Polymorphisms in the *TNFA* gene and the risk of inhibitor development in patients with hemophilia A

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The HLA class I/II alleles and the tumor necrosis factor α *(TNFA)* locus are closely linked in the MHC complex. We have characterized the causative factor VIII mutation, HLA alleles as well as 4 polymorphisms (−827C>T, −308G>A, −238A>G, and 670A>G) in the *TNFA* gene in 164 patients (124 severe, 26 moderate, and 14 mild) in 78 families with hemophilia A enrolled in the Malmö International Brother Study (MIBS). Inhibitors were identified in 77.8% of patients with a single haplotype (Hap 2) and 72.7% of the patients with the TNFA −308 A/A genotype within this haplotype compared with 39.7% for TNFA −308 G/G patients and 46.9% for TNFA −308 G/A heterozygotes (OR 4.0; 95% CI, 1.4-11.5; $P = .008$). The association between the −308 A/A genotype and inhibitors was enhanced in subgroups of patients with severe hemophilia (OR 19.2; 95% CI 2.4-156.5; $P < .001$) and with inversions (n = 75; OR, 11.8; 95% CI, 1.3-105.1; $P = .013$). Associations were found for the HLA A26 and B44 alleles, but these were not consistent in the subgroup analysis. Our data imply that the TNFA −308G>A polymorphism within Hap 2 is a useful marker and potential modulator of the immune response to replacement therapy in patients with hemophilia. (Blood. 2006;108:3739-3745)

Introduction

Alloantibodies neutralizing the hemostatic effect of factor VIII develop in 10% to 15% of patients with hemophilia A during replacement therapy. These antibodies, frequently of the IgG4 subclass, usually appear within the first 25 exposures to factor VIII concentrates.[1,2] A genetically determined predisposition for this side effect of treatment has been suggested in studies of related patients.[3-5] In the Malmö International Brother Study (MIBS), we have described an overall concordance of 78.3% between siblings in 249 families with severe hemophilia A and a higher frequency of inhibitors in African Americans (51.9%) compared with whites (25.8%).[4] We also calculated a relative risk of 3.2 for the development of an inhibitor for a patient whose older brother had previously been diagnosed with an inhibitor compared with that of a patient with an unaffected brother.

Null mutations and large rearrangements of the factor VIII gene appear to confer a higher risk of developing inhibitors compared with point mutations and small insertions/deletions.[6-8] Nonetheless, most patients with these mutations do not develop inhibitors and the concordance rate for inhibitor development between siblings in families with intron 22 inversion and inhibitors was 40.0%, indicating the presence of other genetic determinants.[5] Recently, we reported that inhibitory antibodies were more frequently encountered in patients with allele 134 of the IL10G microsatellite in the promoter region of the *IL10* gene.[9] For subjects with severe hemophilia A, an odds ratio (OR) of 5.4 (95% confidence interval [CI], 2.1-13.7, $P < .001$) was observed. The HLA class I alleles A3, B7, and C7 and the class II alleles DQA0102, DQB0602, and DR15 have all been associated with higher risk for inhibitor development in unrelated patients (relative risk [RR], 1.9-4.0), whereas the HLA C2, DQA0103, DQB0603, and DR13 alleles may be protective.[10,11] The reported associations are weak, however, and not consistently statistically significant. This provides a rationale for additional studies to fully appreciate their importance. No studies of the impact of HLA alleles on inhibitor formation have been performed in siblings. Sibling studies offer advantages when evaluating the genetic contribution to risk because several factors, apart from the causative mutation, are at least partially adjusted for, thereby minimizing the variability introduced by nongenetic influences. Thus, the present study was undertaken to further evaluate these loci. The MHC locus on chromosome 6p21.3 also contains the genes for TNF-α *(TNFA)* and lymphotoxins α and β, flanked by the HLA-B and HLA-DR loci.[12,13] TNF-α is an important cytokine with potent proinflammatory and immunomodulatory functions and polymorphisms in the gene have been associated with autoimmune antibody-mediated diseases.[14-16] Several polymorphisms have been identified in the *TNFA* gene, but few have a reported allelic frequency greater than 5%. The most extensively studied polymorphism with pathophysiologic effects is the biallelic TNFA −308G>A polymorphism in the promoter region, which has been strongly associated with HLA A1, B8, and DR3 alleles.[17] The polymorphism is associated with increased levels of TNF-α in inflammatory bowel diseases and antibody formation in patients with myasthenia gravis (MG) and early onset of the disease.[15,16] DNA constructs corresponding to the normal −308G or variant

