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Inhibitor development in correlation to factor VIII genotypes

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Summary. Alloantibodies (inhibitors) against factor VIII (FVIII) develop in 20–30% of patients with severe haemophilia A and render classical FVIII substitution therapy ineffective. Several studies have shown that genetic factors, the type of FVIII gene mutation and immune response genes (e.g. the Major Histocompatibility Complexes), influence the risk of inhibitor formation. In particular, the type of FVIII gene mutation has proven to be a decisive risk factor. Patients with severe molecular gene defects (e.g. large deletions, nonsense mutations, intron-22 inversion) and no endogenous FVIII synthesis have a 7–10 times higher inhibitor prevalence than patients with milder molecular gene defects (e.g. missense mutations, small deletions, splice site mutations). To date, at least 10 distinct classes of mutations have been shown which have differing risks of associated inhibitor formation. A challenging observation in inhibitor patients is the heterogeneity of the antibody epitopes with respect to their number and their specifity. At least five epitopes in the FVIII molecule have been identified that constitute the targets for antibodies in most inhibitor patients. These epitopes are located in the ar3 region and the A2, A3, C1, C2 domains which correspond to the functional binding sites of the ligands of the FVIII protein. At present, the determinants of the characteristics of these epitopes and the subsequent inhibitor titre are unknown. A relationship of the mutation site and the epitope localization has been shown for some individual patients with mild haemophilia A. However, in severely affected haemophilia A patients, the influence of patient genetics on inhibitor titre and epitope specifity has yet to be elucidated.

Keywords: Haemophilia A, factor VIII, inhibitor, gene mutation, HLA system, epitopes

Introduction

Factor VIII (FVIII) antibodies affect about 30% of patients and usually occur in childhood within the first 10–20 exposure days of therapy [1,2]. The observations that the risk of inhibitor formation is influenced by the degree of severity, the history of inhibitors in family relatives, and ethnicity/race point to an important role of patient genetics in inhibitor formation. Molecular candidates for a genetic predisposition to inhibitor development are the mutation within the FVIII gene [3], genes involved in the immune response as Major Histocompatibility Complex (MHC) class I and II loci, and also other proteins participating in the presentation of antigens [4,5]. As the role of immune response genes may be amplified in the presence of an inflammatory process [6], both the underlying mutation in the FVIII gene and the individual characteristics of the immune system might influence the number of antibody epitopes on the FVIII molecule and the inhibitor titre (Fig. 1). Although, it is widely accepted that the driving forces of inhibitor formation are either the presentation of a novel or an immunologically altered FVIII antigen to the patient's immune system, the pathogenesis of inhibitor formation is only partly understood. Why only one-third of the patients with severe molecular defects develop an inhibitor while two thirds do not is still unknown. What is protecting the latter group against inhibitor formation? Which mechanisms determine the antibody epitopes on the surface of the FVIII protein and what makes an inhibitor become high titre, low titre or transient?

This paper will report on the current knowledge of the genetic background of inhibitor formation by addressing the role of the mutation in the FVIII gene, of the immune

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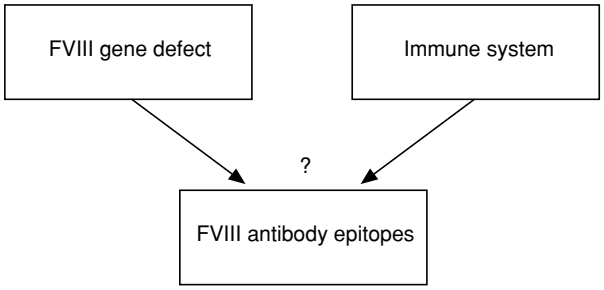

Fig. 1. Do the patient's genetics influence the nature of FVIII antibody epitopes?

response genes and of the antibody epitopes on the FVIII molecule.

FVIII genotype and risk of inhibitor development

An overview of the risk of inhibitor formation with respect to the underlying FVIII gene mutation type is shown in Fig. 2. In general, two groups can be formed. One group is comprised of severe molecular defects – so called null-mutations because they do not make any FVIII protein. These patients with large deletions, nonsense mutations, and intron-22 inversions exhibit an inhibitor prevalence of > 30%. The second group presents with small deletions, missense and splice site mutations which result in loss of function, but not complete absence of the FVIII protein, and exhibit an inhibitor prevalence of less than 10% [3]. Most of these mutation types can be further subdivided according to their risk of inhibitor formation. Data taken from the HAMSTeRS mutation register (*http://europium.csc.mrc.ac.uk*) [7] revealed that patients with *large deletions*, affecting more than one domain of the FVIII protein, are at the highest risk (about 75%) for developing an inhibitor, which is about 3-fold the risk of single domain deletions. *Nonsense-mutations* on the light chain increase the risk of inhibitor formation relative to those on the heavy chain, although the predicted truncated proteins would be shorter in patients with stop codons in the heavy chain. At present, no explanation for this observation exists. The third high risk mutation type with an inhibitor risk of about 30–35% is the *intron-22 inversion*. This mutation is the most prevalent in patients with severe haemophilia A, especially in the subgroup of patients who develop an inhibitor, where it can be found in up to 60% of patients [8].

