

An Allelic Polymorphism within the Human Tumor Necrosis Factor α Promoter Region Is Strongly Associated with HLA A1, B8, and DR3 Alleles

By A. G. Wilson,* N. de Vries,† F. Pociot,§ F. S. di Giovine,*
L. B. A. van der Putte,† and G. W. Duff*

From the *Section of Molecular Medicine, Department of Medicine, University of Sheffield, Royal Hallamshire Hospital, Sheffield S10 2JF, UK; the †Department of Internal Medicine, Division of Rheumatology, University Hospital Nijmegen, 6500 HB Nijmegen, The Netherlands; and the §Steno Memorial Hospital, DK-2820 Gentofte, Denmark

Summary

The tumor necrosis factor (TNF) α gene lies within the class III region of the major histocompatibility complex (MHC), telomeric to the class II and centromeric to the class I region. We have recently described the first polymorphism within the human TNF- α locus. This is biallelic and lies within the promoter region. Frequency analysis of the TNF- α polymorphism, using the polymerase chain reaction and single-stranded conformational polymorphism, in HLA-typed individuals, reveals a very strong association between the uncommon TNF allele and HLA A1, B8, and DR3 alleles. This is the first association between TNF- α and other MHC alleles and raises the possibility that the uncommon TNF- α allele may contribute to the many autoimmune associations of the A1,B8,DR3 haplotype.

The genes for TNF- α and - β lie within a 7-kb stretch of DNA in the class III region of the MHC (1, 2). The TNF locus lies 250 kb centromeric of HLA B and 350 kb telomeric of the class III cluster (3, 4). TNF- α is a central mediator of the inflammatory response and has important immunological activities (5). Because of these features, there has been much speculation that variations within the TNF locus may contribute to the etiology of MHC-related autoimmune diseases.

Regulation of TNF production occurs at the transcriptional and posttranscriptional levels (6, 7). Sequences within the 5' DNA control the rate of transcription (8, 9). We have recently described a biallelic polymorphism in this region of the TNF- α locus that involves the substitution of guanine by adenosine in the uncommon (TNF2) allele (10).

Using the PCR and single-stranded conformational analysis, we have tested the frequency of the TNF alleles in HLA-typed normal individuals from a north European population. We have found the TNF2 allele to be very strongly associated with HLA A1, B8, and DR3 alleles. This raises the possibility that A1,B8,DR3 haplotypic associations with autoimmune diseases may also be related to polymorphism within the TNF- α locus.

Materials and Methods

DNA Preparation. DNA was isolated from anticoagulated PBMC by standard methods (11).

HLA Typing. HLA-A and -B antigens were typed using standard National Institutes of Health microlymphocytotoxicity assays.

DR typing was performed as follows: 49 samples were typed by standard microcytotoxicity tests using antisera to each of the alleles DR1, -2, -3, -4, -5, -w6, -7, -w8, -9, and -w10. Frozen lymphocytes were thawed and incubated overnight at room temperature in RPMI with 20% FCS before separation into B and T cells. Weak results were confirmed by RFLP analysis. The remaining 118 samples were typed for DR β 1 by PCR amplification of DR β 1 exon 2 (sense primer 5'-CCGGTCGACTGTCCCCCAGCACGT-TTC-3'; antisense primer 5'-GAATTCTCGCCGCTGCACTGTG-AAGC-3'). PCR products were analyzed by dot blot hybridization with DR β 1 sequence-specific oligonucleotide probes (12, 13).

PCR Amplification of TNF- α . For sequencing of the TNF- α 5' DNA and exon 1, 985 bp of DNA was amplified by PCR using a 5' primer (5-TCCCCAGGCTTGTCCTGCTACC-3) and a 3' biotinylated primer (5-GATGAAGCTTGGCCAGGCACTCAC-3). For further screening by single-stranded conformational polymorphism (SSCP), 107 bp of DNA was amplified using a 5' primer (5-AGGCAATAGGTTTGTAGGGCCAT-3) and (5-TCCTCCCT-GCTCCGATCCG-3) as the 3' primer. 100 ng of genomic DNA was used for amplification with 0.2 μ M concentrations of each primer in a total volume of 50 μ l containing Taq DNA polymerase 1.25 U (Northumbria Biologicals Ltd., Northumberland, UK), 200 μ M of each dNTP (Boehringer Mannheim, Mannheim, Germany), and PCR reaction buffer at a final concentration of 10 mM Tris-HCl, 1.5 mM MgCl₂, 50 mM KCl, and 0.1% Triton X-100 (Northumbria Biologicals Ltd.). Cycling was performed at 94°C for 3 min, 60°C for 1 min, and 72°C for 1 min, followed by 35 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, with a final cycle of 94°C for 1 min, 60°C for 1 min, and 72°C

for 5 min. PCR products were separated on an agarose gel, stained with ethidium bromide, and visualized with UV light. Negative controls, using water instead of template, were performed in each experiment.