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−308A promoter region have also shown inherent induction of the expression of a CAT reporter gene for the −308A allele.[17] TNFA −827C>T is located at an NF-κB–binding site[18] and the C allele participates in a potential CpG methylation site. The G allele of TNFA −238G>A also creates a CpG site, whereas TNFA 670A>G extends this region with a G allele contributing to a CpG in a MeCP2 consensus-binding site[19] in intron 1. The aim of the current MIBS study was to evaluate whether HLA class I and II alleles, the TNFA −308A allele, or other polymorphisms or haplotypes (Hap) in the *TNFA* gene may confer susceptibility to inhibitor development in a cohort of siblings with hemophilia A.

Materials and methods

Patients

The cohort of 164 patients with hemophilia A from 78 unrelated families enrolled in the MIBS has recently been described[9] and includes 18 of the families evaluated in the study of factor VIII gene mutation and inhibitor risk.[5] Severe hemophilia A was defined as antihemophilic factor (factor VIII:C) less than 1%, moderate as 1 to less than 5%, and mild as a level 5% to 40%. Date of birth, ethnicity, severity, treatment history, inhibitor history including peak titer, and current titer in Bethesda units (BU/mL) were accrued. A high-responding (HR) inhibitor was defined as a historical peak titer greater than 5 BU/mL and a low-responding (LR) inhibitor as one with a peak titer of 5 BU/mL or less.[20] Approval was obtained from the Lund University Institutional Review Board for these studies. Informed consent was provided according to the Declaration of Helsinki.

Methods

Standard methods for the analyses of the factor VIII gene were used including Southern blot and long-range polymerase chain reaction (PCR) for inversion analysis, PCR and mutation screening methods, for example, single-stranded conformation polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), denaturing high-performance liquid chromatography (DHPLC), and direct DNA sequencing.[21] Factor VIII and IX clotting activity was measured according to standard techniques. Inhibitory antibodies were quantified according to the original Bethesda method and the Nijmegen modified assay.[22,23]

TNFA polymorphisms

gDNA was extracted from EDTA-preserved blood using a kit (Qiagen genomic tip, Kebo Lab, Stockholm, Sweden). The TNFA −308G>A (rs1800629) polymorphism was detected using one primer that creates an *Nco*I recognition site in the A allele so that digestion of the PCR product (107 bp) with *Nco*I (Promega, Mannheim, Germany) provides 2 fragments (87 and 20 bp).[14,16] The G allele remains intact. TNFA amplification was performed using a thermal cycler (Techne, PHC-3 Dri-block, Cambridge, United Kingdom). *Nco*I-digested products were subjected to agarose gel electrophoresis in 3% NuSieve GTG at 5 V/cm for 3.5 hours. The surrounding single-nucleotide polymorphisms (SNPs), TNFA −827C>T (rs1799724), −238A>G (rs361525), and 670A>G (rs3093662), in the first intron of TNFA were analyzed using validated SNP genotyping assays from Applied Biosystems on the Prisma 7900HT instrument (Applied Biosystems, Naerum, Denmark) according to standard protocol. Haplotypes were deduced manually.

