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Acquired haemophilia caused by non-haemophilic factor VIII gene variants

Andreas Tiede, Roswith Eisert, Andreas Czwalinna, Wolfgang Miesbach, Inge Scharrer, Arnold Ganser

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Corresponding Author: Dr. Andreas Tiede, M.D., Ph.D.

Corresponding Author's Institution: Hannover Medical School

First Author: Andreas Tiede, M.D., Ph.D.

Order of Authors: Andreas Tiede, M.D., Ph.D.; Roswith Eisert, MD; Andreas Czwalinna, PhD; Wolfgang Miesbach, MD; Inge Scharrer, Prof., MD; Arnold Ganser, Prof., MD

Abstract: The aetiology of anti-factor VIII (FVIII) autoantibody formation in acquired haemophilia remains unknown. We hypothesised that encounter of antigenically different, allogeneic FVIII may challenge inhibitor formation after presentation on MHC class II. Eighteen consecutive cases with acquired haemophilia were enrolled (9 females, 9 males). A control group comprised 50 male and 50 female healthy blood donors. The coding region of the FVIII gene and the HLA-DRB1 genotype were studied. The presentation of foreign FVIII variants on the patient's MHC class II alleles was predicted using SYFPEITHI algorithm. A rare FVIII variant (E2004K) was found in 1 patient with acquired haemophilia after massive transfusion; the 2004K allele was predicted to be presented on the patient's HLA-DRB1*0101. Moreover, distribution of a polymorphism (D1241E) was significantly skewed comparing patients and controls. 3 of 3 patients with transfusion-associated disease carried 1241D in homozygous or hemizygous form and were predicted to present 1241E (foreign), but not 1241D (self), on their HLA-DRB1*0301. Therefore, encounter of 1241E may result in the presentation of a new T cell epitope in these patients. The same conditions were not found in any patient with acquired haemophilia of other causes. The expected frequency in the general Caucasoid population undergoing transfusion is 3 to 4 %. In conclusion, encounter of variant allogeneic FVIII presented on a suitable MHC background could be a risk factor for inhibitor formation.

Response to Reviewers: Dear Professor Eichinger,

Thank you for considering our manuscript for publication in Annals of Hematology. We would also like to thank the reviewer for valuable comments. We have addressed the comments as follows:

* Only 3 cases of transfusion-associated acquired hemophilia: Indeed, acquired hemophilia associated with transfusion has rarely been reported. In our series, 3 cases among 18 consecutive patients are even more than expected from the literature. Our study suggest that immune recognition of certain FVIII variants could play a role in the pathogenesis, but formal proof would require much higher numbers of transfusion-associated cases that, in all probability, will never be collected.

* Transfusion in the other cases: No, transfusion was not documented in any other case of our series. Medical histories were taken by the authors or other well-trained physicians of the department.

We are sure that an association with transfusion would not have been missed in these patients. We have acknowledged this fact in the Methods section (p. 4, first para).

* Frequency of 1241E in blood donations: Based on the known allelic distribution of the 1241E allele and the known sex distribution of blood donors, we determined the frequency of 1241E in single-donor blood products as 21 % (p. 4, last para).

* Transfused units and interval between transfusion and acquired hemophilia: all transfusion-associated cases received multiple transfusions. Time between first transfusion and acquired hemophilia ranged between 5 days and several years (p. 6, first para).

We hope that with the revisions made, our manuscript will be acceptable for Annals of Hematology.

