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Extensive genetic polymorphism in the human tumor necrosis factor region and relation to extended HLA haplotypes

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ABSTRACT **We have identified three polymorphic microsatellites (which we call TNFa, TNFb, and TNFc) within a 12-kilobase region of the human major histocompatibility complex (MHC) that includes the tumor necrosis factor (*TNF*) locus. TNFc is located within the first intron of the TNF-β gene and has only 2 alleles. TNFa and TNFb are 3.5 kilobases upstream (telomeric) of the TNF-β gene and have at least 13 and 7 alleles, respectively. TNFa, -b, and -c alleles are in linkage disequilibrium with alleles at other loci within the MHC, including class I, class II, and class III. TNFa, -b, and -c alleles are also associated with extended HLA haplotypes. These TNF polymorphisms will allow a thorough genetic analysis of the involvement of TNF in MHC-linked pathologies.**

The tumor necrosis factors (TNFs) have been recognized as essential mediators of the inflammatory response (1–3). The genes encoding TNF-α, the major macrophage–monocyte-derived form, and TNF-β, which seems to be produced exclusively by lymphocytes, are located within a 7-kilobase (kb) region (4, 5), which we will henceforth designate as the *TNF* locus. The *TNF* locus is located within the major histocompatibility complex (MHC) of mouse and human (6, 7). In humans, *TNF* maps 320 kb centromeric to HLA-B (class I) and 340 kb telomeric to the C2/BF complex (class III) (8, 9).

The location of *TNF* within the MHC has prompted much speculation about the role of *TNF* alleles in the etiology of MHC-linked diseases, in particular those with an inflammatory or autoimmune component. This hypothesis has been difficult to test because of a lack of genetic markers in the locus: an extensive search for restriction fragment length polymorphisms (RFLPs) has yielded a biallelic *Nco* I RFLP (10–13) and a very rare *Eco*RI RFLP (14). In spite of its limited information content, the *Nco* I RFLP has already allowed some tentative associations between *TNF* alleles and autoimmune diseases (11, 15); recent evidence also suggests that one *Nco* I allele correlates with increased TNF-α and reduced TNF-β production (13, 16).

Microsatellite mapping is a recently developed technique in which the repeat number of a simple sequence element (usually a CA or CT dinucleotide) occurring at a unique location within the genome is measured after amplification by the PCR and used as an allelic marker (17). We have previously used this technique to define a multiallelic polymorphism within the mouse *TNF* locus (18). The present study extends this work to humans and defines three polymorphic regions that are in linkage disequilibrium with alleles at other MHC loci.

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MATERIALS AND METHODS

Microsatellite Mapping. We used the following primers for amplification of the TNFa and TNFb microsatellites: primer 1, GCACTCCAGCCTAGGCCACAGA; primer 2, GCCTCTAGATTTCATCCAGCCACAG; primer 3, CCTCTCTCCCCTGCAACACACA; primer 4, TGTGTGTTGCAGGGGAGAGAGG (complement of primer 3). Approximately 50 ng (1 μl) of genomic DNA was added to 9 μl of PCR buffer (18) containing 1 mM each primer 1 and primer 2 and 1 unit of *Taq* DNA polymerase (Genofit, Geneva). This mixture was subjected to 20 cycles of amplification (94°C for 25 s, 58°C for 60 s, and 74°C for 60 s); this preamplification step ensures that enough template will be available for hybridization with the labeled internal primer. Then 50,000 cpm (^{32}P) of 5′-end-labeled primer 3 (for the TNFa microsatellite) or primer 4 (for TNFb) was added with 1 unit of *Taq* DNA polymerase in a total vol of 2 μl of PCR buffer, and the mixture was subjected to 20 additional amplification cycles. The pattern of amplified fragments was analyzed by electrophoresing an aliquot of the reaction mixture on a denaturing 6% acrylamide gel (sequencing gel) and revealed by autoradiography. For the detection of the TNFc polymorphism, we used two primers (GGGAGGTCTGTCTTCCGCCG and CGTTCAGGTGGTGTCATGGG) flanking the $(CT)_n$ repeat and a one-step amplification procedure (18).