HLA class I and II alleles

The HLA class I and class II genes were investigated by combined locus-specific amplification of gDNA by the PCR and hybridization with sequence-specific oligonucleotide probes (SSOPs). All samples were typed for HLA A, B, Cw, DRB, and DQB alleles by PCR/SSOP Dynal kits (Dynal Biotech, Wirral, United Kingdom) following the manufacturer's recommendations and evaluated by computer software PMP5.4.1 (Dynal Biotech, Hamburg, Germany). All questionable or ambiguous typings as well as HLA-DQB subtyping were submitted to high-resolution investigation performed by the PCR sequence-specific primer kit "One Lambda's High Resolution Trays" (One Lambda, Montpellier, France).

Statistical methods

χ^2 analysis was used for evaluation of the frequency of alleles in patients with and without a history of inhibitory antibodies. For expected numbers below 5, the Fisher exact test was used. RR was calculated in a subgroup analysis where a cell containing zero HLA alleles was observed. In all other cases, ORs and 95% CIs were calculated. Logistic regression analysis was performed to adjust for the association of allele 134 in the IL10G microsatellite. All tests were 2-sided and a P value below .05 was considered to indicate statistical significance. In addition, associations of HLA TNFA alleles with inhibitors were examined using the method proposed by Svejgaard and Ryder.[24]

Results

Inhibitor characteristics of the patients and families are summarized in Table 1.[9] A total of 124 patients (75.6%) had severe (52 HR, 11 LR, 61 no inhibitor), 26 (15.9%) moderate (2 HR, 5 LR, 19 no inhibitor), and 14 (8.5%) mild (3 HR, 4 LR, 7 no inhibitor) hemophilia A. Seventy-seven (47.0%) of the 164 subjects had a history of inhibitors. They belonged to 54 families, 34 of which were discordant and 20 concordant with regard to inhibitor development. The median inhibitor titer was 11 BU/mL (range, 1-3000 BU/mL). The median age of the study cohort was 24 years (range, 3-79 years) and all patients had more than 100 exposures to factor VIII concentrates. All but 4 patients in 2 families were white. Subgroup analysis was performed in 75 patients (45.7%), from 36 unrelated families, with inversions. Forty of these patients (53.3%) in 28 families developed inhibitors. The siblings were inhibitor concordant in 11 of the 28 families and discordant in 17.

HLA alleles

Significant associations were identified for 2 of the class I alleles (Tables 2 and 3), but after correction for multiple comparisons no

Table 1. Inhibitor discordance and concordance between siblings in the study cohort of 78 MIBS families

Inhibitor history in each sibling	Type of hemophilia A			Total
	Severe	Moderate	Mild	
Discordant families, no.				34
HR/No	20	2	1	23
LR/No	5	2	1	8
HR/No/No	1	0	0	1
HR/LR/No	1	0	0	1
HR/No/No/No	0	0	1	1
Concordant families, no.				44
HR/HR	11	0	0	11
LR/LR	0	0	1	1
HR/LR	5	0	1	6
No/No	15	6	1	22
HR/HR/HR	1	0	0	1
LR/LR/LR	0	1	0	1
No/No/No	1	1	0	2
Total. no. (%)	60 (76.9)	12 (15.4)	6 (7.7)	78 (100)

HR, high-responding inhibitor; LR, low-responding inhibitor; No, no inhibitor history.

Table 2. Distribution of HLA A, B, and C alleles in patients with and without inhibitors