Kind regards,
Andreas Tiede

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Title

Acquired Haemophilia Caused by Non-haemophilic Factor VIII Gene Variants

Authors

Andreas Tiede,¹ Roswith Eisert,¹ Andreas Czwalinna,¹ Wolfgang Miesbach,² Inge Scharrer,² and Arnold Ganser¹

Affiliations

- 1 Department of Haematology, Haemostasis, Oncology and Stem Cell Transplantation, Hannover Medical School, Hannover, Germany
- 2 Department of Internal Medicine, University Hospital Frankfurt, Germany

Short title

Factor VIII Gene Variants in Acquired Haemophilia

Correspondence

Andreas Tiede, MD, PhD, Hannover Medical School, Dept. Haematology, Haemostasis, Oncology and Stem Cell Transplantation, Feodor Lynen Str. 5, 30625 Hannover, Germany, tel.: + 49-511-60060430, fax: + 49-511-60060435, email: tiede.andreas@mh-hannover.de

Abstract

The aetiology of anti-factor VIII (FVIII) autoantibody formation in acquired haemophilia remains unknown. We hypothesised that encounter of antigenically different, allogeneic FVIII may challenge inhibitor formation after presentation on MHC class II. Eighteen consecutive cases with acquired haemophilia were enrolled (9 females, 9 males). A control group comprised 50 male and 50 female healthy blood donors. The coding region of the FVIII gene and the HLA-DRB1 genotype were studied. The presentation of foreign FVIII variants on the patient's MHC class II alleles was predicted using SYFPEITHI algorithm. A rare FVIII variant (E2004K) was found in 1 patient with acquired haemophilia after massive transfusion; the 2004K allele was predicted to be presented on the patient's HLA-DRB1*0101. Moreover, distribution of a polymorphism (D1241E) was significantly skewed comparing patients and controls. 3 of 3 patients with transfusion-associated disease carried 1241D in homozygous or hemizygous form and were predicted to present 1241E (foreign), but not 1241D (self), on their HLA-DRB1*0301. Therefore, encounter of 1241E may result in the presentation of a new T cell epitope in these patients. The same conditions were not found in any patient with acquired haemophilia of other causes. The expected frequency in the general Caucasoid population undergoing transfusion is 3 to 4 %. In conclusion, encounter of variant allogeneic FVIII presented on a suitable MHC background could be a risk factor for inhibitor formation.

Abstract word count: 225

Keywords

Acquired haemophilia • Factor VIII • Autoimmunity • MHC presentation • Transfusion

Introduction

Acquired haemophilia is a rare disorder characterised by a sudden onset of severe subcutaneous, muscle or gastrointestinal bleeding. The haemostatic abnormality is due to autoantibodies inhibiting the activity of coagulation factor VIII (FVIII). In approximately 50 % of cases, acquired haemophilia is associated with other autoimmune disorders, malignancy, the peripartum, medication or multiple transfusions [1-4]. The pathogenesis of FVIII autoantibody formation in these cases as well as in idiopathic acquired haemophilia remains largely unknown.

Inhibitory antibodies against FVIII also occur as a complication of protein replacement therapy in congenital haemophilia A. FVIII antibodies occurring in cross-reactive material (CRM)-positive haemophilia A are sometimes specific against allogeneic (foreign, wild-type) FVIII [5]. However, more often these inhibitors cross-react with autologous (self, mutant) FVIII diminishing the patient's basal FVIII activity [6-9].

Current notion of inhibitor formation in CRM-positive haemophilia A is that FVIII missense mutations may alter T cell epitopes presented by the major histocompatibility complex (MHC) class II molecules [7]. This led us to hypothesize that also acquired haemophilia may sometimes result from difference between autologous and allogeneic FVIII. Such difference might induce an immune reaction after presentation of allogeneic FVIII on MHC class II. To proof the principle of such a mechanism, we analysed the FVIII protein sequence and MHC class II genotype in affected patients.

Design and methods

Subjects

We enrolled 18 consecutive patients with acquired haemophilia who were admitted to Hannover Medical School (n=12) or Frankfurt University Hospital (n=6) between 2001 and 2004. Acquired haemophilia was diagnosed due to (i) a recent onset of a bleeding history, (ii) a FVIII activity in plasma below 50 international units (IU) dl⁻¹, and (iii) a FVIII inhibitor ≥ 0.6 Bethesda units (BU) ml⁻¹ measured by the Nijmegen-modified Bethesda assay [10]. Histories were taken by the authors or other physicians trained in disorders of haemostasis and a potential association with transfusion or pregnancy was evaluated in all cases. We also collected blood from the kindred of two patients with acquired haemophilia after delivery and from relatives of patient 5. A control group comprised 50 male and 50 female healthy blood donors (150 alleles). Informed consent was obtained from all study participants.