DNA Sequence Analysis. PCR products were purified using the GeneClean II kit (Strattech Scientific, Luton, UK). Single-stranded DNA template was prepared using streptavidin-coated magnetic beads (Dynal, Liverpool, UK). To 50 μ l purified PCR product was added 50 μ l of dynabeads and 50 μ l solution A, containing 0.17% Triton-X, 100 mM NaCl, 10 mM Tris/HCl, and 1 mM EDTA (all supplied by Sigma Immunochemicals, Dorset, UK). These were mixed and shaken mechanically for 30 min at room temperature. The PCR product/streptavidin-coated beads complex was washed with 100 μ l solution A, and then resuspended in 32 μ l 10 mM Tris/1 mM EDTA (TE) and 8 μ l of 1 M NaOH/4 mM EDTA solution (Sigma Immunochemicals). This was left at room temperature for 5 min. The PCR product/streptavidin-coated beads complex was then precipitated using a magnet, and the supernatant containing the nonbiotinylated strand removed. The beads were again resuspended in 32 μ l TE and 8 μ l of 1 M NaOH/4 mM EDTA solution, and the process was repeated. After this, the complex was again washed with 100 μ l solution A and resuspended in 7 μ l water. Sequencing was performed by the dideoxy chain termination method using a Sequenase Version 2 kit (US Biochemical Corp., Cleveland, OH).

SSCP Analysis. 50 μ l PCR product was denatured with 2.5 μ l 1 M NaOH and heated to 42°C for 5 min. 6 μ l 100% formamide was then added. The samples were loaded on a 9% polyacrylamide gel and electrophoresed for 16 h at 4 V/cm and 4°C. The DNA was stained with ethidium bromide and visualized under UV light.

Southern Blot Analysis. DNA was transferred from an ethidium bromide-stained 9% polyacrylamide gel to a nylon membrane (Zeta-Probe; Bio-Rad Laboratories, Hercules, CA) by electroblotting (4°C, 0.2 A for 12 h). After denaturation and fixation by treatment with 0.4 M NaOH, hybridization with a γ -³²P-end labeled TNF- α oligonucleotide (5-TTCTGGGCCACTGACTGATTTGTG-3, matching positions 3817 to 3840 of the published sequence [1]) was obtained by standard protocols (14).

High stringency washes were performed at 65°C; 2 \times SSC for 15 min, followed by 2 \times SSC for 15 min, 2 \times SSC/0.1% SDS for 30 min, and finally 0.1 \times SSC for 10 min. The membrane was left to dry at room temperature for 10 min. Visualization of the hybridized probe was by autoradiography (Hyperfilm β -max; 2 \times X.OMAT intensifying screens [Genetic Research Ltd., Essex, UK] 6 h exposure).

Statistical Analysis. A 2 \times 2 table was constructed for each DR allele and the corresponding TNF allelic frequencies and the χ^2 value was calculated using Yates' correction or, when appropriate, Fisher's exact test was used to determine statistical significance.

Results and Discussion

A biallelic polymorphism at position -308 was observed involving the substitution of guanine by adenosine in the TNF2 allele (Fig. 1). This is not a highly conserved region between mouse, rabbit, and humans, and does not lie within a currently recognized DNA-binding protein sequence motif.

Using SSCP, 167 normal individuals were screened for the TNF- α polymorphism. Bands corresponding to the two alleles were clearly visible after partial denaturation of the PCR product (Fig. 2 A). Southern analysis of the SSCP with an

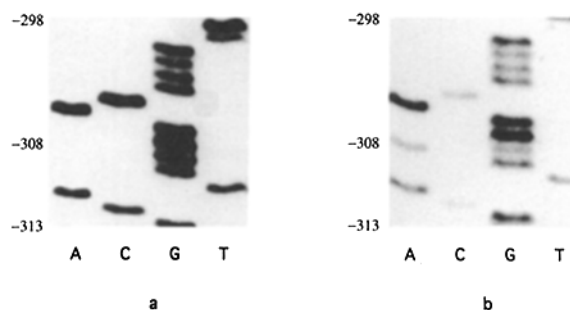


Figure 1. TNF- α polymorphism. (a) G homozygote at position -308 (TNF1 allele); (b) G/A heterozygote at position -308 (TNF1/TNF2 alleles).

internal end-labeled oligonucleotide confirmed the identity of the polymorphic bands (Fig. 2 B).

The TNF2 allele is strongly associated with HLA A1 (Table 1), B8 (Table 2), and DR3 (Table 3) positivity. The association was even stronger when the three alleles were analyzed together (Table 4). The lower degree of association of A1 compared with B8 is most probably due to the greater physical distance from the TNF- α locus. TNF1 is significantly associated with HLA DR4 and DR6 alleles.