Allele	No. patients (no. with severe hemophilia)	
	Inhibitor patients	Noninhibitor patients
HLA A		
*01	16 (13)	18 (17)
*02	37 (29)	49 (28)
*03	19 (14)	19 (15)
*11	14 (13)	14 (10)
*23	4 (3)	8 (7)
*24	12 (11)	17 (15)
*25	5 (4)	4 (3)
*26	0 (0)	6 (3)*
*29	3 (3)	4 (0)
*30	5 (5)	5 (5)
*31	4 (4)	1 (0)
*32	7 (5)	7 (5)
*33	1 (1)	1 (1)
*36	1 (1)	5 (2)
*66	2 (2)	0 (0)
*68	7 (7)	6 (4)
Total	137 (115)	164 (115)
HLA B		
*07	10 (8)	11 (8)
*08	18 (15)	23 (18)
*13	3 (3)	5 (2)
*14	3 (3)	3 (3)
*15	8 (5)	8 (5)
*18	8 (7)	6 (2)
*27	6 (4)	10 (4)
*35	18 (17)	15 (9)
*37	1 (1)	5 (4)
*38	0 (0)	2 (2)
*39	2 (2)	5 (4)
*40	3 (3)	9 (6)
*41	2 (1)	1 (0)
*44	24 (19)†	14 (12)
*45	2 (2)	0 (0)
*47	1 (1)	1 (1)
*49	1 (1)	7 (6)
*50	5 (4)	7 (3)
*51	13 (12)	11 (9)
*52	4 (4)	7 (7)
*53	5 (4)	4 (2)
*55	1 (1)	5 (3)
*57	7 (5)	5 (5)
*58	0 (0)	1 (1)
Total	145 (122)	165 (116)
HLA C		
*01	4 (2)	7 (2)
*02	6 (4)	9 (6)
*03	12 (10)	20 (13)
*04	22 (19)	21 (13)
*05	10 (8)	5 (5)
*06	14 (11)	19 (11)
*07	37 (30)	38 (27)
*08	3 (3)	5 (5)
*12	7 (6)	12 (9)
*13	0 (0)	1 (1)
*14	2 (2)	5 (4)
*15	7 (6)	4 (3)
*16	11 (9)	6 (6)
*17	2 (1)	1 (0)
Total	137 (111)	153 (105)

*RR, 0.96 (95% CI, 0.93-0.99), $P = .033$.
†OR, 2.1 (95% CI, 1.1-4.3), $P = .037$.

significant differences remained.[25,26] The HLA A26 allele was identified only in patients without inhibitors (RR, 0.96; 95% CI, 0.93-0.99; $P = .033$), but the frequency of this allele was very low (n = 6). The HLA B44 allele was associated with a higher risk of inhibitor development and found in 24 (31.2%) of the patients with inhibitors and in 14 (16.1%) without, corresponding to an OR of 2.1 (95% CI, 1.1-4.3; $P = .037$). No significant association with inhibitor development was seen in the subgroup of 124 patients with severe hemophilia A. Interestingly, no trend was seen supporting the notion that the HLA class I alleles A3, B7, and C7 and the class II alleles DQB0602 and DR15 were associated with a higher risk of inhibitor development.[10,11] The haplotype A1-B8-DR3 was found in 16 patients (9.8%), including 7 with inhibitors. Thirteen of the 16 (81.2%) also carried the −308A allele, supporting an association of these HLA alleles with the −308A polymorphism.

TNFA polymorphisms

The observed frequencies of TNFA genotypes in each hemophilia severity subgroup are shown in Table 4. All 4 SNPs studied were in Hardy-Weinberg equilibrium. The only significant association identified for a single SNP with inhibitor formation is that of the −308 A/A genotype. A total of 142 patients (86.6%) carry the −308G allele and 86 (52.4%) the −308A allele compared with 97% and 76% for the G and A alleles, respectively, in the general Swedish population.[16] G/G homozygosity was identified in 47.6% of the patients, which is similar to that found in patients with MG (50%),[17] but less than the 76% described in healthy subjects. Homozygosity for the TNFA −308A allele was identified in 22 individuals (13.4%) compared with 2% in healthy subjects and 10% in patients with MG.[17] Among −308 G/G subjects 31 of 78 had inhibitors compared with 30 of 64 G/A patients (not significant) and 16 of 22 −308 A/A patients (72.7%) yielding an OR of 4.0 (95% CI, 1.4-11.5; $P = .008$) for the A/A genotype using −308 G/G as the reference group. The −308A allele was identified in 46 of the 77 patients (59.7%) with inhibitors and in 40 of the 87 patients (46.0%) without inhibitors (OR, 1.7; 95% CI, 0.9-3.2; $P = .087$). The association between the −308 A/A genotype and inhibitors was consistent in subgroup analysis of the 124 patients with severe hemophilia A (OR, 19.2; 95% CI, 2.4−156.5; $P < .001$), and in the smaller group of 75 patients with inversions (OR, 11.8; 95% CI, 1.3-105.1; $P = .013$). To further evaluate the effect of other genetic and nongenetic factors on the association of the −308 A/A genotype with inhibitors, analysis of data from only the oldest sibling in each family with severe hemophilia was performed. Seven subjects with this genotype were identified, all of whom developed HR inhibitors ($P = .019$).

