Distribution of four polymorphisms in the tumour necrosis factor (TNF) genes in patients with inflammatory bowel disease (IBD)

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SUMMARY
In 153 patients with IBD, 64 with Crohn's disease (CD), and 89 with ulcerative colitis (UC), as well as 54 healthy controls (HC), the frequencies of four known di-allelic polymorphisms in the genes for TNF-α and lymphotoxin alpha (LTα) were investigated. In the Dutch population, the alleles of these four polymorphisms are present in only five combinations, called TNF haplotypes: TNF-C, -E, -H, -I, -P. Furthermore, the relation with the presence of perinuclear anti-neutrophil cytoplasmic autoantibodies (P-ANCA) was studied. A small, but statistically significant, association between the polymorphism at position -308 in the promoter region of the TNF-α gene and UC was found. The frequency of the uncommon TNF-α -308 allele 2 was found to be decreased in patients with UC compared with HC (allele frequency of allele 2 in UC patients 0.15 versus 0.25 in HC, \( P = 0.044 \)). No significant differences in distribution of the TNF haplotypes were found between IBD patients and HC, although there was a tendency towards a higher frequency of the TNF-C haplotype in UC patients compared with controls (haplotype frequency 22% versus 13%; \( P = 0.19 \)). No statistically significant differences in distribution of the TNF haplotypes were observed between P-ANCA-positive and P-ANCA-negative UC patients. The strength of the associations indicates that TNF genes are not markers for the predisposition to suffer from IBD. They may, however, be markers of subsets of patients with UC and CD.

Keywords inflammatory bowel disease Crohn's disease ulcerative colitis HLA association tumour necrosis factor-alpha lymphotoxin α gene polymorphism haplotype

INTRODUCTION
Although the etiology of the IBD, Crohn's disease (CD) and ulcerative colitis (UC), is unknown, several observations have shown that there may be a genetic predisposition underling these diseases. These include the increased familial incidence, the higher prevalence of disease in monozygotic versus dizygotic twins, the higher prevalence in first degree relatives versus spouses, and the differences in prevalence in diverse ethnic groups (for reviews, see [1,2]). In spite of the fact that these observations strongly support the contribution of genetic factors in the pathogenesis of IBD, the relevant genes have not been identified.

In recent years, there have been several studies on HLA associations in IBD. An association of HLA-DR2 with UC has been shown in some studies, especially those of Japanese UC patients [3–7], but was not found in a study by Duerr & Neigut [8]. An association between CD and the HLA-DR1 allele has been found, and was recently confirmed [4,9]. The associations observed are, however, weak and inconclusive. Although striking associations exist between different alleles of the MHC and various autoimmune diseases, the role of MHC class I or II molecules in many of these diseases remains largely unknown. It is even questionable whether these genes are responsible for the predisposition to the disease, rather than markers for other closely linked genes. TNF-α
and lymphotxin alpha (LTo or TNF-β) are potent proinflammatory and immunomodulatory cytokines that play a central role in the initiation and regulation of the immune response. The genes for these cytokines are tandemly arranged in the central region of the MHC, between the HLA-B and HLA-D locus, at the short arm of chromosome 6. This location has prompted much speculation about the role of TNF-α and LTo in the etiology of MHC-associated diseases, especially those with an inflammatory or autoimmune component [10]. In support of this, Jacob et al. showed that HLA-DR2-positive individuals produce less TNF-α than individuals that are HLA-DR3-positive [11]. HLA-linked polymorphic variations within the TNF genes have been claimed to be responsible for differences in secretion. Messer et al. found that a NcoI restriction fragment length polymorphism (RFLP) in the first intron of the LTo gene influences LTo secretion, whereas an association between this polymorphism and production of TNF-α was described by Pociot et al. [12-14]. An AspHI RFLP, also in the first intron of the LTo gene, was described by Ferencik et al. [15]. Wilson et al. showed that a polymorphism at position -308 in the promoter region of the TNF-α gene is strongly linked to the HLA-A1, -B8, -DR3 ancestral haplotype, and suggested that variations at this position might be important in the regulation of TNF-α production [16-17]. A second polymorphism, at position -238 in the promoter region of the TNF-α gene, has been described by D’Alfonso & Momigliano Richiardi [18]. We have recently shown that in the Dutch population, the alleles of these four polymorphisms are present in only five combinations, that have been called TNF haplotypes: TNF-C, -E, -H, -I, -P (Fig. 1) [19].

