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Polymorphism of the tumor necrosis factor beta gene in systemic lupus erythematosus: *TNFB-MHC* haplotypes

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Abstract. We investigated the *Nco* I restriction fragment length polymorphism (RFLP) of the tumor necrosis factor beta (*TNFB*) gene in 173 patients with systemic lupus erythematosus (SLE), 192 unrelated healthy controls, and eleven panel families, all of German origin. The phenotype frequency of the *TNFB*1* allele was significantly increased in patients compared to controls (63.6% vs 47.1%, RR = 1.96, p <0.002). The results of a two-point haplotype statistical analysis between *TNFB* and *HLA* alleles show that there is linkage disequilibrium between *TNFB*1* and *HLA-A1, Cw7, B8, DR3, DQ2,* and *C4A DE.* The frequency of *TNFB*1* was compared in SLE patients and controls in the presence or absence of each of these alleles. *TNFB*1* is increased in patients over controls only in the presence of the mentioned alleles. Therefore, the whole haplotype *A1, Cw7, B8, TNFB*1, C4A DE, DR3, DQ2* is increased in patients and it cannot be determined which of the genes carried by this haplotype is responsible for the susceptibility to SLE. In addition, two-locus associations were analyzed in 192 unrelated healthy controls for *TNFB* and class I alleles typed by serology, and for *TNFB* and class II alleles typed by polymerase chain reaction/oligonucleotide probes. We found positive linkage disequilibrium between *TNFB*1* and the following alleles: *HLA-A24, HLA-B8, DRB1*0301, DRB1*1104, DRB1*1302, DQA1*0501, DQB1*0201, DQB1*0604,* and *DPB1*0101. TNFB*2* is associated with *HLA-B7, DRB1*1501,* and *DQB1*0602.*

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Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease of unknown etiology. Since the first reports from Waters and co-workers and Grumet and co-workers in 1971, several authors have found an association between susceptibility to SLE and different alleles of the human major histocompatibility complex (*HLA*). However, the gene or genes responsible for the primary association have not yet been identified. The gene encoding tumor necrosis factor beta (*TNFB*) is located in tandem with the tumor necrosis factor alpha gene within the *HLA* complex, between the *HLA-B* and *C4A* genes (Nedospasov et al. 1986). TNF-β is a lymphokine which plays an important role in the regulation of the immune response as part of the cytokine network (Goeddel et al. 1986). Because of its localization and the function of its product, we considered the *TNFB* gene as a candidate for the primary association with susceptibility to SLE.

An *Nco* I polymorphic restriction site in the first intron of the *TNFB* gene (Webb et al. 1990; Messer et al. 1991a; Abraham et al. 1991) allows the characterization of two alleles. *TNFB*1* carries the *Nco* I restriction site, while *TNFB*2* lacks it because of a point mutation. The aim of this study was to determine the frequency of *Nco* I-RFLP-*TNFB* alleles and of *HLA-TNFB* haplotypes in the healthy population and to compare them with those of the SLE patients. We also investigated linkage disequilibria between *TNFB* and class I or class II alleles in the healthy population.