Population Sampling and Statistical Analysis. We analyzed a total of 110 DNA samples from HLA-typed individuals, distributed as follows: (*i*) 23 homozygous cell lines from the Tenth International Histocompatibility Workshop (19); (*ii*) 28 families with at least 2 members sharing an HLA haplotype, so that *TNF* alleles could be associated with haplotypes; (*iii*) 15 individuals for which only phenotypes could be obtained. Our population comprised a total of 147 independent HLA haplotypes, among which the frequencies of the most common HLA alleles were very similar to those determined for the French population in general (20). HLA-A, -B, -DR, and -DQ serological types were available for all but two individuals analyzed; HLA-C for 96 of them; HLA-DP cellular typing for 85; and class III allotypes C2, BF, C4A, and C4B for 16, 80, 67, and 67, respectively. All individuals were Caucasians except three who were of Mongoloid origin; most of the families and unrelated individuals originated from southern France.

The statistical analysis included (*i*) allele frequency estimations by $1-\sqrt{1-n}$ (where n is the antigenic frequency) from the phenotypes of all unrelated individuals ($N = 94$), assuming no *TNF* locus null alleles and counting each of the 23 homozygous cell lines as one haplotype, (*ii*) 2 × 2 linkage

Abbreviations: TNF, tumor necrosis factor; MHC, major histocompatibility complex; RFLP, restriction fragment length polymorphism; IDDM, insulin-dependent diabetes mellitus.

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disequilibria and haplotype frequencies: 2 × 2 tables were drawn up from allele counts in unrelated individuals, where +/+ = presence of the two considered alleles, +/− = presence of the TNF allele alone, −/+ = presence of the other MHC allele alone, −/− = absence of both alleles. The 2 × 2 haplotype frequencies (P_{AB}) and gametic associations or linkage disequilibrium (Δ) were calculated according to Mattiuz *et al.* (21) as $P_{AB} = P_A \times P_B + \Delta_{AB}$, where P_A and P_B are, respectively, the allele frequencies and $\Delta_{AB} = \sqrt{F4} - \sqrt{(F2 + F4)(F3 + F4)}$; F2, F3, and F4 represent the frequency of +/−, −/+, and −/−, respectively. Fisher's exact test was used to determine the significance of the deviation from 0 of the Δ value. The extended HLA haplotypes were deduced from the family data and from this 2 × 2 analysis.

RESULTS AND DISCUSSION

The region surrounding the human *TNF* locus contains at least three microsatellites, which we designated TNFa, TNFb, and TNFc. TNFc is located in the first intron of the TNF-β gene (nucleotides 1238–1260 in ref. 5), while TNFa and TNFb are adjacent to each other in a region 3.5 kb upstream of the TNF-β mRNA start site (22). The primers that we used measure variations in the number of CA (TNFa) or CT (TNFb) repeats within this region.

The 110 DNA samples that we typed for TNFa, TNFb, and TNFc cover most of the HLA haplotypes commonly found in Caucasian populations and include enough families to ascertain inheritance patterns. The prominent characteristics of the *TNF* alleles can be summarized as follows:

(*i*) Number of alleles. A preliminary study on randomly sampled blood donors revealed a limited, biallelic polymorphism of the TNFc microsatellite (data not shown). Further analysis of 74 DNA samples from HLA-typed workshop lines and families failed to reveal any additional TNFc alleles. Based on the length of the poly(CA) or poly(CT) repeats, we could identify 12 TNFa and 6 TNFb alleles in our sample population (Fig. 1; Tables 1 and 2). The length of consecutively numbered TNFa alleles differed by 2 nucleotides (one repeat), while the TNFb alleles differed by only 1 nucleotide. Although they were identified in other individuals (22), the TNFa9, TNFa15, and TNFb6 alleles were absent from our French sample. In addition, we have evidence for the existence of null alleles: the DNAs from two individuals with one shared HLA haplotype (mother and son) each contained a single, and different, observable TNFa allele. Whether null alleles result from deletions in the TNFa region or mutations in the sequences complementary to the oligonucleotide primers remains to be determined.