Logistic regression analysis revealed that these ORs remained largely unchanged after adjustment for the IL10G promoter allele. The explained fraction (R^2) for this model was 0.13 in the entire cohort. The corresponding figure was 0.20 in the subgroup of patients with the severe form of the disease compared with 0.07 and 0.11 for the individual *TNFA* and *IL10* genes, respectively. No significant associations with inhibitor formation were seen with any of the other 3 SNPs studied.

Characteristics of the 22 subjects in 16 families with the TNFA −308 A/A genotype are summarized in Table 5. Only 1 of the 6 unaffected siblings with this genotype suffered from severe hemophilia. In this discordant inhibitor family (no. 12) with an intron 22 inversion, the oldest subject who was −308 G/A heterozygous had developed a low-titer inhibitor with a

Table 3. Distribution of HLA DR and DQ alleles in patients with and without inhibitors

Allele	No. patients (no. patients with severe hemophilia A)	
	Inhibitor patients	Noninhibitor patients
HLA DR		
*01	9 (8)	16 (11)
*03	26 (22)	20 (15)
*04	20 (12)	28 (19)
*07	21 (18)	21 (14)
*08	7 (7)	4 (4)
*10	2 (1)	6 (3)
*11	20 (16)	18 (10)
*12	0 (0)	1 (0)
*13	18 (15)	21 (13)
*14	5 (4)	5 (4)
*15	21 (18)	29 (25)
*16	5 (5)	5 (4)
Total	154 (126)	174 (122)
HLA DQ		
*02	35 (31)	32 (23)
*03	53 (39)	57 (35)
*04	3 (3)	5 (5)
*0501	13 (11)	18 (11)
*0502	7 (7)	5 (4)
*0503	4 (3)	5 (4)
*0601	5 (5)	5 (5)
*0602	17 (14)	25 (21)
*0603	8 (6)	10 (5)
*0604	5 (3)	6 (4)
*0609	0 (0)	1 (0)
*0619	0 (0)	1 (1)
Total	150 (122)	170 (118)

Numbers of patients with severe hemophilia A are shown in parentheses.

peak titer of 3 BU/mL, whereas the 22-year-old −308 A/A homozygous brother has no identified inhibitor activity to date. All other unaffected siblings with the −308 A/A genotype suffered from mild or moderate hemophilia.

TNFA haplotype analysis

Two major haplotypes became evident due to the presence of 48 and 18 homozygotes, allowing Hap assignment to all combinations with more than 2 subjects. Tables 6 and 7 indicate that either recombination or spontaneous mutations occur within this 1.5-kb distance. The −827T allele is present only in Hap 3 and never together with −308A. When comparing inhibitor frequency in Hap 1-3 and Hap 2-3 with Hap 1-1 and Hap 2-2, respectively, −827T appears to protect against inhibitor formation. The relatively infrequent Hap 4 with −308G and 670G may have a similar protective effect compared to Hap 1, but the numbers are too small to permit reliable evaluation.

