with the FVIII light chain.

Acknowledgements. We gratefully acknowledge Dr. Margus Pooga for a helpful discussion. We thank Prof. Dr. F. H. Herrmann and Dr. R. Ljung for perfoming DNA analyses of haemophilia A patients. The study was in part supported by a Nordic coagulation grant.

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IV

Kallas A, Talpsep T.
The von Willebrand factor
collagen-binding activity assay: clinical application.
Annals of Hematology 2001; 80 (8): 466–471

Ann Hematol (2001) 80:466–471
DOI 10.1007/s002770100329

ORIGINAL ARTICLE

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The von Willebrand factor collagen-binding activity assay: clinical application

Received: 16 May 2000 / Accepted: 22 March 2001 / Published online: 30 May 2001

Abstract A collagen type III based collagen-binding assay was developed for measuring the functional activity of the von Willebrand factor. The assay had a low coefficient of variance (4.8%) for normal values under optimized conditions. The results of the collagen-binding activity (CBA) assay correlated with ristocetin cofactor activity tested in normal plasma samples (n=29). We found that the CBA of blood group O is lower than that of other blood groups. The test was used for the diagnosis of von Willebrand's disease (VWD) and for estimating the response to treatment with DDAVP (1-deamino-D-arginine-8 vasopressin) and factor VIII concentrate. A mean ratio of VWF antigen (VWF:Ag) to CBA of 1.5 indicated type 1 and of 2.7 indicated type 2 VWD. The increase in the collagen-binding activity of VWF released in type 1 VWD patients (n=7) after treatment with DDAVP was higher than the increase in the VWF antigen; this is characteristic of very high multimers with greater functional activity. Factor VIII concentrate Koate-HP (Bayer) administered to a patient with VWD type 3 had a mean residence time of 12.6 h for VWF:Ag and 11.2 h for CBA. These findings suggest that the collagen-binding assay is a useful test for measuring the functional activity of VWF in plasma samples, factor VIII concentrates, as well as for estimating the outcome of treatment.

Keywords Collagen · Factor VIII · Factor VIII concentrate · von Willebrand's disease · von Willebrand factor

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Introduction

The von Willebrand factor (VWF) is a glycoprotein with an essential role in primary haemostasis and in blood coagulation. VWF mediates the adhesion of platelets to subendothelium at the site of vascular injury and stabilizes factor VIII by forming a noncovalent complex [24]. VWF comprises multimers ranging from 500 kDa to 20,000 kDa linked together by disulfide bridges. Each subunit of the VWF carries binding sites for collagen, factor VIII, and the platelet receptor glycoprotein Ib/IXa and IIb/IIIa [24].

Different defects of the VWF molecule cause the most common inherited bleeding disorder, von Willebrand's disease (VWD), with a prevalence of 0.83% in the general population [23]. The simplified classification of VWD distinguishes between three groups: partial quantitative deficiency (type 1), qualitative deficiency (type 2), and total quantitative deficiency (type 3). The highly variable clinical picture and the presence of many different defects in the molecule of VWF complicate the diagnosis of VWD. To date, no single test that gives appropriate information about the various functions of VWF is available. Several analyses, such as the ristocetin cofactor aggregation method, the VWF antigen assay, assay of factor VIII coagulation activity, and others, are required for diagnosing VWD and for characterizing the defect in the particular patient. Factor VIII coagulation (F VIII:C) activity indirectly reflects the level of its stabilizing protein VWF [24]. The von Willebrand factor antigen level is measured by an ELISA method for which polyclonal VWF antibodies are used and which therefore has no effect on the functional activity of VWF. The assay of ristocetin cofactor (RCof) activity has been used widely for measuring the VWF ability to mediate platelet aggregation. The dimeric form of the antibiotic ristocetin is able to induce platelet aggregation by binding to both VWF and platelets [26]. The disadvantages of this test include the poor sensitivity (50%) [22], difficulties in standardization [3], and the lack of a physiological analogue. The monoclonal VWF antibody

based commercial ELISA test for describing RCof-like activity is less effective in discriminating VWD subtypes than the collagen-binding assay is [8]. A recently introduced simple ELISA test for collagen-binding activity (CBA) is sensitive and seems useful for screening VWD patients [3, 21, 27]. The binding of VWF to the collagen in the subendothelium is the first step in the initiation of primary haemostasis. The native conformation of VWF is essential for the interaction with collagen, but limited reduction of multimers to their dimeric form results in decreased binding to collagen [20]. We developed an ELISA assay using collagen type III for measuring VWF binding to collagen in different plasma samples and in different factor VIII concentrates. The test was applied in the diagnosis of VWD and for estimating the response of VWD patients to treatment with DDAVP (1-deamino-D-arginine-8 vasopressin) and factor VIII concentrates.