Since 1990, several authors have reported the presence of perinuclear anti-neutrophil cytoplasmic antibodies (P-ANCA) in the serum of the majority of patients suffering from UC [20]. In most of these studies, the presence of P-ANCA in UC patients does not correlate with disease activity or extent or the disease, and is detectable even years after colectomy [21]. This, together with the observation that this autoantibody has been found in asymptomatic relatives of patients with UC, suggests that its presence is not secondary to inflammation. Therefore, it has been suggested that P-ANCA may serve as a marker for a genetically controlled immunoregulatory disturbance [21,22]. The finding that P-ANCA positivity was mainly found in a group of HLA-DR2-positive UC patients strengthened this hypothesis [23].

We have previously studied the TNF-α -308 polymorphism in IBD patients [24]. In the present study we report on the distribution of all four polymorphisms and the TNF haplotypes in IBD patients and healthy controls. We have also investigated whether the presence of P-ANCA is correlated with these polymorphisms or the TNF haplotypes.

**SUBJECTS AND METHODS**

Blood samples were obtained from 153 selected IBD patients, 64 with CD (44 females, 20 males; mean age 39 years, range 19–67 years) and 89 with UC (43 females, 46 males; mean age 45 years, range 20–84 years), attending the Department of Gastroenterology over a 2-year period. Blood samples from 54 healthy controls (HC) (24 females, 30 males; mean age 36 years, range 24–60 years) served as controls. All subjects were recruited from The Netherlands and were Caucasian. Diagnosis of CD or UC was based on the conventional clinical, radiological, endoscopic, and pathological criteria, as described by Lennard-Jones et al. [25].

**TNF-α and LTo polymorphisms**

Genomic DNA was extracted from anti-coagulated blood by a conventional proteinase K digestion/phenol-chloroform extraction method. Typing of the di-allelic RFLP at position -308 in the TNF-α promoter (TNF-α -308) was performed by polymerase chain reaction (PCR) amplification (using a 5’ primer 5’-AGGCAATAGGTTTTGGGC TT-3’ and 3’ primer 5’-TCCTTGCCAGTTGG-3’ as the 3’ primer) and NcoI digestion as described by Wilson et al. [16]. A single-strand conformation polymorphism method was optimized for the detection of di-allelic polymorphisms at both positions -308 and -238 in the TNF-α gene.

PCR fragments spanning sequences from position -396 to -69 were generated using a 5’ primer 5’-TCTTCTGCGATCCCGT TGTCTGGA-3’ and a 3’ primer 5’-CAGCGGAAAATT TCTGCTGG-3’ as described by D’Alfonso & Momigliano Richiardi [18]. The 328 base pair (bp) fragments were denatured and run on pre-cast non-denaturing 12.5% polyacrylamide PhastGels. Horizontal electrophoresis and silver staining were performed semi-automatically with the PhastSystem (Pharmacia, Uppsala, Sweden).

PCR amplification with primers located in the 5’ untranslated region and third intron of the LTo gene (5’ primer 5’-CCGTCCTGCTGCTTGGACTA-3’ and 3’ primer 5’-AGAGCTCTGAGGGGACATGTCTG-3’) resulted in 740 bp fragments that were either cut (allele LTo NcoI*1) or remained intact (allele LTo NcoI*2) following incubation with the restriction enzyme NcoI [12] (Fig. 2). For typing the AspHI RFLP described by Ferencik et al., the 740-bp PCR fragments were digested with the isoschizomer BsiHKai (New England Biolabs, Beverly, MA) [15]. This resulted in 425-bp and 315-bp fragments (allele LTo AspHI*1) or the undigested 740-bp fragment (allele LTo AspHI*2). Fragments from individual digests were analysed by electrophoresis in 0.1% etidium bromide-stained 1.5%-agarose gels.

**Indirect immunofluorescence technique**

The standard ANCA indirect immunofluorescence technique

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Fig. 2. Lymphotoxin alpha (LTα) NcoI-restriction patterns in three individuals. The sizes of the fragments after NcoI digestion are given on the left: 740 bp for the LTα NcoI*2 allele; 555 bp and 185 bp for the LTα NcoI*1 allele. Lane 1, PstI digest of phage λ DNA as a molecular weight marker; lanes 2, 3 and 4, an individual homozygous for allele 2, homozygous for allele 1, and a heterozygous person, respectively.