DNA based assays

Genomic DNA was isolated from EDTA-anticoagulated blood using a DNA extraction kit (Amersham Pharmacia, Freiburg, Germany) and stored at -20 °C. DNA concentration and purity was assessed by absorbance at 260 and 280 nm. Sequencing of the entire coding region and exon/intron boundaries of the FVIII gene was performed using the 310 ABI Prism Genetic Analyser and Big Dye Terminator sequencing chemistry (Perkin Elmer, Weiterstadt, Germany). The numbering of nucleotides is according to GenBank accession number M14113, of amino acid positions according to accession number P00451. Genetic analysis of the human leukocyte antigen (HLA)-DRB1 locus was performed using polymerase chain reaction (PCR) with locus-specific primers and immobilised sequence-specific oligonucleotide probe (SSOP) methods.

Prediction of MHC-presented peptides

A motif pattern-based prediction algorithm together with the SYFPEITHI database [11] was used to predict FVIII-derived peptides presented on HLA-DRB1 alleles. For other MHC class II genes, data were not available. Searches were performed either for the complete B domain (regarding D1241E) or A3 domain (regarding E2004K). All peptides were ranked according to the prediction score. Relative ranks range from 0.1 % to 99.9 % with lower numbers representing better scores. According to Rammensee *et al.*, the top 2 % of peptides (relative rank <2 %) represent naturally occurring T cell epitopes in 90 % of the predictions [11].

Probability calculations

We first calculated the frequency of 1241E and 1241D phenotypes in blood donations assuming the determined allelic frequency ($f_{1241D}=0.84$, $f_{1241E}=0.16$) and a female/male sex distribution of 0.35/0.65 among blood donors (Hannover Medical School Blood Bank):

$$p [1241D] = 0.65 \times 0.84 + 0.35 \times (1 - (1 - 0.84)^2) = 0.89$$

$$p [1241E] = 0.65 \times 0.16 + 0.35 \times (1 - (1 - 0.16)^2) = 0.21$$

We then estimated how frequently the assumptions made for the proposed pathomechanism are fulfilled in the general population undergoing transfusion assuming the HLA-DRB1 phenotype frequency among West European Caucasoids as given in the Database of Blood and Kidney Donors of Essen, Germany (accessible at www.allelefreqencies.net): *0301 (0.22), *0701 (0.17), and *1501 (0.33).

Results

Patients

Clinical and laboratory data of the patients are summarised in Table 1. Acquired haemophilia was associated with malignancy (n=5), delivery (n=3), transfusion (n=3), and bronchial asthma or chronic obstructive lung disease (n=2). Of the three patients, who developed acquired haemophilia after transfusion, one patient (no. 5) had first been massively transfused seven years earlier because of traumatic liver rupture. Now, on the day before an elective wound revision, a slightly prolonged activated partial thromboplastin time (APTT, 41 s, reference range 33-40 s) was documented. During the next 13 days, 32 units of red blood cells, 20 units of fresh frozen plasma and 1 platelet aphaeresis concentrate were administered due to bleeding and an increasingly prolonged APTT was noted. The other two patients received multiple units of red blood cells because of bleeding after percutaneous coronary intervention (no. 13) and trauma (no. 17, who also had von Willebrand disease type 2A). The exact number was not reported by the referring hospitals. APTT prolongation was first documented 5 and 12 days after first transfusion, respectively.