Materials and methods

Collagen-binding assay

Acid-soluble collagen type III from human placenta (C 4407, Sigma, USA) was dissolved in 3% acetic acid. The collagen solution was rapidly diluted to a final concentration of 10 µg/ml in PBS buffer, pH 7.3, and then immobilized on a microtitre plate. We used different polystyrene plates for coating: Nunc immunoplates PolySorp™, MaxiSorp™ (Nunc, Denmark), Nunclon™, and Titertek™ soft microplate (Titertek, USA). The collagen solution (100 µl) was incubated for 1 h at room temperature. After the plate was coated, it was blocked with 5% bovine serum albumin (BSA) in PBS with Tween (0.05%). A normal plasma pool from 22 donors was used as a local standard and calibrated against RCof activity of the 3rd International standard for factor VIII and von Willebrand in plasma (91/666). The standard plasma dilutions 1:10 to 1:120 corresponding to 0.1 to 2.0 IU/ml of CBA were used for constructing a standard curve. Investigated plasma samples were diluted 1:20, 1:40, and 1:60 and incubated in duplicate on a microtitre plate for 1 h at room temperature. Thereafter a peroxidase (HRP) conjugated rabbit antihuman VWF (P226 Dako, Denmark) in dilution 1:2000 was added and incubated for 1 h. The reaction was visualized by the addition of OPD substrate solution (*o*-phenylenediamine in 0.1 M citrate–phosphate buffer, pH 5.0, containing 0.03% H_2O_2). The reaction was stopped by the addition of 3 M H_2SO_4. The absorbance was read at 450 nm on a microplate reader, Labsystem Multiscan Plus (Labsystem, Finland).

Standards

The local plasma pool from 22 donors was used as a standard and calibrated against the WHO 3rd standard for factor VIII and VWF in plasma (code 91/666). The 4th International standard for blood coagulation F VIII:C concentrate (88/804) was used when factor VIII concentrates were studied.

Investigated samples

Collagen-binding activity was studied in different samples. Plasma samples of 29 blood donors (8 from each blood group except blood group AB with 5 samples), plasma samples from 30 diagnosed VWD patients, as well as samples of 5 different factor VIII concentrates were included in the study. Investigated factor VIII concentrates were Haemoctin SDH 250 IU (Biotest, Germany), Koate-HP 310 IU (Bayer, USA), lyophilized cryoprecipitate Kryo-AHG 400 IU (North Estonian Blood Centre, Estonia) and Haemate 1000 IU (Aventis Behring, USA). The factor VIII concentrates were diluted according to the manufacturers' instructions. Blood samples were obtained by vein puncture and centrifuged at 2000 *g*. As anticoagulant, 0.129 M sodium citrate was used (1 part anticoagulant to 9 parts blood). All samples were stored at –70 °C until assayed.

Assay for F VIII:C activity

F VIII:C activity was measured by a chromogenic method (Coatest F VIII:A, Chromogenix Instrumentation Laboratory, SpA, Italy). Factor VIII concentrates were prediluted to a final activity of 1 IU/ml of F VIII:C in TBS buffer containing 1% BSA.

ELISA assay for VWF:Ag

Rabbit antihuman VWF antibody (A082, Dako, Denmark) was used as a coating antibody and HRP rabbit antihuman VWF antibody (P226, Dako, Denmark) was used as a conjugate.

Ristocetin cofactor assay

Ristocetin cofactor activity of VWF was determined with the use of formaldehyde fixed washed platelets (FWP) for the agglutination test [14]. The FWP reagent was made as described previously [6].

VWF multimer analyses

The analysis of VWF multimers was done by SDS–agarose gel electrophoresis with 1.9% gel, according to Metzner et al., by the discontinuous submarine technique [18]. VWF multimers were detected with VWF antibody A082 (Dako) and goat anti-rabbit IgG alkaline phosphatase conjugate (Sigma). The bands were developed with substrate solution BCIP/NBT.