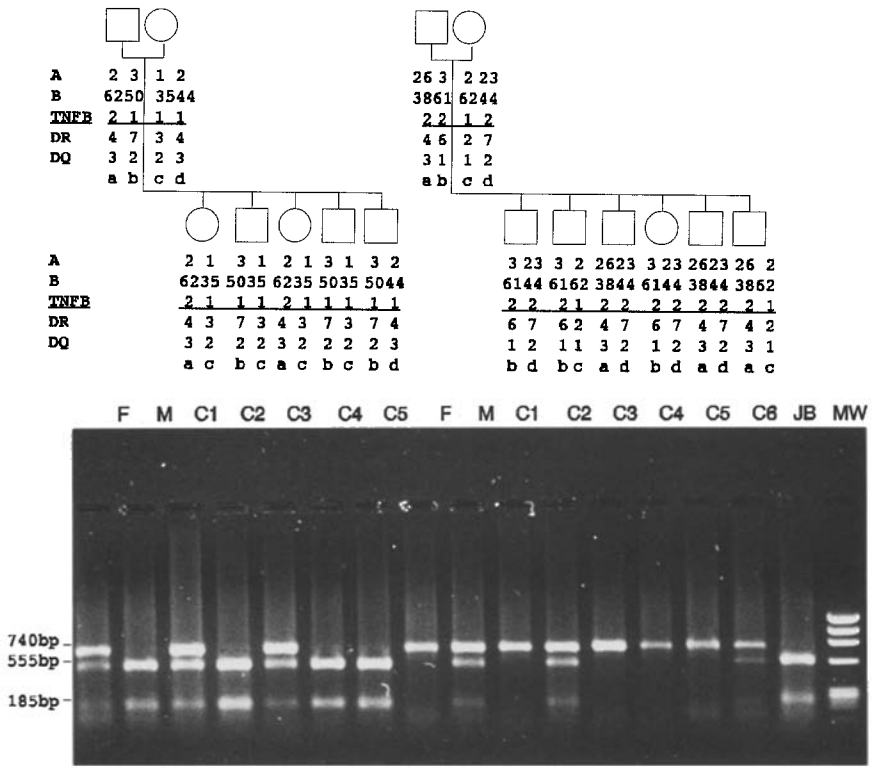

Fig. 1. Segregation pattern of *HLA* class I (*–A* and *–B*), class II (*–DR* and *–DQ*) and *TNFB* alleles in two panel families. Separation by gel electrophoresis of the fragments obtained after PCR amplification of a 740 bp segment of the *TNFB* gene and digestion with *Nco* I. Each lane corresponds to the individual shown immediately above. *TNFB*1* homozygotes show two bands of 555 and 185 bp, respectively; *TNFB*2* homozygotes, one band of 740 bp. In heterozygotes the three bands are present. JB is a *TNFB*1* homozygous cell line and MW is the molecular weight marker Φ × 174/*Hae* III.

Materials and methods

Patients. The study encompassed 173 SLE German patients whose diagnosis fulfilled the revised American Rheumatism Association (ARA) criteria for SLE (Tan et al. 1982). The patients were a randomly selected subset of the 417 involved in the German multicenter SLE study (Hartung et al. 1989). They were serologically typed for the class I *MHC* loci *HLA-A, B,* and *C* and for the class II loci *HLA-DR* and *-DQ* (Hartung et al. 1991). In the class III region they were tested for the complement loci *C4A* and *C4B* at the DNA level (P. Schneider, personal communication).

Controls. One-hundred-and-ninety-two unrelated healthy individuals were included as control group. Eleven panel families were also studied. These samples are part of the panel of the Immunogenetics Laboratory in Munich. They were serologically typed for class I antigens, RFLP typed for class II (Andreas et al. 1989) and for *C4A* and *C4B* (Keller et al. 1991), and using polymerase chain reaction (PCR)/oligonucleotide probes for *DRB1, DQA1, DQB1,* and *DPB1.*

Determination of the Nco I-polymorphism of the TNFB gene. Genomic DNA was obtained from peripheral blood using a salting out method (Miller et al. 1988). The typing was achieved by applying a PCR/RFLP protocol described by Messer and co-workers (1991 b). Briefly, a 740 base pair (bp) fragment of the *TNFB* gene, which extends from exon 1 to intron 3 and includes the polymorphic *Nco* I restriction site, was amplified using PCR. The primers used were: TNF-β L: 5′ CCG TGC TTC GTG CTT TGG ACT A 3′ and TNF-β R: 5′ AGA GCT GGT GGG GAC ATG TCT G 3′. The amplification product was digested with the restriction enzyme *Nco* I. The fragments obtained after amplification and digestion were detected by performing an electrophoresis in an agarose gel and staining with the fluorescent dye ethidium bromide.

Statistical methods. Fisher's exact test was used for the comparison of phenotype and genotype frequencies between patients and controls. The relative risk was calculated by Woolf's method with Haldane's modification. Haplotype frequencies for two-locus haplotypes were estimated from the phenotype of random individuals by an iterative statistical method (Arnold and Albert 1978) using a computer program developed by Baur and Danilovs (1980).

