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

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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.

Materials and Methods

DNA Preparation. DNA was isolated from anticoagulated PBMC by standard methods (11).
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 (Stratech Scientific, Luton, UK). Single-stranded DNA template was prepared using streptavidin-coated magnetic beads (Dynal, Liverpool, UK). To 50 μl purified PCR product was added 50 μl of dynabeads and 50 μl 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 μl solution A, and then resuspended in 32 μl 10 mM Tris/1 mM EDTA (TE) and 8 μl 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 μl TE and 8 μl of 1 M NaOH/4 mM EDTA solution, and the process was repeated. After this, the complex was again washed with 100 μl solution A and resuspended in 7 μl water. Sequencing was performed by the dideoxy chain termination method using a Sequenase Version 2 kit (US Biochemical Corp., Cleveland, OH).

**SSCP Analysis.** 50 μl PCR product was denatured with 2.5 μl 1 M NaOH and heated to 42°C for 5 min. 6 μl 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 × SSC for 15 min, followed by 2 × SSC for 15 min, 2 × SSC/0.1% SDS for 30 min, and finally 0.1 × 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 × XOMAT intensifying screens [Genetic Research Ltd., Essex, UK] 6 h exposure).

**Statistical Analysis.** A 2 × 2 table was constructed for each DR allele and the corresponding TNF allelic frequencies and the χ² 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 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...
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^{-4}; OR = 8.9.

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.

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 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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