In vivo studies

The effect of DDAVP on release of VWF was investigated on seven patients with type 1 VWD. In plasma samples taken before and 10 min after i.v. injection of 0.3 µg/kg Octostim (Ferring, Sweden), F VIII:C activity, VWF:Ag, RCof, and CBA activities were measured. Multimeric composition before and after treatment was measured by Western blotting and bleeding time was measured by the Ivy method with the Simple II device.

Pharmacokinetic study

Factor VIII concentrate Koate-HP (Bayer) (dose 45 IU/kg) was injected into a patient (27 years old, body weight 51 kg) with type 3 VWD. Plasma samples were collected before and 10 min, 30 min, 1, 3, 6, 9, 12, 24, 26, 28, 48 h after administration. The collagen-binding activity, VWF:Ag, and F VIII:C were measured in all samples. The study was repeated after 12 months on the same patient and under the same conditions. A model-independent method was used for the calculation of the pharmacokinetic parameters (AUC, MRT) [17]. The half-life was calculated as $T_{1/2}$=MRT/1.443. In vivo recovery was calculated as a percentage of the measured post-infusion rise to expected rise. The expected rise was calculated by division of the injected dose by the estimated plasma volume (45 ml plasma to 1 kg body weight).

The Ethics Committee of the University of Tartu, Estonia approved the study protocol.

Statistical analysis

Results are expressed as the mean and standard deviation (SD) with 95% confidence interval. The differences between the results

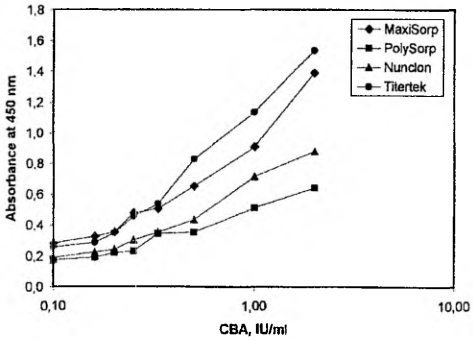

Fig. 1 The effect of different microplates on the collagen-binding assay. The results given are the mean values obtained in at least two replicated experiments

of the different blood groups were analysed by the *t* test for independent samples; a *P* value of 0.02 was considered significant. Calculation of the correlation between VWF:Ag and RCof activity was based on the Spearman rank order correlation coefficient.

Results

Optimization of the collagen-binding assay

VWF collagen-binding activity was measured by the ELISA assay. Microplates were coated with collagen type III from human placenta and incubated with standard plasma dilutions. The bound VWF was detected with polyclonal VWF antibody. Different polystyrene immunoplates were tested for suitability for the assay. VWF had the highest binding ability to collagen immobilized on the Titertek microplate (Fig. 1). With the PolySorp immunoplate, the assay had poor sensitivity, indicating a lower number of active binding sites on collagen available for the interaction with VWF. Next, we compared the use of different collagen concentrations for the coating of microplates. At a collagen concentration of 20 μg/ml the results were not significantly better than at 10 μg/ml. In the following experiments we used a collagen solution of 10 μg/ml for coating the Titertek microplates. Under these conditions the absorbance curve was linear at CBA values from 0.25 IU/ml to 2.0 IU/ml. The samples investigated were diluted so that the results could be read from the linear part of the standard graph. The intra-assay coefficient of variance was 4.8% for 1 IU/ml of collagen-binding activity and 14% for 0.5 IU/ml of CBA.

Reference range

The collagen binding of VWF was measured in plasma samples of blood donors with the aim to establish normal reference values. The mean collagen-binding activity of 29 plasma samples was 0.92 IU/ml (Table 1). The results of the collagen-binding assay correlated with the VWF antigen values (Spearman rank order correlation coefficient r=0.76) and with ristocetin cofactor activity (r=0.67). Blood group O had the lowest values for VWF:Ag, RCof, and CBA (Table 1), compared to blood groups A, B, and AB; however, we could not find a statistically significant difference, because of the small number of samples representing each blood group. All blood donor samples had the normal multimeric structure of VWF measured by Western blotting.