Results

Digestion with *Nco* I of the amplificate obtained from the *TNFB*1* allele yielded two fragments of 555 and 185 bp, respectively. In the case of TNFB**2* the amplificate suffered no change.

Typing of panel families. Eleven panel families, typed serologically for *HLA* class I and by RFLP as well as by PCR/oligonucleotide probes for *HLA* class II, were tested for polymorphism of the *TNFB* gene. The pattern of segregation obtained in the families studied coincided with the hypothesis of a diallelic system. Furthermore, the three possible phenotypes (*TNFB*1* homozygote, *TNFB*2* homozygote, and the heterozygote) could be clearly distinguished. No problems of partial digestion, which could lead to false heterozygote assignment, were found. Figure 1 shows the results obtained in two families.

Table 1. Phenotype and gene frequencies of *TNFB* alleles in patients and controls.

Allele	Patients		Controls		p*	Relative risk
Phenotype frequencies						
*TNFB*1*	110/173	63.6%	90/191	47.1%	<0.002	1.96
*TNFB*2*	147/173	85.0%	170/191	89.0%	ns	0.70
Gene frequencies						
*TNFB*1*	136/346	0.393	111/382	0.291	<0.004	1.6
*TNFB*2*	210/346	0.607	271/382	0.709	<0.004	0.6

* p is the two-sided probability from Fisher's exact test.

Table 2. Two-point-haplotype analysis of *HLA* alleles associated with susceptibility to SLE*.

	HLA-A1	*HLA-Cw7*	*HLA-B8*	*TNFB*1*	*HLA-DR3*	*HLA-DQ2*
HLA-A1	**Patients = >**	1637	1726	1854	1602	1750
		761	1232	901	981	837
		0.49	0.71	0.62	0.53	0.52
		42.1 ±	183.8 ±	31.0 ±	89.5 ±	50.5 ±
	Controls ⇓					
HLA-Cw7	761	**Patients = >**	1857	2489	1849	1755
	439		1073	884	723	473
	0.38		0.73	0.39	0.46	0.21
	11.8 ±		90.5 ±	18.6 ±	34.1 ±	10.9 ±
		Controls ⇓				
HLA-B8	664	783	**Patients = >**	2253	2005	2028
	525	596		1345	1389	1187
	0.67	0.89		0.95	0.81	0.79
	46.9 ±	34.8 ±		72.5 ±	193.1 ±	107.8 ±
			Controls ⇓			
*TNFB*1*	649	814	867	**Patients = >**	2218	2209
	208	171	601		1195	851
	0.19	0.11	0.93		0.76	0.42
	3.0	1.1	38.5 ±		48.9 ±	18.9 ±
				Controls ⇓		
HLA-DR3	476	521	592	942	**Patients = >**	2625
	301	253	483	594		1664
	0.30	0.25	0.59	0.70		0.96
	12.5 ±	4.5 ±	48.5 ±	30.4 ±		193.7 ±
					Controls ⇓	
HLA-DQ2	579	816	667	852	1071	
	258	307	468	304	830	
	0.24	0.19	0.71	0.39	0.94	
	5.4	3.8	27.2 ±	14.9 ±	72.6 ±	

* For each two-point haplotype the table contains a block of four figures as follows:
1) Haplotype frequency per 10000.
2) Absolute delta value per 10000.
3) Relative delta value.
4) Chi square.
Symbols following the Chi square value indicate the nominal level of significance:
† = $p < 0.05$
± = $p < 0.005$

Table 3. Restricted analysis of *TNFB*1* in comparison with *HLA-A1, -Cw7, -B8, -DR3, -DQw2,* and *C4ADE.*