Statistical analysis
The results are presented as genotype and allele frequencies for each gene polymorphism. The haplotypes are shown as haplotype frequencies. For the comparison of allele frequencies as well as TNF haplotype frequencies, Fisher's exact test was performed using the Graphpad InStat software package on a personal computer. A two-sided P value < 0.05 was considered statistically significant.

RESULTS
Genotype frequencies and allele frequencies of the four TNF polymorphisms are shown in Table 1. As can be seen from the Table, there was a small, but statistically significant association between the TNF-α -308 polymorphism and UC when allele frequencies were compared. The frequency of the uncommon TNF-α -308 allele 2 was found to be decreased in patients with UC compared with HC (P = 0.044; odds ratio (OR): 1.86; 95% confidence interval (CI): 1.025–3.39).

The frequencies of the TNF haplotypes are shown in Fig. 3. No significant differences in the distribution of the TNF haplotypes were found between IBD patients and HC.

Table 1. Genotype and allele frequencies of four TNF polymorphisms in patients with IBD and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>CD (n = 64)</th>
<th></th>
<th>UC (n = 89)</th>
<th></th>
<th>HC (n = 54)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotypes (%)</td>
<td>A.F.</td>
<td>Genotypes (%)</td>
<td>A.F.</td>
<td>Genotypes (%)</td>
<td>A.F.</td>
</tr>
<tr>
<td>TNF-α -308 *1,1</td>
<td>42 (66)</td>
<td>0.8</td>
<td>65 (73)</td>
<td>0.85</td>
<td>31 (57)</td>
<td>0.75</td>
</tr>
<tr>
<td>TNF-α -308 *1,2</td>
<td>18 (28)</td>
<td>0.2</td>
<td>21 (24)</td>
<td>0.15</td>
<td>19 (35)</td>
<td>0.25</td>
</tr>
<tr>
<td>TNF-α -308 *2,2</td>
<td>4 (6)</td>
<td>0.08</td>
<td>3 (3)</td>
<td>0.05</td>
<td>4 (7)</td>
<td>0.05</td>
</tr>
<tr>
<td>TNF-α -238 *1,1</td>
<td>56 (88)</td>
<td>0.92</td>
<td>81 (91)</td>
<td>0.95</td>
<td>49 (91)</td>
<td>0.95</td>
</tr>
<tr>
<td>TNF-α -238 *1,2</td>
<td>8 (12)</td>
<td>0.13</td>
<td>8 (9)</td>
<td>0.13</td>
<td>5 (9)</td>
<td>0.13</td>
</tr>
<tr>
<td>TNF-α -238 *2,2</td>
<td>0 (0)</td>
<td>0.00</td>
<td>0 (0)</td>
<td>0.00</td>
<td>0 (0)</td>
<td>0.00</td>
</tr>
<tr>
<td>LTα NcoI*1,1</td>
<td>9 (14)</td>
<td>0.33</td>
<td>12 (13)</td>
<td>0.37</td>
<td>8 (15)</td>
<td>0.38</td>
</tr>
<tr>
<td>LTα NcoI*1,2</td>
<td>27 (42)</td>
<td>0.42</td>
<td>41 (46)</td>
<td>0.63</td>
<td>25 (46)</td>
<td>0.62</td>
</tr>
<tr>
<td>LTα NcoI*2,2</td>
<td>28 (44)</td>
<td>0.48</td>
<td>36 (40)</td>
<td>0.63</td>
<td>21 (39)</td>
<td>0.62</td>
</tr>
<tr>
<td>LTα AspH1*1,1</td>
<td>7 (11)</td>
<td>0.25</td>
<td>11 (12)</td>
<td>0.37</td>
<td>7 (13)</td>
<td>0.38</td>
</tr>
<tr>
<td>LTα AspH1*1,2</td>
<td>30 (47)</td>
<td>0.49</td>
<td>43 (48)</td>
<td>0.63</td>
<td>27 (50)</td>
<td>0.62</td>
</tr>
<tr>
<td>LTα AspH1*2,2</td>
<td>27 (42)</td>
<td>0.46</td>
<td>35 (40)</td>
<td>0.63</td>
<td>20 (37)</td>
<td>0.62</td>
</tr>
</tbody>
</table>

n, Number of individuals; A.F., allele frequency; CD, Crohn's disease patients; UC, ulcerative colitis patients; HC, healthy controls; LTα, lymphotoxin alpha.

† P = 0.044; odds ratio: 1.86; 95% confidence interval: 1.025–3.39 (compared with HC).