E2004K missense mutation

In one patient (no. 5), a hemizygous missense mutation at nucleotide 6238 (G>A) was found causing a substitution in the A3 domain (E2004K). This mutation does not cause congenital haemophilia A as demonstrated by the patient's unremarkable bleeding history prior to the onset of disease and a FVIII activity >100 % after eradication of the inhibitor. The mutation was inherited as it was found in a heterozygous state in the patient's mother (FVIII activity 196 IU dl⁻¹) and in two of his three sisters (FVIII activity not determined). Mutations of this residue were not found in any of the 100 controls enrolled in this study and have not been reported in the haemophilia A database [12]. The patient developed acquired haemophilia after multiple transfusions. A peptide containing E2004 (the wild-type variant foreign to the patient) ranked among the top 1 % of peptides predicted to be presented on the patient's MHC class II allele HLA-DRB1*0101.

D1241E polymorphism

A polymorphism was found at nucleotide 3951 (C>G) causing a substitution in the B domain (D1241E). Among 100 controls, the allelic frequency of 1241D and 1241E was 84 % and 16 %, respectively. Peptides containing 1241D or 1241E were predicted to be presented on HLA-DRB1 alleles *0701 (relative rank 1.1 %) and *1501 (1.5 %). Allele *0301 was predicted to present a peptide containing 1241E (1.8 %), but not 1241D. Alleles *0101, *0401 and *1101 were predicted not to present peptides containing 1241D or E.

We hypothesized that immunisation might happen in patients with either 1241D or 1241E in homozygous form (females) or hemizygous form (males) together with an HLA-DRB1 allele

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4 predicted to present the foreign variant. As shown in Table 2, three of three patients with
5 transfusion-associated disease were homozygous or hemizygous for 1241D (self) and were
6 predicted to present 1241E (foreign) on HLA-DRB1*0301. In these patients, HLA-DRB1*0301
7 was the only HLA allele predicted to present 1241E. Therefore, transfusion of 1241E (foreign)
8 is predicted to result in a new T cell epitope because 1241D (self) cannot be presented.
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11 We hypothesized that encounter of foreign FVIII variants may also happen during fetomaternal
12 transfusion in pregnancy. Three of three patients with post-partum disease were
13 homozygous for 1241D. In one patient, HLA-DRB1*1501 was predicted to present both 1241D
14 (self) and 1241E (foreign). The remaining two patients had HLA-DRB1 alleles for which
15 prediction was not available. Thus, the hypothesized pathomechanism cannot be ruled out in
16 these patients.
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20 In patients with acquired haemophilia due to other causes, data for prediction were complete
21 in eight of twelve. Six of eight patients were either heterozygous for D1241E or had HLA-DRB1
22 alleles predicted not to present the foreign variant. Two patients were hemizygous for 1241D
23 or 1241E and had HLA-DRB1 alleles predicted to present both variants of the polymorphism.
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26 We assumed that immunisation against 1241E is particularly likely in transfusion recipients
27 who are homozygous or hemizygous for 1241D and carry HLA-DRB1*0301, because this MHC
28 class II molecule is predicted to present 1241E, but not 1241D. We calculated the probability
29 of this assumption met in West European Caucasoids assuming the frequency of 1241D in
30 males ($f_{1241D}=0.84$) and 1241D/D in females ($f_{1241D/D}=0.84 \times 0.84=0.70$), the phenotypic
31 frequency of HLA-DRB1*0301 ($f_{*0301}=0.22$) and the probability of a 1241E phenotype in a
32 single-donor blood product ($p_{1241E}=0.21$, see Design and Methods):
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$$36 \quad p = f_{1241D} \times f_{*0301} \times p_{1241E} = 0.04 \text{ (males)}$$

$$37 \quad p = f_{1241D/D} \times f_{*0301} \times p_{1241E} = 0.03 \text{ (females).}$$

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41 Thus, the assumptions made above are expected to be fulfilled in 3 to 4 % of the general
42 Caucasoid population undergoing transfusion. In transfusion-associated acquired haemophilia,
43 these assumptions were met in 3 of 3 cases.
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Discussion

We hypothesised that inhibitor formation in acquired haemophilia may sometimes be challenged by allogeneic FVIII variants encountered by administration of blood products or fetomaternal transfusion. Our hypothesis was applicable in all patients with exposure to allogeneic FVIII and prediction of MHC presentation available: in three out of three transfusion-related and one out of three pregnancy-related cases. In the remaining two pregnancy-related cases, the same mechanism cannot be ruled out because prediction of MHC presentation was not available. In one patient with transfusion-related acquired hemophilia we found an additional, rare non-haemophilic FVIII variant (E2004K) presented on the patient's MHC class II.