The TNFA −308A allele was found primarily in Hap 2 (104 of 108 alleles). Of all homozygous Hap 2 subjects (n = 18), 14 (77.8%) had inhibitors, all with high levels. Among Hap 1 homozygotes (n = 48), only 20 (42.7%) had inhibitors and 25% had high levels. Hap 2-2 (including −308 A/A) was also present in 3 of 7 cases of mild hemophilia without inhibitors and in 1 of 7 mild cases with inhibitors, but was not observed in moderate cases.

Discussion

TNF-α is an important mediator of inflammatory responses and has crucial immunomodulatory activities. Because the TNFA locus is located in the HLA class III region of the MHC complex, genetic effects at these loci can be difficult to distinguish. Numerous TNFA SNPs have been studied in animals and experimental systems, among which we selected the few SNPs reported to have more than 10% heterozygosity in whites[27] and for which BLAST[28] analysis indicated unique results. We also expanded the analysis to evaluate possible haplotype effects. The selected SNPs have the added feature that they involve potential CpG methylation sites. Furthermore, methylated TNFA −827C and −238G are potential and TNFA 670G is a probable binding site for MeCP2,[29] a protein that can block transcription while inducing methylation of histone

Table 4. Characteristics of patients and families with the TNFA −308A/A genotype

Family no., by type of hemophilia A	TNF-α-308G>A genotype in sibling 1/2/3/4	Type of inhibitor in sibling 1/2/3/4	Peak titer in sibling 1/2/3/4, BU/mL	Factor VIII gene mutation
Severe				
1	AA/AA	High/High	282/6	Small deletion (del T5574)
2	AA/GA	High/High	38/300	Intron 22 inversion*
3	GA/AA	High/High	15/8	Intron 22 inversion
4	AA/GA	High/High	840/72	Large deletion (exon 1-6)*
5	AA/AA/AA	High/High/High	1100/1560/164	Intron 22 inversion
6	AA/GA	High/Low	31/2	Intron 22 inversion
7	AA/GG	High/Low	153/1	Intron 22 inversion
8	GA/AA/GA	Low/High/No	1/70/—	Small deletion (del T4159)†
9	GG/AA	No/High	—/150	Nonsense (Arg427stop)
10	AA/GG	High/No	560/—	Intron 1 inversion*
11	GA/AA	No/High	—/22	Intron 22 inversion
12	GA/AA	Low/No	3/—	Intron 22 inversion
Moderate				
13	AA/AA	Low/No	1/—	Missense (Pro1761Gln)†
14	AA/GG/GG	No/No/No	—	Missense (Arg2163His)*
Mild				
15	GG/AA/GG/AA	No/No/No/High	—/—/—/6	Missense (Tyr2105Cys)*
16	AA/AA	No/No	—	Missense (Arg1696Gly)

The siblings are numbered in consecutive order, sibling 1 being the oldest brother.
— indicates not apllicable.
*Mutation previously associated with inhibitor.
†Novel mutation.

Table 5. Frequencies of TNFA genotypes according to severity and inhibitor status