In view of the close physical proximity of the TNF locus to these genes, the above associations are almost certainly due

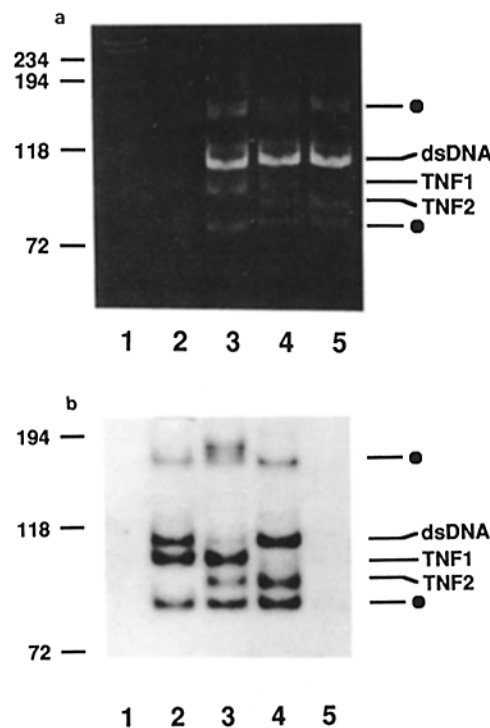


Figure 2. (a) SSCP analysis of position -308. Lanes: 1, PhiX174/HaeIII ladder; 2, negative control; 3, homozygote TNF1; 4, heterozygote TNF1/TNF2; 5, homozygote TNF2. (b) Southern probing of SSCP with TNF- α probe. Lanes 1, PhiX174/HaeIII ladder; 2, homozygote TNF1; 3, heterozygote TNF1/TNF2; 4, homozygote TNF2; and 5, negative control. The bands corresponding to the two TNF alleles and double-stranded DNA are indicated. (●) Two nonspecific, but constant, bands.

Table 1. Frequency of TNF Alleles in HLA DR-typed Individuals

DR allele	TNF1	TNF2	p	OR*
DR1	9	0	NS	
DR2	40	12	NS	
DR3	6	37	$p < 10^{-10}$	22
DR4	53	8	$p < 10^{-3}$	-4
DR5	19	10	NS	
DR6	44	8	$p < 10^{-2}$	-3
DR7	27	10	NS	
DR8	9	2	NS	
DR9	3	2	NS	
DR10	2	0	NS	

* Odds ratio.
n = 161.

Table 2. Association of HLA A1 Allele with TNF2

	TNF2 ⁻	TNF2 ⁺
A1 ⁺	18	28
A1 ⁻	69	12

n = 127; $p < 10^{-6}$; OR = 8.9.

to linkage disequilibrium. Previously described polymorphisms at the TNF locus have all involved the TNF- β gene (15, 16), or have been in the corresponding upstream DNA (17, 18).

The polymorphism that we describe here lies within the region of the TNF- α gene believed to be important in regulation of transcription. Recent reports have demonstrated that a single base polymorphism within a promoter region may alter both the rate of gene transcription and the rate of protein production (19). This can occur even when the polymorphism does not alter the binding of *trans*-activating factors at the altered base position and may be related to changes in the secondary structure of DNA that affect access of transcription factors (20).

Stable interindividual production rates for TNF- α have been demonstrated (21), and in addition, production rate has been shown to correlate with DR alleles. DR2-positive individuals produce low levels, whereas DR3- and DR4-positive

Table 3. Association of HLA B8 Allele with TNF2

	TNF2 ⁻	TNF2 ⁺
B8 ⁺	8	25
B8 ⁻	82	12

n = 127; $p < 10^{-10}$; OR = 21.3.

Table 4. Association of HLA A1,B8,DR3 with TNF2

	TNF2 ⁻	TNF2 ⁺
A1 B8 DR3 ⁺	1	20
A1 B8 DR3 ⁻	95	24

n = 140; $p < 10^{-10}$; OR = 79.

individuals produce high levels of TNF- α (22). The major control of TNF- α production seems to be at the posttranscriptional level (23). Untranslated sequences in the fourth exon are central to the control of translational efficiency (24), and it will be interesting to see if genetic variation in this region may also contribute to the phenotypic associations with DR alleles.

The association of autoimmune diseases with different DR alleles has been widely recognized for some time. HLA haplotypes that include DR3 (especially HLA A1,B8,DR3) are known to be associated with a wide range of autoimmune diseases including insulin-dependent diabetes mellitus, systemic lupus erythematosus, Graves' disease, and celiac disease (25). A previous report has implicated the TNF locus on DR3 haplotypes in diabetes (26). Whether this association is due to the products of these alleles directly or to genes in linkage disequilibrium with them, is not clear. If the TNF2 allele can be shown to have a higher relative risk than class I and II alleles in these diseases, then the case for the involvement of TNF- α as a genetic predisposition factor will be strengthened.

TNF- α has been implicated as a pathogenic mediator in many inflammatory, infectious, and immune diseases (27). The present report is the first to link allelic polymorphism in TNF- α to the MHC class I and class II regions. In view of this, it is interesting to speculate that TNF- α might contribute causally to the many autoimmune diseases associated with the HLA A1,B8,DR3 haplotype.

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Address correspondence to Anthony G. Wilson, Section of Molecular Medicine, Department of Medicine, University of Sheffield, Royal Hallamshire Hospital, Sheffield S10 2JF, UK.

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