Within the group of low-risk mutations, the mutation type of *small deletions/insertions* is of special interest, because it shows an unexpectedly low risk of inhibitor formation of 7.4% [3]. From the nature of the mutation type – most of the small deletions/insertions lead to a frameshift with a subsequent stop codon – an inhibitor risk similar to the nonsense mutations would be expected. Any explanation of this phenomenon was reported by Young *et al.* [9] who discovered an endogenous restoration of the reading frame by polymerase errors during DNA replication/RNA transcription in patients with small deletion/insertion mutations that were located at stretches of adenines. The resulting small amounts of endogenous FVIII protein (usually less than 0.01 IU mL^{-1} and often only visible by means of the thrombelastogram) protect against inhibitor development (Table 1) [10]. These patients have almost no risk for inhibitor development. In contrast, patients

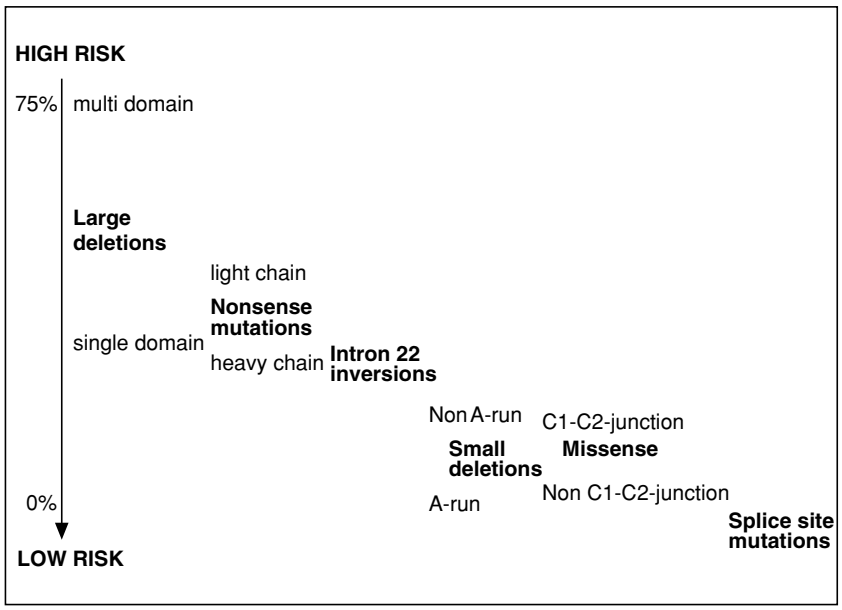

Fig. 2. Mutation types and risk of inhibitor development.

Table 1. Coagulation parameters of patients with small deletion/insertion mutations in exon 14 (upper part) and corresponding age patients with standard (stop codon, intron-22 inversion) mutations according to Oldenburg *et al.* [10]

Patients Mutation	FVIII:C Thrombelastogram		
	(IU/mL)	r (min)	r + k (min)
Group A			
HD25, Exon 14 DelACAC, Codon 1187/88	< 0.01	29	36
HD26, Exon 14, DelA, Codon 1192	< 0.01	26	39
HD26, Exon 14, DelA, Codon 1192	< 0.01	48	71
HD26, Exon 14, DelA, Codon 1192	< 0.01	26	35
Group B			
HP141, Exon 14, CGC(Arg)1689TGC(Stop)	< 0.01	158	199
HP131, Exon 13, TAC(Tyr)636TAG(Stop)	< 0.01	134	202
HP127, Exon 12, CGA(Arg)583TAG(Stop)	< 0.01	175	224
HI36, Proximale Intron 22 Inversion	< 0.01	178	259
HI40, Distale Intron 22 Inversion	< 0.01	159	219

with small deletions/insertions at other sites may well have a remarkable risk of inhibitor formation which, according to the data of HAMSTeRS, is 20.5% (17 of 83 patients).