Phenotype		Patients	Controls	Odds ratio		p*
HLA-A1	*TNFB*1*					
+	+	62	30	++ vs −−	3.1	$<10^{-4}$
+	−	8	21	+− vs −−		ns
−	+	47	55	−+ vs −−		ns
−	−	52	78			
-Cw7	*TNFB*1*					
+	+	71	26	++ vs −−	5.7	$<10^{-7}$
+	−	22	22	+− vs −−		ns
−	+	19	52	−+ vs −−		ns
−	−	32	67			
-B8	*TNFB*1*					
+	+	70	29	++ vs −−	3.9	$<10^{-6}$
+	−	1	1	+− vs −−		ns
−	+	38	58	−+ vs −−		ns
−	−	60	96			
C4A DE	*TNFB*1*					
+	+	36	27	++ vs −−	3.8	$<10^{-3}$
+	−	0	2	+− vs −−		ns
−	+	26	44	−+ vs −−		ns
−	−	26	74			
-DR3	*TNFB*1*					
+	+	64	35	++ vs −−	3.4	$<10^{-5}$
+	−	7	7	+− vs −−		ns
−	+	35	54	−+ vs −−		ns
−	−	50	92			
-DQw2	*TNFB*1*					
+	+	72	42	++ vs −−	3.6	$<10^{-5}$
+	−	26	36	+− vs −−		ns
−	+	26	39	−+ vs −−		ns
−	−	32	68			

* p is the two-sided probability from Fisher's exact test.

Phenotype frequencies. The phenotype and gene frequencies of the *TNFB* alleles are listed in Table 1. *TNFB*1* is significantly more frequent in patients than in controls (63.6% in patients and 47.1% in controls; $p < 0.002$). The relative risk, which gives a measure of the strength of the disease association, is 1.96. The phenotype frequency of the *TNFB*2* allele is not significantly different between both groups (85% in patients vs 89% in controls).

Restricted analysis of associated alleles. There is an association of susceptibility to SLE with different genes of the *MHC* in the group of patients studied: *HLA-A1, B8, Cw7, DR3, DR2, DQ2,* and *C4A DE.* (Hartung et al. 1992). *TNFB*1* must be now added to this list. There is a strong linkage disequilibrium between *HLA-A1, B8, Cw7, DR3, DQ2,* and *C4A DE* (Baur and Danilovs 1980). *TNFB*1* is also in linkage disequilibrium with these alleles, as is shown by the results of the two-point haplotype statistical analysis summarized in Table 2. In order to establish whether *TNFB*1* is the gene primarily involved in the susceptibility to SLE, we analyzed the combined presence of each of the mentioned alleles and *TNFB*1* in patients and controls. As shown in Table 3, *TNFB*1* is significantly increased in patients compared to controls only in the presence of the other alleles which are part of the haplotype *A1, Cw7, B8, TNFB*1, C4A DE, DR3,* and *DQ2.*

Two-point haplotypes between TNFB and HLA alleles in the healthy German population. Two-locus associations were analyzed in 192 unrelated healthy controls for *TNFB* alleles and class I antigens typed by serology and for *TNFB* and class II alleles typed by PCR/oligonucleotide probes. Table 4 summarizes the statistically significant associations for class I and class II, respectively.

Discussion

We have found that *TNFB*1* is significantly increased in SLE patients, as has been already described by Fug-

Table 4. Linkage disequilibrium between *TNFB* and *HLA* class I and class II alleles.

	*TNFB*1*				*TNFB*2*			
	f	Δ	Δr	χ2	f	Δ	Δr	χ2
class I								
HLA-A24	670	328	0.40	9.0†	428	–328	0.40	9.0†
HLA-B7	81	–330	–0.80	7.6†	1322	330	0.80	7.6†
HLA-B8	881	608	0.92	36.8‡	50	–608	–0.92	36.8‡
class II								
*DRB1*1501*	38	–325	–0.89	8.5†	1184	325	0.89	8.5†
*DRB1*0301*	845	547	0.78	28.7‡	155	–547	–0.78	28.7‡
*DRB1*1104*	261	170	0.79	8.4†	44	–170	–0.79	8.4†
*DRB1*1302*	426	228	0.49	7.2†	241	–228	–0.49	7.2†
*DQA1*0501*	1251	518	0.30	12.5‡	1207	–518	–0.30	12.5‡
*DQB1*0201*	982	434	0.32	10.8†	925	–434	–0.32	10.8†
*DQB1*0602*	123	–250	–0.67	4.9*	1175	250	0.67	4.9*
*DQB1*0604*	298	155	0.44	4.5*	199	–155	–0.44	4.5*
DPB1**0101*	320	194	0.64	8.3†	108	–194	–0.64	8.3†

f is the haplotype frequency per 10000; Δ is the absolute delta value per 10000; Δr is the relative delta value and χ2, Chi square. Symbols following the Chi square value indicate the nominal level of significance:
* = $p < 0.05$; † = $p < 0.01$; ‡ = $p < 0.005$.