The probability of our results being a chance finding has been estimated using a control group. Concerning the E2004K variant, this mutation is exceptionally rare. The mutation introduces a major change in a T cell epitope, which is likely to elicit an immune response. Concerning the D1241E polymorphism, both variants were sufficiently frequent and some of the most prevalent MHC class II variants were capable of presenting. Therefore, alloimmunisation might be expected with a certain frequency in the general population undergoing transfusion. However, the epitope change introduced by the D1241E polymorphism seems to be minor since both amino acids are similar in charge and size. This may explain why most individuals undergoing transfusion do not develop a detectable immune response. It was striking that all individuals with transfusion-associated acquired haemophilia carried HLA-DRB1*0301. This allele was predicted to present 1241E, but not 1241D. Encounter of 1241E may result in a new T cell epitope in these patients, because the patient's self allele, 1241D, cannot be presented and central tolerance might not be achieved during T cell maturation in the thymus. In the general population, the same conditions are expected at a very low frequency. However, this frequency is still considerably higher compared to the incidence of acquired haemophilia. Similar to inhibitor formation in congenital haemophilia, the actual risk of inhibitor formation might be influenced by additional factors including immune response genes, danger signals and the dose of antigen encountered.

The immune reaction resulting in acquired haemophilia is apparently both autoreactive (as evidenced by the patient's decreased FVIII activity) and alloreactive (as demonstrated by the lack of response to FVIII protein replacement). Our hypothesis of inhibitor formation through alloimmunisation implies a switch from an initially alloreactive immune response towards a response that is both alloreactive and autoreactive. Current notion of inhibitor formation in CRM-positive haemophilia A is that allogeneic FVIII-derived peptides presented on MHC class II trigger a specific, alloreactive CD4 T cell response [13]. Subsequently, armed CD4 T helper cells can stimulate reactive B cells to produce alloreactive antibodies. However, the normal B cell

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4 repertoire contains a broad spectrum of autoreactive B cells including anti-FVIII B cells [14]. As
5 B and T cell epitopes may reside in different regions of a protein, armed alloreactive T cells can
6 stimulate any B cell presenting the allogeneic T cell epitope, regardless of the B cell epitope.
7 As a result, autoreactive B cells can be stimulated by alloreactive T cells (Figure 1). This
8 mechanism is also known as *determinant spreading* from other autoimmune disorders [15].
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11 Similar mechanisms might be at work in post-transfusion purpura, a syndrome characterised
12 by sudden onset of thrombocytopenia after blood transfusion. Patients have been shown to
13 be sensitised to allogeneic platelet antigens, most often HPA-1a. Pan-reactive platelet
14 antibodies including autoantibodies have been detected in the acute phase of disease [16]
15 indicating that a primarily alloreactive immune response resulted in loss of self tolerance.
16 Another example is the development of factor V inhibitors in patients receiving topical bovine
17 thrombin either directly or as a component of fibrin glue [17]. These preparations also contain
18 bovine factor V and have been reported to frequently induce autoreactive antibodies against
19 factor V [18]. Factor V inhibitors have also been reported following the use of human
20 thrombin preparations containing human factor V [19]. Therefore, some of the many known
21 factor V polymorphisms may be antigenically relevant in patients with appropriate MHC class II
22 alleles and trigger inhibitory immune responses. Recently, the development of pure red cell
23 aplasia has been reported due to erythropoietin autoantibodies in patients treated with
24 recombinant human erythropoietin [20, 21]. Although glycosylation differences between the
25 native and recombinant hormone are currently suspected to promote autoantibody formation
26 [21], it might be useful to look for rare erythropoietin variants in these patients.
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34 In summary, our study suggests that primary structure variants of the FVIII protein presented
35 on a suitable MHC class II background may trigger inhibitor formation after exposure to
36 allogeneic FVIII. The mechanism suggested here may not only be relevant for acquired
37 haemophilia. Generally, patients carrying variant or polymorphic proteins could develop
38 autoreactive inhibitors after therapeutic administration of antigenically different proteins.
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Figure legend