Missense mutations represent another very interesting group of genetic defects with respect to inhibitor formation because beside its occurrence in severe haemophilia A, missense mutations are the main mutation type in patients with a nonsevere form of the disease. Most of these patients synthesize some endogenous protein. However in severe haemophilia patients the nonfunctional FVIII protein is sufficient to induce immune tolerance in most of the patients. Consequently, the inhibitor prevalence in this mutation type is as low as 4.3%. Interestingly, for patients with missense mutations in the C1 and C2 domains, the risk of inhibitor formation is 4-fold higher (in HAMSTeRS 17.7%, 36 of 203 patients), thus suggesting that this part of the FVIII molecule is especially immunogenic. This assumption is supported by several publications. Suzuki *et al.* [11] reported that a mutation at amino acid 2153 modifies the antigenicity of the C2 domain. Investigating 26 patients with nonsevere haemophilia A and inhibitor development, Hay *et al.* [12] found that in nine of 11 families in which the FVIII gene defect was known, the mutation affects the C1/C2 junction (amino acids 2009–2229). Jacquemin *et al.* [13] described that mutations within the C1-domain (Ile2098Ser, Asn2129Ser, Arg2150His, Trp2229Cys, Gln2246Arg) are associated with a normal function of the FVIII molecule except that the FVIII binding to vWF is reduced, causing the decrease of FVIII:C. Lui *et al.* [14] found very similar results in 10 out of 14 missense mutations in the C1- and C2-domains. Based on the crystallographic structure of the C2-Domain [15], they demonstrated that eight of the 10 affected amino acids were exposed on the surface of the FVIII molecule. Comparing their data with HAMSTeRS, the investigators found that mutations in this region are often associated with inhibitor formation. From these data it can be hypothesized that the C1/C2 domains significantly contribute to the binding of FVIII protein to VWF. During FVIII activation VWF is cleaved off and the same C1/C2 region bind to the phospholipid membrane to constitute the tenase complex. Any changes in the three dimensional structure of this critical part of the FVIII molecule may affects its immunogenicity.

Beside the small deletions/insertions at series of adenine nucleotides *splice site mutations* represent the second mutation type that bear almost no risk for inhibitor formation. The likely explanation is that a very small number of FVIII molecules are still spliced normally and these few molecules are sufficient to induce immune tolerance.

One of the most important questions for the pathogenesis of inhibitor formation is: why do not all patients with a single type of null mutation develop an inhibitor? The intron-22 inversion may be a good example to discuss this aspect. It is a completely uniform mutation type and from the pathomechanism, the intron-22 inversion appears to be a true null mutation which results in almost no endogenous FVIII protein in the patient's circulation. Why do only one-third of patients with this mutation type develop an inhibitor while two-thirds do not? Speculations on potential reasons may include some maternal FVIII that is presented *in utero* to the fetal immune system thus inducing immune tolerance towards substituted FVIII. Also, the individual characteristics of a patients polymorphic immune system may either increase or decrease risk of inhibitor development. It also

may be speculated that an immune response is arising in almost all patients with null mutations, but it is down-regulated in two-thirds of the patients by still unknown mechanisms. Solving the question of penetrance of inhibitor formation in haemophilic patients will be the key to understanding the pathomechanism of inhibitor development.

Genetics of the immune system and risk of inhibitor development

Presently, two studies provide indirect evidence that genes participating in the immune response considerably influence the risk of inhibitor development. Scharrer *et al.* [16] showed a meta-analysis of three USA studies (Kogenate [17], Recombinate [18] and US retrospective study [19]) that clearly demonstrate the influence of race on inhibitor formation. In the ethnic group of American-Africans, the inhibitor incidence in severe haemophilic patients was doubled (51.9%, 14 of 27) when compared to that of Caucasians (25.8%, 51 of 191). Cox-Gill [20] compared the incidence of inhibitor formation in haemophilic siblings to more extended haemophilic relatives and found a much higher incidence in siblings (50%) than in extended haemophilic relatives (9%). Since the genetic defects of the FVIII gene are expected to be similar in both studies, the observed difference in inhibitor incidence should be caused by genetic variations of the immune system. Candidates for this immunogenetic determinant of inhibitor formation are the MHC classes and other polymorphic genes (e.g. cytokines) that participate in the immune response.