ger and co-workers (1989) in a small group of patients. Messer and co-workers (1991 a) have reported that there is not only a structural but also a functional difference between both *TNFB* alleles. Upon stimulation with phytohemagglutinen (PHA) of peripheral blood mononuclear (PBMN) cells in vitro, *TNFB*1* homozygotes are higher responders than *TNFB*2* homozygotes. This, coupled with the fact that the *TNFB* gene is on the *MHC,* could explain the association of the *MHC* with SLE.

However, coinciding with previous reports from Dawkins and co-workers (1989), Badenhoop and co-workers (1989), and Fugger and co-workers (1989), we have found that the linkage disequilibrium of the *A1, B8, C4A DE, DR3,* and *DQ2* haplotype also includes the *TNFB*1* allele. *TNFB*1* is significantly increased in patients only as part of this haplotype. It is not possible to determine which gene (or genes) carried by this haplotype is (or are) primarily responsible for conferring susceptibility to SLE.

Finally, the analysis of the two-locus haplotypes in the control individuals rendered the following results:

*TNFB*1* is associated with different alleles which can be grouped into three common Caucasian haplotypes:

1. *HLA-B8, DRB1*0301, DQA1*0501, DQB1*0201,* and *DPB1*0101.*
2. *DRB1*1104* and *DQA1*0501.*
3. DRB1**1302* and *DQB1*0604.*

*TNFB*2* is associated with *HLA-B7, DRB1*1501,* and *DQB1*0602,* which are also part of a common Caucasian haplotype.

References

Abraham, L. J., Du, D. C., Zahedi, K., Dawkins, R. L., and Whitehead, A. G. Haplotypic polymorphisms of the *TNFB* gene. *Immunogenetics 33:* 50–53, 1991

Andreas, A., Chandanayingyong, D., Attatippaholkum, W., Sirikong, M., Klaythong, R., Keller, E., and Albert, E. D. Unusual HLA-DR/DQ haplotypes: two different breakpoints in two different DR2-DQw3 haplotypes. *Immunogenetics 30:* 141–144, 1989

Arnold, A. and Albert E. D. Computer Programm zur Berechnung von Haplotypfrequenzen des HLA-Systems. *In* Proceedings of the 7th ISBN, pp. 407–415, 1978

Badenhoop, K., Schwartz, G., Trowsdale, J., Lewis, V., Usadel, K. H., Gale, E. A. M., and Bottazzo, G. F. TNF-α gene polymorphisms in Type I (insulin dependent) diabetes mellitus. *Diabetologia 32:* 445–448, 1989

Baur, M. P. and Danilovs, J. A. Population analysis of HLA-A,B,C,DR and other genetic markers. *In* P. I. Terasaki (ed.): *Histocompatibility Testing 1980,* pp. 955–1004, UCLA Tissue Typing Laboratory, Los Angeles, 1980

Bettinotti, M. P. Untersuchungen an zwei mit dem Systemischen Lupus Erythematosus assoziierten HLA-Haplotypen: Dissertation (Humanbiologie) der Medizinischen Fakultät der Ludwig-Maximilians-Universität München, 1992

Dawkins, R. L., Leaver, A., Cameron, P. U., Martin, E., Kay, P. H., and Christiansen, F. T. Some disease-associated ancestral haplotypes carry a polymorphism of TNF. *Hum Immunol 26:* 91–97, 1989

Fugger, L., Morling, N., Ryder, L. P., Georgsen, J., Jakobsen, B. K., Svejgaard, A., Andersen, V., Oxholm, P., Pedersen, F. K., Friis, J., and Halberg, P. NcoI restriction fragment length polymorphism (RFLP) of the tumor necrosis factor (TNFα) region in four autoimmune diseases. *Tissue Antigens 34:* 17–22, 1989

