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Allelic Variation in the TNF-β Gene Does Not Explain the Low TNF-β Response in Patients With Primary Biliary Cirrhosis

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Messer G, Spengler U, Jung MC, Honold G, Eisenburg J, Scholz S, Albert ED, Pape GR, Riethmüller G, Weiss EH. Allelic Variation in the TNF-β Gene Does Not Explain the Low TNF-β Response in Patients With Primary Biliary Cirrhosis. Scand J Immunol 1991;34:735–40

Autoimmune disorders in humans are often associated with particular alleles of major histocompatibility genes. However, the chronic inflammatory liver disease primary biliary cirrhosis (PBC) has not been found to be correlated with certain haplotypes so far.

Interestingly, an impaired production of tumour necrosis factor β (TNF-β) upon mitogen stimulation was observed for PBC patients, especially in the immunologically active stages of the disease. Furthermore, the identification of alleles of the TNF-β gene which differ in one unique amino acid, and in the production of TNF-β after phytohaemagglutinin stimulation, has prompted the idea of a possible linkage between the impaired TNF-β response in PBC and the genetic prevalence of a certain TNF haplotype.

We report here a rapid method for typing the TNFB*1 and TNFB*2 genes by a standard polymerase chain reaction. PBC patients ($n = 60$) as well as randomized healthy controls ($n = 179$) of the Munich area were studied for the occurrence of the TNF alleles. No deviation was found in the PBC collective (0.7) for the TNFB*2 distribution when compared with the control (0.67).

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Primary biliary cirrhosis (PBC) is a chronic cholestatic liver disease of unknown aetiology characterized by the destruction of intrahepatic bile ducts. Tissue damage is thought to be the result of an autoimmune process [1–5]. The disease is associated with many immunological defects including autoantibodies [6–10] and T lymphocyte immunoregulatory abnormalities which involve both $CD4^+$ and $CD8^+$ cells [5, 11, 12]. We have recently shown that T lymphocytes and T-cell lines from patients with PBC produce much lower amounts of tumour necrosis factor β (lymphotoxin, TNF-β) in response to phytohaemagglutinin (PHA) than T cells of controls [13, 14]. Since reduced inducibility of TNF-β in PBC patients was also reflected in the amount of TNF-β mRNA produced, this dysregulation must be controlled at least in part at the level of transcription.

The localization of the tumour necrosis factor genes between the major histocompatibility complex (MHC) class I and class III loci [15–18] has prompted the idea that a polymorphism of the TNF genes might be linked to autoimmune diseases. Several groups have investigated possible associations between MHC genes and PBC [19–22], but no strong associations with class I or class II loci have been found so far.

We have recently characterized two TNF-β alleles (TNFB*1 and TNFB*2) distinguished by a polymorphic *Nco*I restriction site in the first intron of the TNF-β gene, which differ in primary structure and in the inducibility upon stimulation with PHA [23]. The TNFB*1 allele (5.3-kb *Nco*I

TNF-β gene fragment) codes for asparagine at position 26 and presents with a significantly higher TNF-β mRNA and protein synthesis upon stimulation of peripheral blood mononuclear cells than the TNFB*2 allele (11-kb *Nco*I TNF-β gene fragment) coding for threonine at position 26.

We analysed a large panel of PBC patients in order to find out whether the reduced TNF-β response of these patients might be linked with the TNFB*2 allele. In addition, we present a rapid method for typing the TNF-β *Nco*I polymorphism and the result of a collective of 60 caucasoid PBC patients from the Munich area compared with 179 healthy individuals.

MATERIALS AND METHODS

DNA extraction and RFLP typing. DNA was extracted from fresh or cryopreserved peripheral blood lymphocytes either by lysis in 4 M guanidinium thiocyanate followed by phenol/chloroform/isoamylalcohol extraction and ethanol precipitation or alternatively by protease K digestion, NaCl precipitation of peptide debris and ethanol precipitation of the chromosomal DNA. For genomic southern blot analysis, 10 μg of DNA was digested with the restriction enzyme *Nco*I. The digests were separated on a 0.7% agarose gel, transferred to a nylon membrane (Hybond-N$^+$, Amersham & Buchler, Braunschweig, Germany) and hybridized with the 2.4-kb TNF-β *Eco*RI fragment detecting either a 11-kb *Nco*I fragment or a 5.7-kb and a 5.3-kb *Nco*I fragment [23].