(*ii*) Informativity of the polymorphism. The *TNF* alleles are not distributed uniformly in the population (Tables 1 and 2) but show a very high level of heterozygosity, particularly for TNFa. The calculated polymorphism information content scores are 0.290 for TNFc and 0.861 for TNFa (maximum for a biallelic RFLP, 0.38), underscoring the advantages of microsatellite mapping over RFLP analysis. It should also be noted that we never detected more than 2 alleles in one individual, even in those carrying HLA haplotypes reported to contain duplications of the region containing the *TNF* locus (23). The existence of null alleles could lead to an underestimate of the heterozygosity of the population, since apparent homozygotes could in fact carry a null allele; likewise, it prevents haplotype assignments in apparent homozygotes when family data are not available. However, we identified 3 cases of demonstrable TNFa homozygosity (i.e., where null alleles could be excluded) among the 12 possible homozygous individuals who were not also homozygous for the rest of their MHC. All of the 23 MHC homozygous workshop cell lines scored as homozygotes for TNFa, -b, and -c.

(*iii*) Inheritance. As expected, the *TNF* alleles segregated in a codominant manner with HLA haplotypes in each of the 8 families for which we had enough members to follow inheritance patterns. In one case with a crossover between C4B (class III) and HLA-DR (class II), the *TNF* alleles segregated with the class I–class III region, consistent with the physical mapping of the *TNF* locus.

(*iv*) Linkage disequilibria. Pairwise analysis of allelic associations between TNFa, TNFb, TNFc, and other polymorphic MHC loci uncovered several strong linkage disequilibria (Tables 1 and 2). Associations were not stronger with the closely linked class I (HLA-B, HLA-C) or class III (C4A, BF)

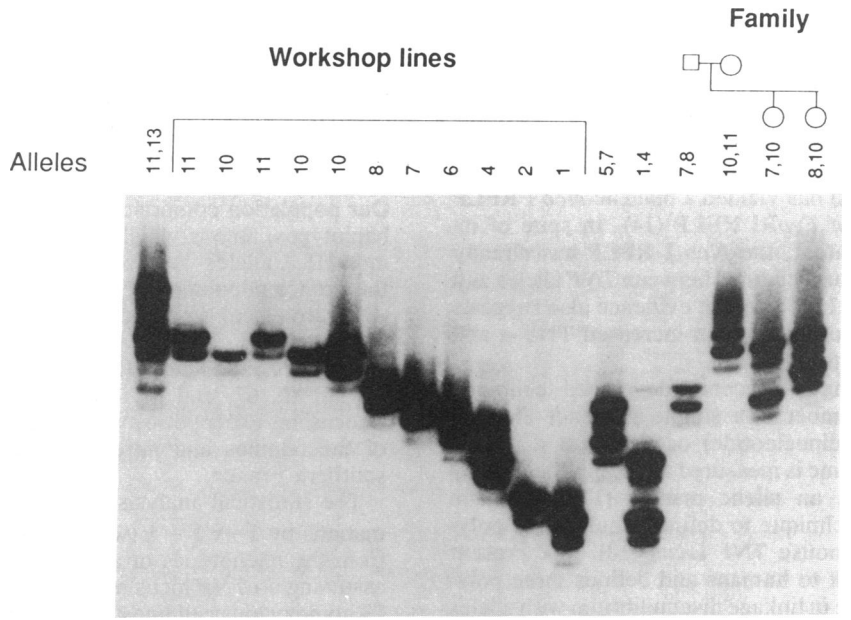

FIG. 1. Multiplicity and codominant inheritance of the TNFa alleles. DNA samples were amplified and analyzed as described. Three heterozygous individuals (with alleles 11 and 13, 5 and 7, 1 and 4), 11 HLA homozygous workshop lines, and a family with 2 children, in which both children inherited the same maternal allele (allele 10) but a different paternal allele (allele 7 or 8) are shown. Alleles 3, 9, and 12 are not represented among the samples shown. The autoradiogram was slightly overexposed in order to visualize the 2-nucleotide "shadow" ladder that is always seen when using this technique (18).