Figure 1 Different B cell and T cell epitopes resulting in *determinant spreading* of an immune response. (A) Normal situation: preformed autoreactive B cells recognizing a B cell epitope in autologous FVIII. Incorporation and MHC class II presentation of peptides does not result in B cell activation due to lack of autoreactive T cells. (B) After exposure to allogeneic FVIII. Allogeneic FVIII is incorporated through the same B cell epitope. A distant, allogeneic T cell epitope is recognised by alloreactive T cells. Following activation, these T cells can reciprocally stimulate autoreactive B cells resulting in both alloreactive and autoreactive anti-FVIII antibody production.

Table 1: Patient characteristics

Patient no.	Age (yr), sex (m/f)	Disease association	Bleeding manifestations	FVIII (IU dl ⁻¹)	Inhibitor (BU ml ⁻¹)
1	35, f	Post partum	Vaginal, subcutaneous, knee	<1	48
2	67, f	None	Muscle, haematuria	10	8
3	28, f	Post partum	Vaginal, subcutaneous	8	13
4	35, f	Post partum	Subcutaneous	<1	13
5	20, m	Surgery, multiple transfusion	GI, wound bleeding	<1	2.1
6	76, m	None	Subcutaneous	1.1	3.1
7	78, m	Lymphoma	After bone marrow biopsy	<1	>1000
8	79, f	COPD	Subcutaneous	<1	40
9	77, m	Colorectal cancer	Subcutaneous	<1	5.8
10	9, m	Atopy, nephrotic syndrome	Subcutaneous, ankle	6	7.6
11	81, f	None	Subcutaneous	<1	188
12	78, f	Colorectal cancer	Subcutaneous, muscle	<1	8
13	73, m	Transfusion	After percutaneous coronary intervention	4	1.5
14	74, m	None	Muscle	5	8.5
15	71, m	Colorectal cancer	Haematuria	<1	28
16	82, m	Colorectal cancer	Muscle	9.3	13
17	80, f	VWD, trauma, transfusion	Muscle, haematuria	1.6	68
18	72, f	Allergic asthma	Subcutaneous	1.5	6.6

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Table 2: Prediction of MHC class II presentation of the D1241E polymorphism

Patient no.	FVIII D1241E polymorphism	HLA-DRB1	Prediction result ¹
<i>Transfusion</i>			
5	D	0101, 0301	1241E on *0301 ²
13	D	0101, 0301	1241E on *0301
17	D/D	0301, 0401	1241E on *0301
<i>Post-partum</i>			
1	D/D	0801, 1103	-
3	D/D	0101, 1501	1241 D and E on *1501
4	D/D	1103, 1201	-
<i>Other</i>			
2	D/E	0301, 0701	Negative
6	E	0301, 0801	-
7	E	0301, 1103	-
8	D/D	0101, 1101	Negative
9	D	0401	Negative
10	E	0401, 0901	-
11	D/E	0101, 1601	Negative
12	D/E	1301, 1501	Negative
14	E	0701, 1501	1241 D and E on *1501
15	D	1301, 1501	1241 D and E on *1501
16	D	No data	-
18	D/D	0401, 0404	Negative

1 The mode of presentation is indicated as predicted by SYFPEITHI. 'Negative' indicates patients in whom the proposed pathomechanism could be ruled out because both HLA-DRB1 alleles were predicted *not* to present D1241E containing peptides or because the patient is heterozygous for D1241E. No result ('-') is given if presentation could be neither predicted nor ruled out due to lack of information for at least one of the HLA-DRB1 alleles.

2 This patient had an additional rare FVIII sequence variant (E2004K) presented on HLA-DRB1*0101.

Figure 1
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