For polymerase chain reaction (PCR) typing of the TNF-β *Nco*I restriction fragment length polymorphism (RFLP), the following primers were used: TNF-βL (5′-CCGTGCTTCGTGCTTTGGACTA-3′) located in the 5′ untranslated region, and TNF-βR (5′-AGAGCTGGTGGGGACATGTCTG-3′) within the second intron of the TNF-β gene (see Fig. 1), resulting in the amplification of a fragment of 740 bp. The amplification was carried out in a 20 μl reaction for 30 cycles with 0.5 to 1.0 μg genomic DNA and 1.4 U Taq polymerase (Amersham & Buchler) in a Hybaid thermocycler (Biometra, Göttingen, Germany). The initial denaturation step was increased to 6 min and each PCR cycle contained 1 min of denaturation, 1 min of annealing at 55°C and 1 min of extension reaction at 72 C. For optimal amplification the Mg^{2+} concentration of the reaction buffer was adjusted to 1 mM [24]. Five microlitres of amplification product were restricted in 50 μl of *Nco*I reaction buffer with 0.5 U of the enzyme *Nco*I and loaded on a 1.5% agarose gel. The presence of the *Nco*I recognition sequence in intron 1 of the TNF-β gene (TNFB*1 allele) resulted in two cleavage products of 555 bp and 185 bp, whereas the TNFB*2 allele was detected as the intact amplified 740-bp fragment. In the study, control genomic DNA samples homozygous for either one or the other TNFB allele were amplified and restricted in parallel with the samples tested. Eight individuals of the controls and 9 patients of the PBC group were tested both by digestion of genomic DNA and by RFLP analysis of the PCR amplification product, and no deviation was detected.

Patients and controls. Six male and 54 female patients of PBC aged 32 to 64 years were collected from two referral centres of the Munich area. Diagnosis of PBC was established by clinical, biochemical and histological criteria and all patients had significant titres of antimitochondrial autoantibodies. Additional criteria for histological staging were employed according to Scheuer [25].

As the control group, 179 healthy, unselected and unrelated individuals were typed for the TNFB *Nco*I RFLP, either by genomic southern blot analyses or by PCR typing. Owing to the random selection, the distribution of females versus males was equal.

HLA serology. HLA typing for HLA-A, -B, -C antigens was carried out by standard microlymphotoxicity testing. Twenty-nine PBC patients and 44 individuals of the control group were typed for HLA class I and II antigens. Allele frequencies were compared with data from reference tables [26].

Statistical analyses. TNFB allele frequencies (Table 1) were calculated by gene counting of the observed phenotypes. The control group was analysed by Hardy-Weinberg equilibrium and presented no significant deviation. Relative risks were estimated according to Haldane's formula. Contingency tables were analysed by the Chi-square test [19].

RESULTS

Amplification of a 740-bp exon 1/intron 3 carrying fragment of the human TNF-β gene was performed from genomic DNA (Fig. 1). The amplification primers were chosen such that the *Nco*I restriction site was in an excentric position. Thus, amplification and *Nco*I restriction of the TNFB*1 allele resulted in two fragments of 185 bp and 555 bp in length. The TNFB phenotype frequencies of 179 healthy, randomly selected individuals are given in Table I. The gene frequencies were 0.33 for the TNFB*1 allele and 0.67 for TNFB*2. No deviation from the expectations according to Hardy-Weinberg equilibrium was found. The distribution of the TNFB alleles did not differ significantly between female and male individuals.

In PBC patients, no difference in the frequency of TNFB*1 phenotypes (10%) was found compared with the control (11.2%) (see Table I, in brackets). Heterozygosity was decreased (40%) and the TNFB*2 phenotype was increased in the patients (50%) compared with the controls (43.6% and 45.2%, respectively). The overall

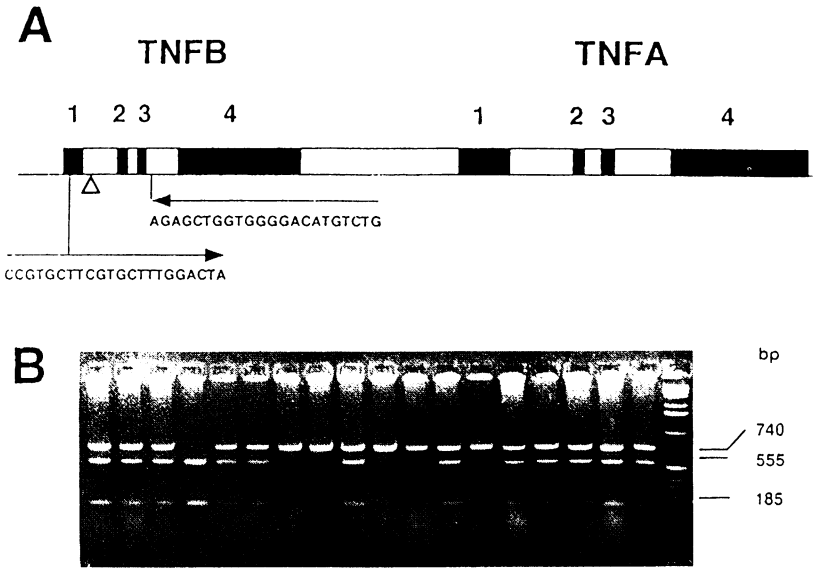

FIG. 1. Strategy for PCR-typing of the *Nco*I RFLP of the human TNF-β gene. The genomic structure of the linked human TNF-β and TNF-α genes is shown in A. Exons are indicated as black boxes with the numbers above. The position and the sequence of the oligonucleotide primers used for PCR amplification of genomic DNA resulting in a 740-bp fragment are depicted underneath. The open triangle indicates the *Nco*I restriction site present in the first intron of the TNFB*1 allele.