Genotypes	Severe hemophilia A, no. patients (%)		Moderate/mild hemophilia A, no. patients (%)		Total no. patients (%)
	Inhibitor positive	Inhibitor negative	Inhibitor positive	Inhibitor negative	
TNFA −827					
−827 C/C	52 (82.5)	50 (83.3)	12 (85.7)	21 (80.8)	135 (82.8)
−827 C/T	11 (17.5)	10 (16.7)	2 (14.3)	5 (19.2)	28 (17.2)
Total	63	60*	14	26	163
TNFA −308					
−308 G/G	24 (38.1)	33 (54.1)	7 (50.0)	14 (53.8)	78 (47.6)
−308 G/A	25 (39.7)	27 (44.3)	5 (35.7)	7 (26.9)	64 (39.0)
−308 A/A	14 (22.2)†	1 (1.6)	2 (14.3)	5 (19.2)	22 (13.4)
Total	63	61	14	26	164
TNFA −238					
−238 G/G	55 (88.7)	55 (90.2)	11 (78.6)	23 (88.5)	144 (88.3)
−238 A/G	7 (11.3)	6 (9.8)	3 (21.4)	3 (11.5)	19 (11.7)
Total	62*	61	14	26	163
TNFA 670					
670 A/A	52 (82.5)	54 (88.5)	11 (78.6)	19 (73.1)	136 (82.9)
670 A/G	11 (17.5)	7 (11.5)	3 (21.4)	7 (26.9)	28 (17.1)
Total	63	61	14	26	164
Haplotypes					
2-2	13 (20.6)	1 (1.6)	1 (7.1)	3 (11.5)	18 (11.0)
2-X	21 (33.3)	25 (41.0)	4 (28.6)	6 (23.1)	56 (34.1)
X-X	29 (46.0)	35 (57.4)	9 (64.3)	17 (65.4)	90 (54.9)
Total	63	61	14	26	164

*Data for one subject were missing.

†OR, 19.2 (95% CI, 2.4-156.5; $P < .001$) for presence of TNFA −308 A/A among patients with severe hemophilia A and inhibitors.

proteins, thus contributing to transcriptional shutdown.[19] The −827C>T polymorphism is immediately 3′ of an NF-κB binding site, and binding of NF-κB1 homodimers has a negative effect on transcription.[18] Presumably, methylation of −827C might affect this binding. Thus, the −827T allele could be biologically protective, compatible with a slight protective effect conferred by Hap 3 on heterozygous carriers.

In this study, homozygosity for Hap 2 (Hap 2-2) had a significantly higher risk for the development of inhibitors than Hap 1-1, and the only difference between these 2 haplotypes is the rare TNFA −308A allele, known to have a pathophysiologic role in certain antibody-mediated autoimmune diseases. The association with this allele was strongest in the subgroup of patients with severe hemophilia A (OR, 19.2; $P < .001$), and was also observed in the group of 75 patients with inversions—that is, in a cohort of patients with the same risk for developing inhibitors based on the causative factor VIII mutation itself. This association was furthermore independent of the presence of allele 134 of IL10G.[9]

The −308A allele coexists with 670A in risk Hap 2, compatible with the concept that the 670G allele might confer protection via the MeCP2 mechanism, and the few heterozygous carriers of the 670G allele have lower inhibitor frequencies than corresponding haplotypes with the 670A allele, although the association is not significant. Methylation of all of the potential CpG methylation sites has been reported in leukemia cells.[30] Thus, further investigation of the genetic influence on methylation in TNFA and consequences for transcriptional regulation, inflammation, or immune response is of interest. There are inherent weaknesses in retrospective cohort studies, but our data strongly suggest that Hap 2 of the *TNFA* gene and −308 A/A may be important markers or determinants for the risk of inhibitor development. Although the −308A allele frequency seems to be relatively consistent among European Americans (0.15), African Americans (0.12), and Asians (0.12) (Cheryl A. Winkler, National Cancer Institute, Frederick, MD, unpublished data, June 2006), the frequency of the −308 A allele and that of the A/A genotype were higher than those described for a healthy white Swedish population, and similar to those found in a cohort of patients with the autoimmune disease MG.[16,17] This may be indicative of a causal relationship of −308 A/A with inhibitor formation and the high frequency related to selection of inhibitor patients (47.0%) for study in the MIBS cohort, or to other factors inherited along with Hap 2. However, the possibility that the −308

Table 6. TNFA haplotypes identified in the cohort of 164 MIBS patients

Haplotype	TNFA polymorphism			
	−827	−308	−238	670
Hap 1	C	G	G	A
Hap 2	C	A	G	A
Hap 3	T	G	G	A
Hap 4	C	G	A	G
Hap 5	C	G	G	G
Hap 6	C	A	A	G
Hap 7	C	A	G	G