Table 1 Von Willebrand factor activity in samples of blood donors. Results shown are the mean and standard deviation (95% CI)

Blood group	*n*	CBA IU/ml (95% CI)	VWF:Ag IU/ml (95% CI)	RCof IU/ml (95% CI)
A	8	1.04±0.47 (0.64–1.43)	1.21±0.42 (0.86–1.57)	0.97±0.28* (0.74–1.20)
O	8	0.70±0.26* (0.48–0.92)	0.92±0.22* (0.73–1.10)	0.67±0.27* (0.44–0.90)
B	8	0.99±0.08* (0.97–1.07)	1.14±0.17* (0.99–1.29)	0.84±0.18 (0.69–0.99)
AB	5	0.96±0.06* (0.89–1.04)	1.05±0.24 (0.76–1.34)	1.06±0.24 (0.75–1.35)
Mean		0.92±0.31	1.05±0.29	0.87±0.28

*Statistical difference compared with group O (P<0.02)

Table 2 Von Willebrand factor in plasma samples of VWD patients

Type of VWD	*n*	CBA IU/ml	VWF:Ag IU/ml	RCof IU/ml	F VIII:C IU/ml
1	17	0.52±0.33	0.63±0.30	0.57±0.21	0.60±0.27
2A	10	0.42±0.25	0.62±0.32	0.43±0.14	0.68±0.48
3	3	0.02±0.01	0.09±0.02	0.14±0.04	0.05±0.03

Collagen-binding activity in samples of VWD patients

We screened samples of 30 patients diagnosed with VWD to evaluate the ability of the collagen-binding assay to detect high molecular weight multimers of VWF (Table 2). Type 1 VWD was diagnosed in 17 patients, type 2A in 10 patients, and type 3 in 3 patients. Diagnosis was based on the values of VWF:Ag, F VIII:C, and RCof activities and on the agarose gel electrophoresis pattern of the VWF multimers. The results of the collagen-binding assay correlated well with the values of the VWF antigen (r=0.76). The mean ratio VWF:Ag/CBA was 1.5±0.9 for type 1, 2.7±1.9 for type 2A, and 7.1±3.9 for type 3 VWD.

The effect of DDAVP on the release of functional VWF

Patients with VWD can be treated with DDAVP or with factor VIII concentrates. The effect of DDAVP on the re-

Table 3 Von Willebrand factor activity in different factor VIII concentrates

Factor VIII concentrate	*n*	F VIII:C IU/ml	VWF:Ag IU/ml	RCof IU/ml	CBA IU/ml	VWF:Ag/CBA	No. of multimers
Haemoctin SDH (Biotest)	6	58.4±3.5	31.8±4.6	13.3±3.2	8.5±1.9	4.0±1.6	14
Koate-HP (Bayer)	6	114.4±39.2	251.4±79.5	118.8±1.7	96.5±42.0	2.4±0.4	16
Kryo-AHG (North Estonian Blood Centre)	6	8.5±2.2	12.2±3.1	8.43±1.7	10.2±3.1	1.4±0.4	16
Haemate (Aventis Behring)	1	47.6	89.1	85.5	81.8	1.1	17

lease of functional VWF was measured in seven patients with type 1 VWD by collagen-binding assay. Treatment with DDAVP increased VWF binding to collagen 4.6±2.5 times, while the VWF antigen level increased 2.4±1.1 times. The ratio VWF:Ag/CBA decreased from 1.23±0.68 to 0.67±0.30 after treatment. In four patients the bleeding time decreased. High molecular weight multimers of VWF appeared or their concentration increased after DDAVP treatment; this is in good agreement with the collagen-binding activity of VWF.

Collagen-binding activity of VWF in factor VIII concentrates

The collagen-binding activity was investigated in different factor VIII concentrates containing VWF available in Estonia. Also included in the study were locally produced single-donor lyophilized cryoprecipitate (Kryo-AHG) and one sample of Haemate concentrate (Aventis), which is indicated for the treatment of VWD. All investigated concentrates were prediluted with TBS buffer with 1% bovine serum albumin. No significant changes were seen when plasma from a VWD type 3 patient was used instead of the buffer in the collagen-binding assay. The highest VWF content was in Koate-HP concentrate (Table 3), with a good correlation between the results of F VIII:C activity and VWF:Ag (r=0.94), and also between the results of the VWF antigen and the collagen-binding activity assays (r=0.96). Factor VIII concentrate Haemoctin SDH (Biotest) showed correlation only between the results of the VWF:Ag and RCof activity (r=0.94). The ratio VWF:Ag/CBA of factor VIII concentrate Haemate was 1.1, while that for the lyophilized cryoprecipitate (Kryo-AHG) was 1.4±0.4; this indicates the presence of functional VWF in both products.