Fig. 1B shows the result of the *Nco*I-restricted products obtained from 18 different PBC patients. The 1-kb molecular weight marker (BRL, Karlsruhe, Germany) was co-electrophoresed in the last lane on the right. The sizes of the fragments after *Nco*I restriction are given on the right side (740 bp for the TNFB*2 allele; 555 bp and 185 bp for the TNFB*1 allele). The patient four from the left is homozygous for the TNFB*1 allele.

distribution of the TNFB*1 allele was insignificantly reduced in PBC (0.3) compared with the controls (0.33), and the relative risk of 1.16 for the TNFB*2 allele did not reveal an association of the more frequently found TNFB*2 allele to the disease. To further evaluate whether a correlation with HLA haplotypes could be found, we investigated HLA class I and class II antigens in the patients who were available for typing (PBC $n=29$, and control $n=44$). HLA typing of the patients did not show a strong association of specific HLA haplotypes with PBC (data not shown). In general, a similar increase for certain HLA class I antigens has been observed as previously reported by Prochazka *et al.* [22]. Gene frequencies in the control group were identical to those obtained by the population analysis of Baur *et al.* (in Ref. 26), demonstrating that no bias due to the sampling procedure was present.

DISCUSSION

The high frequency of HLA-B8 and HLA-DR3 antigens among patients with autoimmune diseases, and the tight linkage of the TNFB*1 allele with the B8-DR3 haplotype [27–29], has raised the possibility that variations of the TNF genes might be related to the individual disease susceptibilities. Thus, several groups have studied the association of the diallelic *Nco*I RFLP indicative for the two TNF-β alleles, although often only a small collective of patients, with the following diseases: diabetes mellitus (IDDM, Refs 27–29), Grave's disease [30], systemic lupus erythematosus (SLE), pauciarticular juvenile rheumatoid arthritis (P-JRA), rheumatoid arthritis (RA) and primary Sjögren's syndrome (pSS, Ref. 31). No increase in the frequency of one allele was found when comparing with HLA-matched controls.

TABLE I. Distribution of the TNFB phenotypes in 60 patients with PBC (primary biliary cirrhosis) in comparison to 179 healthy randomly selected individuals. The phenotype is given in numbers and as calculated percentage (in brackets). Expected values of the control group are calculated according to the Hardy-Weinberg equilibrium for the distribution of the TNFB*1 and TNFB*2 alleles and are given under the observed number. The gene frequencies of TNFB*1 and TNFB*2 are given underneath. Relative risk for PBC in the TNFB*2 group (RR = 1.1) is calculated by Haldane's formula; the 2 × 2 tables are calculated as shown in Tiwari & Terasaki [19].

	TNFB*1	TNFB*1,2	TNFB*2
PBC	6 (10%)	24 (40%)	30 (50%)
($n = 60$)	0.3		0.7
Controls	20 (11.2%)	78 (43.6%)	81 (45.2%)
Expected	19.5 (10.8%)	78.8 (44.2%)	80.4 (44.9%)
($n = 179$)	0.33		0.67

Chi-square = 0.4; $P > 0.05$.

Only a higher number of heterozygotes among patients with IDDM was observed [27, 29]. When additional HLA-associated susceptibilities such as ankylosing spondylitis [32, 33], Hashimoto's thyroiditis [34], multiple sclerosis and optic neuritis [35] were analysed, again no significant deviation in gene or phenotype frequencies was found.

Although no strong association was detected between HLA alleles with PBC, the possibility remained that the presence of a particular TNF allele might contribute to the pathogenesis of the disease. Fugger *et al.* reported a significantly decreased frequency of the TNFB*2 allele in 22 patients with PBC [36]. The analysis of 60 patients presented here demonstrates no association of one particular TNF-β allele with the disease. The gene frequencies are the same as in the control group and in agreement with the distribution described in previous reports [27, 29, 32, 36]. Thus, no association can be found between PBC and the *Nco*I RFLP. The question has been raised as to whether differences exist in the TNF genes between healthy individuals and patients which cannot be detected by RFLP analyses. In our study of the linkage of the *Nco*I RFLP with the amino acid substitution at position 26 and of the TNF-α promoter region, we have amplified and sequenced the relevant segments of PBC patients, homozygous for the *Nco*I RFLP (5 patients for the 5′ gene segment of the TNF-β gene and 3 patients for the TNF-α gene promoter), and no further nucleotide differences have been found [23].

The results presented here argue against a particular TNF-β allele being involved in the pathogenesis of PBC. Moreover, the observed low production of TNF-β mRNA and protein in patients with PBC [5, 13, 14] cannot be explained by a unique structure of the TNF-β gene in patients, but might be caused by a disease-specific inability in the activation of transcription factors regulating TNF-β gene expression. In this context, it was of interest to learn that the low TNF-β response of T lymphocytes derived from PBC patients may be due to the low interleukin 2 (IL-2) production by these cells [37]. Moreover, mice used as model systems for autoimmune diseases (NZB × NZW)F1 in which autoimmunity has been attributed to low TNF-α synthesis [38], or MRL/Lpr mice, might also have a reduced IL-2 response [39–41].

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