Table 7. Association of inhibitors with each TNFA haplotype identified

Haplotype	Total no. of patients	Inhibitor positive, %	High-responding inhibitors, %
Hap 1-1	48	42.7	25.0
Hap 2-2	18	77.8	77.8
Hap 1-2	43	41.9	30.2
Hap 1-3	14	28.6	21.4
Hap 2-3	12	58.3	50.0
Hap 1-4	8	37.5	12.5
Hap 2-4	7	57.1	57.1
Hap 1-5	4	25	0
Hap 2-5	2	50	50
Hap 2-6	2	50	0
Hap 2-7	2	50	50
Hap 3-4	2	100	100

A allele could be more prevalent in hemophilia patients with inhibitors than in those not developing inhibitors and in healthy subjects needs to be evaluated in multiethnic population-based cohorts. The development of inhibitory antibodies against exogenous factor VIII is usually considered to be a Th2 cell-induced immune response, whereas TNF-α is primarily linked to Th1 cells. However, the cytokine profile clearly indicates the formation of inhibitors to be a mixed Th1 and Th2 cell response, which further underscores the fact that the level of TNF-α may also modulate the immune response to the deficient factor in patients with hemophilia.[31]

The −308A allele has been associated with increased constitutive and inducible transcription levels[32] and with increased production and secretion of TNF-α in patients with autoimmune diseases and healthy controls[15,16] compared to the −308G allele. In a study by Wilson and colleagues,[17] TNFA −308A was strongly associated with the HLA A1, B8, and DR3 alleles. However, although our findings support an association between the −308A polymorphism and these alleles, none of them were more frequently seen in patients with inhibitors in our cohort, among whom B44 was the only enriched allele, with an OR of 2.1—that is, similar to that described by Oldenburg and colleagues (34.5% versus 16.7%; OR, 2.2).[10] The HLA class I alleles A3, B7, and C7 and the class II alleles DQB0602 and DR15, which in previous reports have been more frequently observed in patients with inhibitors were, however, equally distributed between the 2 patient groups in our study. This was also the case for the C2, DQB0603, and DR13 alleles, which have been observed in a lower frequency in inhibitor patients suggesting a protective effect.[10,11] The class II molecules, which present the antigen to the T-helper cells, are indiscriminate in that a range of peptide sequences may be bound.[33] Based on our family data, we therefore believe that a consistent association between HLA class I and II alleles and inhibitor formation for use as a risk indicator for patients will be difficult to attain. No known linkage between TNFA −308, HLA A26, or HLA B44 has been described, and the present data from our small study population should be interpreted with caution. The impact of the TNFA −308G>A polymorphism on various diseases has been debated, and because cytokines rarely manifest their effects in isolation but rather in complex regulatory networks, the mechanisms by which this polymorphism may influence the immune response in patients with hemophilia remains to be further evaluated.[34] It is, however, tempting to believe that TNFA variants will be useful as markers for estimating the risk of inhibitor development. It is also interesting to note that a number of trials have shown that a clinically available chimeric monoclonal antibody against TNF-α is an effective therapeutic agent in patients with various immunologic diseases.[35] It is, however, too early to speculate whether this agent could be of any benefit for patients with hemophilia. The current findings, together with family data of inhibitor concordance in siblings carrying the same causative mutation[5] and the recently described IL10G association with inhibitors,[9] imply that the development of inhibitors is a polygenic complex process that to some extent may be influenced by environmental factors. Further studies expanding the MHC locus haplotype and including, for example, DNA methylation status may contribute to a clearer understanding of this process.

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Authorship

The authors declare no competing financial interests.

A complete list of the members of the Malmö International Brother Study (MIBS) appears in Document S1, available at the *Blood* website; see the Supplemental Document link at the top of the online article.

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