Table 1. Allele frequencies, 2-point haplotype frequencies, and significant 2 × 2 linkage disequilibria (Δ) between TNFa alleles and alleles at other MHC loci

Allele	Allele frequency	Linkage	Δ × 10^4	Haplotype frequency × 10^4	Fisher's *P*
1	0.020	C5	244.7	266.01	2.87×10^{-3}
		BFF1	197.9	202.74	1.48×10^{-5}
		DR3	148.4	171.33	2.72×10^{-3}
		B18	153.9	164.99	4.18×10^{-4}
2	0.215	A1	606.4	1112.97	2.97×10^{-5}
		C4AQ0	556.8	854.60	7.85×10^{-4}
		B8	527.1	771.61	2.02×10^{-6}
		B17	164.4	264.75	2.14×10^{-2}
3	0.034	A9	170.8	230.07	2.06×10^{-3}
		B5	139.4	165.19	1.02×10^{-2}
4	0.040	B14	150.3	164.01	1.39×10^{-3}
5	0.040	C4A2	206.3	232.34	7.00×10^{-3}
		B21	198.4	220.66	3.14×10^{-4}
		BFSO.7	193.8	203.36	2.96×10^{-4}
6	0.101	DR8	185.8	227.21	2.85×10^{-3}
		DQw4	165.4	192.12	9.45×10^{-3}
7	0.134	BFF	478.5	840.07	2.48×10^{-3}
		DQw2	409.2	784.13	3.65×10^{-3}
		DR7	482.9	731.03	6.92×10^{-5}
		B12	401.3	606.10	3.04×10^{-4}
		A29	190.9	264.25	1.75×10^{-2}
		B13	144.6	171.95	6.09×10^{-3}
8	0.040	BFF	265.5	374.01	6.88×10^{-3}
		B12	227.7	289.13	1.41×10^{-3}
		DR7	215.6	290.08	4.50×10^{-3}
		A29	193.4	221.45	8.99×10^{-4}
10	0.154	NS			
11	0.128	DQw1	543.8	1257.60	1.38×10^{-3}
		C7	512.4	916.43	6.91×10^{-3}
		B7	474.4	591.01	2.61×10^{-7}
		A9	362.0	587.32	2.06×10^{-3}
		DR2	220.8	346.84	1.35×10^{-2}
12	0.074	NS			
13	0.013	NS			

Sample size, 94 unrelated individuals. All disequilibria with *P* values $<5 \times 10^{-2}$ are shown. The values for the allele frequencies, Δ, the haplotype frequency (2-point), and Fisher's *P* were calculated as described. NS, no significant disequilibria detected.

genes than with the physically more distant class II genes. Thus, studies that have linked the potential of human monocytes to synthesize TNF with the HLA-DR alleles of the donors (24–26) could in fact have detected linkage disequilibria between class II and TNF. Surprisingly, the associations between TNFa and TNFb alleles were not significantly higher than those between TNF and other MHC loci (Tables 1 and 2), suggesting that the TNFa and TNFb microsatellites evolved independently in spite of their tight physical linkage. The data in Table 1 also show that the TNFa alleles 7 and 8 are associated with the same set of other MHC alleles; this strongly argues for a relatively recent divergence of alleles 7 and 8 in the population from which our sample was drawn.

Within the limits imposed by the small sample of this study, we also sought associations between *TNF* alleles and the most common extended haplotypes. The most characteristic TNF associations with extended HLA haplotypes are shown in Table 3. More could probably be described with a larger sample size. It is worthwhile to note that the TNFa5 allele, which is found in two families on a haplotype bearing the rare BF allele SO.7, is also carried by a BFS11 haplotype, as BFS11 is a variant derived from SO.7 (27). Of particular interest is the association of the TNFa1 allele with the B18, BFF1, C4A3, C4BQ0, DR3, DQw2 haplotype. This haplotype is known to be associated with an increased risk of insulin-dependent diabetes mellitus (IDDM) and is common in southern Europe, particularly in the Basque population (28, 29). Within our initial sample, the TNFa1 allele was found in all three B18, BFF1, DR3 individuals and in no others. A more recent analysis of multicase IDDM families has confirmed and strengthened this association (unpublished observations).