The influence of the genes of the MHC classes on inhibitor formation has been addressed in various studies [21–24]. The MHC class II genes DQ, DR and DP are of particular interest because their function is to present extracellular antigens – as substituted FVIII – to the patient's immune system. However, with respect to the presentation of endogenously truncated and/or immunologically altered FVIII, the MHC class I genes that process intracellular antigens may also play an important role in the genesis of inhibitor formation. The results of the former studies had been inconclusive and sometimes even contradictory. One problem of these studies was that they did not consider the patient's mutation type. As a consequence, the influence of specific immune response genes on inhibitor formation might have been masked by the strong influence of the FVIII gene defect. In a recent study, the influence of the MHC class I/II genotype on inhibitor formation was exclusively investigated in patients with the homogenous intron-22 inversion [4]. A condensate of the results is shown in Table 2. The MHC class I/II alleles A3, B7, C7, DQA0102, DQB0602 and DR15 could be assigned as risk alleles (relative risk 1.9–4.0), because they occurred more often in inhibitor than in noninhibitor patients. In contrast the MHC class I/II alleles C2, DQA0103, DQB0603 and DR13 could be assigned as protective alleles (relative risk 0.1–0.2) because they occurred less often in inhibitor than in noninhibitor patients. These MHC class I/II alleles belonged to extended haplotypes (A3-B7-C7-DQA0102-DQB0602-DR15 and C2-DQA0103-DQB0603-DR13) that were also frequent and less frequent, respectively, in the normal population. However our number of patients was too small to reach a clear statistical significance. Moreover, the inheritance as haplotypes might mask those MHC class I/II alleles that are decisive for the risk or protection of inhibitor formation. In

Table 2. Common (upper part) and rare (lower part) MHC class I and II alleles in severe haemophilia A patients with intron-22 inversion and inhibitor formation according to Oldenburg *et al.* [4]. For the MHC class I loci A, B, C the number of individuals and for the MHC class II loci DR, DQA, DQB the number of chromosomes is given

Allele	Patients with inhibitor		Patients without inhibitor		Relative Risk
	No.	%	No.	%	
Common MHC-alleles in patients with inhibitor					
A3	11	37.9	9	21.4	2.2
B7	14	48.3	8	19.1	4.0
C7	17	58.6	16	38.1	2.3
DQA0102	20	34.5	16	19.1	2.2
DQB0602	18	31.0	12	14.3	2.7
DR15	19	32.8	17	20.2	1.9
Rare MHC-alleles in patients with inhibitor					
C2	1	3.4	6	14.3	0.2
DQA0103	1	1.7	12	14.3	0.1
DQB0603	0	0.0	6	7.1	0.1
DR13	1	1.7	9	10.7	0.1

this context, a finding of Chics *et al*. [25] was of interest. The investigator found a 16 amino residues peptide from the FVIII light chain (amino acids 1706–1721) that could be eluted from a DR15 cell line. The peptide, located on the surface of the FVIII molecule, was bounded by two functional cleavage sites. It may be hypothesized that some peptides of the FVIII protein are especially efficient when presented to the patient's immune system, thus leading to the determination of FVIII antibody epitopes. Notably, Hay *et al*. [5] found the same MHC class II alleles to be associated at similar frequencies with inhibitor formation in patients with intron-22 inversions, thus supporting the concept that the MHC is of some significance for the risk of inhibitor formation. Both studies, however, failed to achieve significant results. Beside the arguments already presented, this may be due to the fact that additional genes, not only those belonging to the MHC, constitute the immunogenic risk profile for inhibitor formation.

Patients' genetics and characteristics of FVIII antibody epitopes

During the last years, several FVIII antibody epitopes have been characterized. An overview of the epitopes of inhibitory antibodies is shown in Fig. 3. The FVIII A2, A3, C2 domains are the most immunogenic while the A1 and B domains are at best poorly immunogenic. Inhibitory epitopes have been assigned to the ar1 region (aa 351–365) [26], the A2 domain (aa 484–508) [27], the ar3 region (aa1687–1695) [28,29], the A3 domain (aa 1778–1823) [30] and the C2 domain (aa 2181–2243) [31] (aa 2248–2312) [32]. Inhibitors against the C1 domain also have been reported [33]. Interestingly, the antibody epitopes correlate very well with the functional epitopes of ligands that interact with the FVIII protein (Fig. 4) [reviewed in 34].

In severe haemophilia A, little is known about the relationship of the type and site of the mutation and the localization of the FVIII antibody epitopes. Epitopes have been shown to vary among inhibitor patients with respect to their number and specifity [35,36]. Most haemophilic patients have shown a complex immune response with two or three epitopes contributing significantly to the inhibitor titre [36]. The mechanisms determining the heterogeneous patterns of the various FVIII antibody epitopes are not known. A correlation of the type and site of the mutations to epitope characteristics may help to understand this relationship.