Pharmacokinetic studies

Factor VIII concentrate Koate-HP (Bayer) was chosen for the in vivo experiments, because previous investigations indicated that this concentrate has a high content of functional VWF. Pharmacokinetic parameters were studied in the patient with type 3 VWD. A dose of 46 IU/kg of F VIII:C (97 IU/kg of VWF:Ag and 38 IU/kg of CBA) was administered and the results were compared with another experiment carried out 12 months later on the same patient and under the same conditions (the second dose was 50 IU/kg of F VIII:C, 113 IU/kg of VWF, and 43 IU/kg of CBA). In the meantime, the patient's bleeding (mainly in the target left knee joint) was successfully treated with the same concentrate. The first and second infusions did not differ with regard to dose and response. The decreasing VWF:Ag, CBA activity, and F VIII:C after administration of the second dose are shown in Fig. 2. The average mean residence time (MRT) calculated from the VWF:Ag results was 12.6 h and from the CBA assay results was 11.2 h. The VWF administered was able to stabilize factor VIII, and the second peak of F VIII:C was seen within 12 h after the first injection. The mean residence time for F VIII:C was 18 h. The mean half-lives for CBA, VWF:Ag, and F VIII:C were 7.7 h, 8.7 h, and 12.6 h, respectively. The mean in vivo recovery calculated from the VWF:Ag values was 53.1%, and was 50% for the CBA results. High molecular weight multimers appeared in the plasma sample taken 10 min after injection. The multimeric composition of VWF was similar to VWF in the Koate-HP and correlated with the collagen-binding activity of VWF.

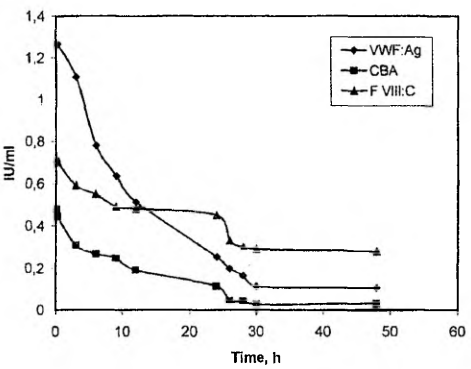

Fig. 2 The decrease in VWF and factor VIII after administration of Koate-HP (Bayer) to the patient with type 3 VWD. The results were obtained after the second injection. The injected dose was 50 IU/kg of F VIII:C, 113 IU/kg of VWF, and 43 IU/kg of CBA

Discussion

We optimized the collagen-binding assay to measure VWF activity for discrimination of VWD subtypes and for estimating the response to treatment with DDAVP and factor VIII concentrates. The assay gives information about the functional activity of VWF, indicating the presence or absence of high and intermediate molecular weight multimers. Results of the optimized collagen-binding assay correlated well with VWF antigen values and ristocetin cofactor activity when normal plasma samples were tested.

Our results demonstrate the importance of optimizing the assay conditions to reach an excellent calibration curve and a low intra-assay coefficient of variance. The different polystyrene microplates had different effects on the collagen–VWF interaction: some microplates caused a lower number of collagen-binding sites to be available for interaction with the VWF molecule. Previous studies have used the covalent linkage of collagen onto the surface, which overcomes the problem of steric hindrance of the large VWF molecule [9, 27]. We had good results in the CBA assay with the Titertek non-activated low-cost immunoplate. Microplates were coated with collagen type III, which contains the most critical binding site for VWF in the sequence 541–558 of the α1(III) chain [28]. Compared to type I collagen, type III forms less thick fibrils and more surface is available for interaction with large ligands such as VWF [20]. The collagen quaternary structure and the native conformation of the VWF domains are both essential for effective collagen–VWF interaction [4]. Surface plasmon technology shows excellent binding of collagen type III to VWF [27]. Because of these advantages, collagen type III has been widely used in CBA assays [7, 9, 27]. An optimal collagen concentration (10 μg/ml) provided a low variation coefficient (4.8% for normal values) and good discrimination between different types of VWD. The borderline value of the ratio VWF:Ag/CBA distinguishing type 1 and type 2A VWD was 2.7. The results of this study agree with those from previous ones that indicated the capability of the test for diagnosing subtypes of VWD [3, 7].