Goeddel, D. V., Aggarwal, B. B., Gray, P. W., Leung, D. W., Nedwin, G. E., Palladino, M. A., Patton, J. S., Pennica, D., Shepard, H. M., Sugarman, B. J., and Wong, G. H. W. Tumor necrosis

factors: gene structure and biological activities. *Cold Spring Harbor Symposia on Quantative Biology, LI:* 597–609, 1986

Grumet, F. C., Coukell, A., Bodmer, J. G., Bodmer, W. F., and McDevitt, H. Histocompatibility (HL-A) antigens associated with systemic lupus erythematosus. *N Engl J Med 285:* 193–196, 1971

Hartung, K., Fontana, A., Klar, M., Krippner, H., Jörgens, K., Lang, B., Peter, H. H., Pichler, W. J., Schendel, D., Robin-Winn, M., and Deicher, H. Association of class I, II and III MHC gene products with systemic lupus erythematosus. *Rheumatol Int 9:* 13–18, 1989

Hartung, K., Coldewey, R., Krapf, F., Lang, B., Specker, Ch., Schendel, D., Schneider, P., Seuchter, S., Stangel, W., Albert, E., and Deicher, H., and the members of the Deutsche SLE-Multizenter Studie. Hetero- and homozygosity of MHC class II gene products in systemic lupus erythematosus. *Tissue Antigens 38:* 165–168, 1991

Hartung, K., Baur, M. P., Coldewey, R., Kalden, J. R., Lakomek, H. J., Peter, H. H., Schendel, D., Schneider, P. M., Seuchter, S. A., Stangel, W., and Deicher, H. R. G., and the members of the SLE Multicenter Study Group. MHC-haplotypes and complement C4-alleles in systemic lupus erythematosus – Results of a multicenter study. *J Clin Invest,* in press, 1992.

Keller, E., Andreas, A., Scholz, S., Dörr, H. C., Knorr, D., and Albert E. D. Prenatal Diagnosis of 21-Hydroxylase Deficiency by RFLP analysis of the 21-hydroxylase, complement C4 and HLA class II genes. *Prenatal Diagnosis 11:* 827–840, 1991

Messer, G., Spengler, U., Jung, M. C., Honold, G., Blömer, K., Pape, G. R., Riethmüller, G., and Weiss, E. H. Polymorphic structure of the tumor necrosis factor (TNF) locus. An NcoI polymorphism in the first intron of the human TNF-β gene correlates with a variant amino acid in position 26 and a reduced level of TNF-β production. *J Exp Med 173:* 209–215, 1991a

Messer, G., Spengler, U., Jung, M. C., Honold, G., Eisenburg, J., Scholz, S., Albert, E. D., Pape, G. R., Riethmüller, G., and Weiss, E. H. Allelic variation in the TNF-beta gene does not explain the low TNF-beta response in patients with primary biliary cirrhosis. *Scand J Immunol 34:* 735–740, 1991b

Miller, S. A., Dykes, D. D., and Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Res 16:* 1215, 1988

Nedospasov, S. A., Shakhov, A. N., Turetskaya, R. L., Mett, V. A., Azizov, M. M., Georgiev, G. P., Kowliko, V. G., Dobzynin, V. N., Filippov, S. A., Bystiov, N. S., Boldyeva, E. F., Chuvpilo, S. A., Chumakov, A. M., Shingarova, L. N., and Ovchinmikov, Y. A. Tandem arrangement of genes coding for tumor necrosis factor (TNF-α) and lymphotoxin (TNF-β) in the human genome. *Cold Spring Harbor Symposia on Quantitative Biology LI:* 611–624, 1986

Tan, E. M., Cohen, A. S., Fries, J. F., Masi, A. T., McShane, D. J., Rothfield, N. F., Schaller, J. G., Talal, N., and Winchester, R. J. 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum 25:* 1271–1277, 1982

Waters, H., Konrad, R., and Walford, R. L. The distribution of HL-A histocompatibility factors and genes in patients with systemic lupus erythematosus. *Tissue Antigens 1:* 68–73, 1971

Webb, G. C. and Chaplin, D. D. Genetic variability at the human tumor necrosis factor loci. *J Immunol 145:* 1278, 1990