In mild haemophilia patients, a direct relationship of the mutation site and the antibody epitope has been clearly established for some patients. Fijnvandraat *et al*. [37] described a mild haemophilia A patient with an Arg593Cys mutation in whom the FVIII antibodies were directed against substituted FVIII but not against the mutated endogenous FVIII protein. This led to the interesting phenomenon that DDAVP is able to induce a considerable increase of the FVIII activity while infused replacement FVIII did not. Peerlinck *et al*. [36] described two mild haemophilia A patients with a residual FVIII:C of 0.09 IU mL^{-1} despite inhibitor titres of 300 and 6 Bethesda Units (BU), respectively. Both patients had the mutation Arg2150His and produced inhibitory antibodies directed against a FVIII domain encompassing the mutation site. By expression studies and functional tests, the mutation at residue 2150 was shown to lead to reduced FVIII binding to vWF that subsequently decreased FVIII:C [13].

Conclusion

The risk of inhibitor development in haemophilia A is considerably determined by the genetic characteristics of the patients with respect to the underlying mutation in the FVIII gene and the individual polymorphic immune system. Although a relationship of the mutation site and the epitope localization has been shown for some single patients with mild haemophilia A, it still remains to be elucidated, whether titre and epitope specifity of the inhibitors are influenced by the genetics of the patients.

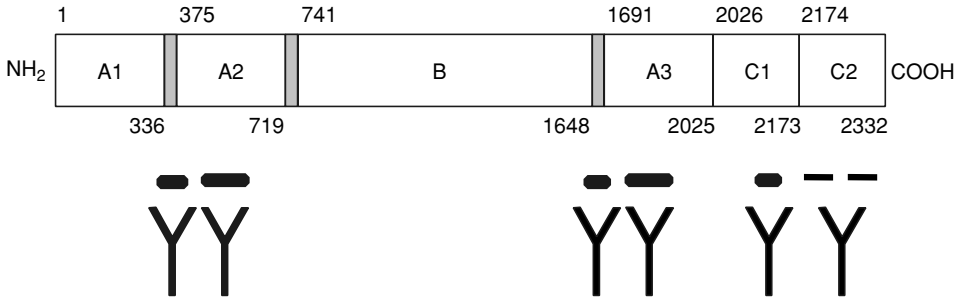

Fig. 3. Inhibitor epitopes on the FVIII protein (ar1 region [26], A2 domain [27], ar3 region [28,29], A3 domain [30], C1 domain [33] and C2 domain [31,32]).

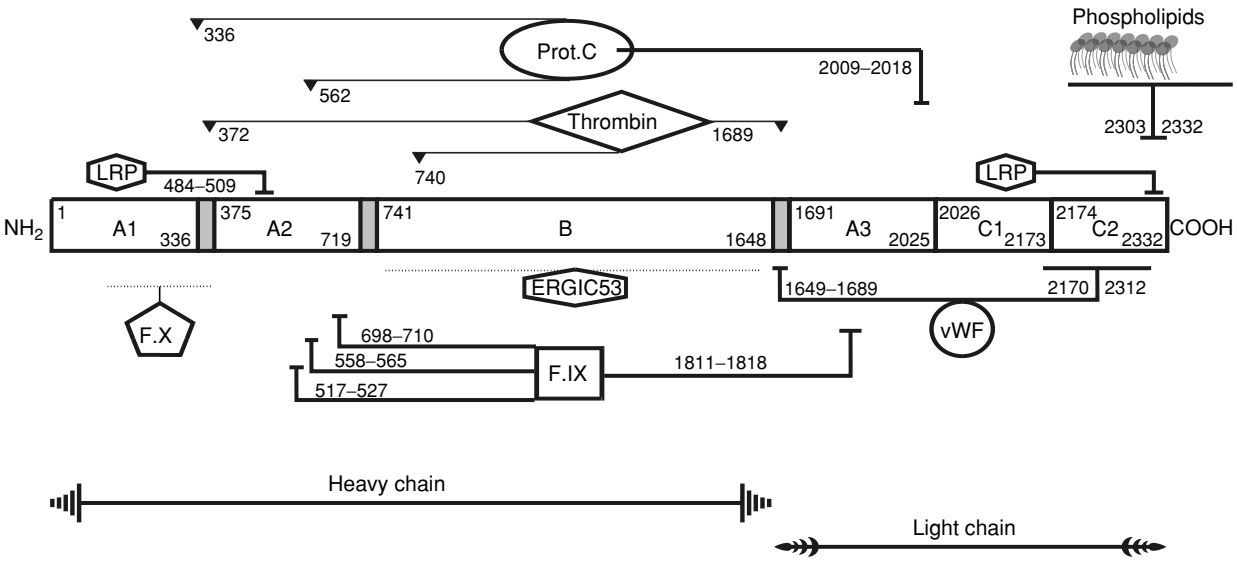

Fig. 4. Interactions of the FVIII protein with other components of the clotting cascade.

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