Favaloro recommended including plasmas of type 2A and 2B VWD patients as controls in every test [7]. Increasing the number of abnormal controls is only reasonable if the cost of analysis is low. Our CBA assay makes it possible for a few plasma samples to be analysed at a time, because only the necessary number of microplate strips need to be coated with collagen. Therefore, this test can be used for the diagnosis and treatment-response evaluation in laboratories of haemophilia centres with a low number of patients at a reasonable price.

The treatment preferred for VWD type 1 patients is administration of DDAVP, which causes the release of the endogenous VWF from Weibel-Palade bodies [29]. Compared to VWF antigen values, the CBA values of released VWF were higher (VWF:Ag/CBA before treatment 1.23, after treatment 0.67). The increased interaction between VWF and collagen may be associated with the appearance of ultra-high molecular weight multimers, which have the highest thrombogenic potential and have been found in the plasma of a VWD type 2A patient 15 min after DDAVP treatment [2].

The sensitivity of the CBA assay in the detection of high molecular weight multimers was tested by analysing VWF containing factor VIII concentrates. The correlation between VWF:Ag and CBA values as well as the ratio VWF:Ag/CBA provide information about the degree of degradation of VWF. Ramasamy and co-workers demonstrated the capability of the CBA assay to characterize VWF in a small-scale manufacture of the concentrate, but they used collagen type I in the assay and the assay had a coefficient of variance of 12.5% for normal values [21]. European Pharmacopoeia (EP) proposes the CBA method for the determination of the biological activity of VWF in factor VIII concentrates [5]. Our homemade CBA assay can be used for estimating the content of the functional VWF of factor VIII concentrate in vitro and for evaluating the outcome of administered factor VIII concentrate in vivo. The in vivo recovery of VWF measured in samples taken 10 min after injection, calculated from values of the collagen-binding assay, was 50%. Proteolytic degradation of the subunit reduces the size of the largest VWF multimer in plasma, resulting in intermediate and low molecular weight multimers [10]. Injected VWF is probably exposed to proteolytic cleavage and the concentration of high molecular weight multimers decreases. The mean residence time (MRT) was used for characterizing the outcome of the administered factor VIII concentrate, because it gives information on how long the injected substance is kept in circulation. MRT measured by the collagen-binding assay was 12 h; this indicates that functionally active VWF circulates in plasma for this amount of time.

Among VWD type 1 patients, 70% belonged to blood group O [11, 19]. Ristocetin cofactor activity, F VIII:C, VWF:Ag, as well as factor VIII antigen are about 25% lower in healthy persons with blood group O [1, 11, 16, 23]. VWF contains *N*-linked oligosaccharides in the A1 domain [15], which are essential for VWF binding to GP Ib/IXa receptors on the platelet surface. Removal of A and B group antigens from the VWF decreases ristocetin cofactor activity, but binding to collagen remains unchanged [25]. The main binding sites of the fibrillar collagens type I and III are located in the A3 domain, while the collagen type VI binding site is in the A1 domain of the VWF molecule [12, 13, 20]. We found lower CBA values in blood group O plasma samples. Probably there is another mechanism responsible for the lower CBA. However, the use of ABO-adjusted ranges for VWF levels might not be essential for diagnosis, because bleeding symptoms may depend on the VWF level regardless of the ABO type [19]. In addition to low levels of the VWF antigen and activity, the bleeding history as well as inheritance are essential for the diagnosis and treatment of VWD type 1.

In conclusion, our results suggest that the ratio VWF:Ag/CBA can be used for the diagnosis of VWD

before analysis of the multimeric composition of VWF. Establishing an international standard for collagen-binding activity could make this method a standardized procedure for measuring the functional activity of VWF in plasma samples, for estimating the dose of VWF containing concentrates, and for evaluating the response to treatment.

Acknowledgements We thank the staff of Tartu University Children's Hospital for helping with pharmacokinetic studies, Kadi Krinal for technical assistance, and Dr. Ravindra Sarode for useful discussions.

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ISSN 1024–395X
ISBN 9985–56–654–8