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Fig. 3. Representation of the haplotype frequencies of the five TNF haplotypes (TNF-C, -E, -H, -I, -P) in Crohn’s disease (CD) patients (■), ulcerative colitis (UC) patients (□), and healthy controls (HC; □). No statistically significant differences in the distribution of the haplotypes were observed between patients and controls.

Although there was a tendency towards a higher frequency of the TNF-C haplotype in UC patients compared with controls (haplotype frequency 22% vs 13%: P = 0.19; OR: 1.95; 95% CI: 0.762-4.969), the TNF haplotype frequencies and carrier frequencies were identical, only haplotype frequencies are shown in the figure.

Relation between TNF haplotypes and P-ANCA

Sixteen UC patients were P-ANCA-negative, and 56 were P-ANCA-positive. Of the CD patients, six were P-ANCA-positive and 50 P-ANCA-negative.

In Fig. 4, the frequencies of the TNF haplotypes in P-ANCA-negative and P-ANCA-negative UC patients are shown. As can be seen, no statistically significant differences in distribution were seen between P-ANCA-positive and P-ANCA-negative patients.

Discussion

There is ample evidence to support the necessity of a genetic predisposition to suffer from IBD [2,27]. Genetic studies directed to unravel the biological basis of this predisposition are essential to understand the pathogenesis of these diseases. Studies in different populations have found an association between the HLA-DR2 allele in UC and the DR1 allele in CD [3-7,9]. The associations observed are, however, not strong. In view of their particular localization within the central region of the MHC, as well as their potent proinflammatory and immunomodulatory properties, the TNF genes could be involved in the association between HLA and various HLA-associated diseases. Therefore, in a search for stronger genetic associations and markers for disease, we evaluated the distribution of four previously described polymorphisms in the TNF genes in patients with IBD and HC. We previously showed that in the Dutch population, the alleles of these four polymorphisms occur in only five combinations (Fig. 1). In the present study, we found a weak, but statistically significant association between the polymorphism at position -308 in the promoter region of the TNF-α gene and UC (Table 1). The uncommon allele 2 was found to be decreased in UC patients compared with HC. This decreased frequency was also reflected in the distribution of the TNF haplotypes. Although not statistically significant, the TNF-C haplotype was found more often in UC patients, whereas the TNF-E haplotype was found to be decreased in UC patients compared with controls (Fig. 3).

Wilson et al. showed that allele 2 of the TNF-α -308 gene polymorphism is in strong linkage disequilibrium with the HLA-A1-B8-DR3 ancestral haplotype [16,17]. We studied the relation between the TNF haplotypes and HLA-DR alleles in 100 IBD (59 UC, 41 CD) patients. We found that 88% of the HLA-DR3-positive IBD patients were carriers of allele 2 of the TNF-α -308 polymorphism. Furthermore, we found a striking association between the DR1 allele and the TNF-I haplotype: 68% of the DR1-positive individuals carried the TNF-I haplotype, whereas only 33% of the DR1-negative individuals had the TNF-I haplotype (P < 0.01). The TNF-P haplotype was found to be associated with the HLA-DR2 allele, as the frequency was 81% versus 49% in DR2-negative individuals (P < 0.01).

Yang et al. found that P-ANCA-positive UC patients had an increased frequency of HLA-DR2 compared with P-ANCA-negative individuals [23]. We could not confirm the association of P-ANCA with HLA-DR2 in UC patients in our study population (Oudkerk Pool et al., submitted). In relation to the present results, it can be concluded that P-ANCA is not associated with the TNF haplotypes. This suggests that in our...
population. P-ANCA is an immunological marker rather than an immunogenetic marker of a subset of patients with UC.

What is the biological relevance of the TNF haplotypes for IBD? The strength of the associations indicates that these genes are not markers for disease predisposition. They may, however, be markers of subsets of patients with UC and CD. From the clinical point of view, UC and CD are heterogeneous diseases [28,29]. Individual CD or UC patients can differ enormously in their course of disease, prognosis, and response to medical treatment. In a previous study we investigated the in vitro secretion of TNF-α and IL-10 by peripheral blood mononuclear cells (PBMC) in IBD patients and HC, and found large inter-individual differences in the intrinsic capacity to produce these cytokines [30]. In a subsequent study we showed that TNF-α and IL-10 secretion is strongly correlated with the TNF haplotypes [31]. This suggests that a different genetic background may determine the height of the immune response, and might therefore be responsible for different disease manifestations in IBD. Further studies are necessary to determine the clinical relevance of the TNF genes in the identification of groups of IBD patients with different prognosis and response to medical treatment.

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