(*v*) Possible implications. Clear causal relationships have been demonstrated between defects in class III genes coding for complement components (C2, C4) and the appearance of systemic lupus erythematosus or other immune deficiencies (30). These conditions had been previously described as HLA-linked diseases. The genes coding for the major heat shock protein hsp70 (31), which has been shown to play a major role in the maturation of newly synthesized proteins, and for two proteins that are probably involved in the transport of immunogenic peptides into the endoplasmic reticulum (32–34) have recently been mapped to the MHC. Deficiencies in either of these types of genes could also lead

Table 2. Allele frequencies, 2-point haplotype frequencies, and significant 2 × 2 linkage disequilibria (Δ) between TNFb and TNFc alleles and alleles at other MHC loci

Polymorphism	Allele	Allele frequency	Linkage	Δ × 10^4	Haplotype frequency × 10^4	Fisher's *P*
TNFb	1	0.112	B14	146.1	188.33	2.22×10^{-2}
			B15	215.5	319.91	1.4×10^{-2}
			TNFa3	143.2	185.01	2.76×10^{-2}
	2	0.022	DR6	156.4	199.63	1.67×10^{-2}
	3	0.164	TNFa2	606.4	937.22	6.14×10^{-6}
			DR3	375.3	573.93	6.83×10^{-4}
	4	0.418	TNFa10	539.1	1131.70	2.13×10^{-3}
			TNFa11	539.3	1069.44	5.2×10^{-4}
			DR2	389.0	823.16	4.75×10^{-3}
	5	0.224	TNFa6	395.88	613.16	7.7×10^{-4}
			DR1	327.59	636.71	9.7×10^{-4}
	7	0.060	B21	265.5	316.31	2.52×10^{-4}
			B14	162.4	184.94	3.2×10^{-3}
			TNFa5	220.7	247.40	2.4×10^{-4}
			TNFa4	220.7	247.40	3.4×10^{-4}
TNFc	1	0.772	C4B1	1267.8	7614.60	3.54×10^{-2}
	2	0.228	DQw3	666.7	1255.90	1.14×10^{-3}
			DR4	430.5	639.19	4.09×10^{-3}
			B5	316.3	491.53	1.24×10^{-2}

The sample and analysis are identical to those of Table 1.

Table 3. Most characteristic extended HLA haplotypes including TNFc and TNFa alleles

Allele	HLA-A	Cw	B	TNFa	TNFb	TNFc	BF	C4A	C4B	DR	DQw
1	A1	Cw7	B8	2	3	1	BFS	C4AQ0	C4B1	3	w2
2	A29		B12	7			BFF			7	w2
3		Cw7	B7	11	4	1	BFS			2	w1
4	A30	Cw5	B18	1	2		BFF1	C4A3	C4BQ0	3	w2
5			Bw50	5			BFSO.7	C4A2	C4B1	7	w2
6				6						w8	w4
7	A1	Cw7	B17	2			BFS	C4A6	C4B1	7	w2
8	A30	Cw6	B13	7			BFS	C4A3	C4B1	7	w2
9	A28	Cw8	B14	4	1,7	2	BFS	C4A3	C4B1	7	w2

The alleles known to be part of the extended haplotypes but not in significant linkage disequilibrium with the other alleles in our sample are underlined. Each entire haplotype was represented at least twice in the population studied and up to nine times when considering the 4-point haplotypes including the HLA-B, BF, TNFa, and HLA-DR loci.

to manifestations of autoimmunity. In contrast, there is still very little evidence for MHC class I or class II alleles (instead of alleles of other genes in linkage disequilibrium with them) being directly responsible for the development of particular pathologies. A possible exception is the strong circumstantial evidence linking specific amino acid residues in the DQβ or DRβ chains to susceptibility or resistance to IDDM and rheumatoid arthritis (41).

The identification of several multiallelic polymorphisms in very close physical linkage to the human *TNF* locus opens the way to a systematic study of associations between *TNF* alleles and the development of MHC-linked diseases: if *TNF* alleles should turn out to represent higher relative risk factors than class I or class II alleles in some pathologies, the case for an involvement of TNF would obviously be strengthened. Combined typing for TNFa, -b, and -c and for the *Nco* I RFLP could further increase the precision of this analysis by extending it to *TNF* haplotypes (Table 3). There is direct evidence for the involvement of TNF (or TNF deficiencies) in the etiology of several autoimmune diseases, including systemic lupus erythematosus-associated glomerulonephritis (25, 35) and the pancreatic insulitis that leads to IDDM (16, 36–39). In addition, a genetic factor mapping near or within the MHC class III region and acting independently of HLA-DQ has been associated with IDDM susceptibility (40). Since *TNF* alleles are in linkage disequilibrium with both MHC class I and class II alleles, it is certainly possible that some of the MHC disease associations found so far do in fact result from structural or regulatory defects in the *TNF* genes.

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