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Novel Compound Tetra-, Dinucleotide Microsatellite Polymorphism in the Tumor Necrosis Factor/Lymphotoxin Locus

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A polymorphic $(TGCG)_n$ tetranucleotide repeat was discovered juxtaposed to the $(GT)_n$ dinucleotide repeat that comprises the tumor necrosis factor a microsatellite (TNFa) located telomeric to the tumor necrosis factor/lymphotoxin gene cluster. The degree of complexity of this compound tetra-, dinucleotide microsatellite consists of 16 potential alleles of combined length ranging from 24 to 54 bp. The pattern of frequencies of individual alleles belonging to the compound TNFa microsatellite was established from 52 healthy volunteers and was found to be highly heterogeneous. The data diverges significantly from previously published statistics that recognized only a simple variable dinucleotide tandem repeat. The newly recognized compound tetra-, dinucleotide TNFa microsatellite polymorphism establishes a more accurate genetic basis to explore potential linkage with disease susceptibility genes located within this region of the class III major histocompatibility complex. In addition, variable tumor necrosis factor and lymphotoxin production may reflect the more complex polymorphic nature of this microsatellite region. Finally, compound microsatellites probably exist elsewhere, throughout the human genome. Recognition of their presence may have a considerable impact on the validity of past and future microsatellite-based genetic analyses.

Microsatellite markers, variable number tandem repeats of 2- to 5-bp units, are employed extensively in linkage analyses to study the molecular basis of genetic diseases. Insertion-deletion and restriction fragment length polymorphisms (RFLP) also provide information for tracking the inheritance of genetic diseases in affected families. However, these markers occur relatively infrequently, possess few alleles, and thus tend to be less informative, and the technology needed to identify them is complicated and time-consuming. Microsatellites are more abundant and are easier to find, identify, and type by PCR-coupled gel and capillary electrophoresis methodology. It is estimated that there are approximately 500,000 microsatellites distributed throughout the human genome with an estimated average spacing of 7,000 bp. Finally, microsatellites tend to have multiple alleles. Therefore, odds are excellent that many genes may have several linked, highly informative microsatellites.

Amplification by PCR and adaptation of automated DNA sizing technology for locus genotyping have promoted the popularity of using microsatellite markers in genetic analyses (22, 45). However, microsatellite loci can be complex, exhibiting features of imperfect and compound tandem repeats, and several microsatellite loci may coexist closely juxtaposed. Frequently, microsatellite loci are characterized by sequence data derived from a single or a small number of reference cases. Inaccurate characterization and underestimation of microsatellite locus complexity may result in the incorrect assignment of polymorphism frequencies in population studies among related microsatellite alleles and, consequently, the generation of unreliable genetic linkage analyses. Thus, the true nature and degree of microsatellite polymorphism are determined only after a rigorous compilation of sufficient preliminary sequencing data.

Spanning the short arm of chromosome 6, located telomeric to the DR locus and juxtaposed between the class I and II major histocompatibility complex (MHC) genes, the class III genes consist of approximately 36 genes thus far identified within a 680-kb stretch of DNA (28). Many genes code for proteins involved in inflammation and immune regulation, including tumor necrosis factor alpha and lymphotoxin (TNF-α and TNF-β, respectively) (Fig. 1). These cytokines possess a broad range of immunoregulatory properties, and the localization of the TNF-α and TNF-β genes within the MHC close to the HLA-B locus has raised speculation that the TNF locus might participate in the association between HLA and various autoimmune diseases. A polymorphic *Nco*I restriction enzyme site, generating 5.5- or 10.5-kb restriction fragment lengths, is located in the first intron of the TNF-β gene, and the 5.5-kb *Nco*I fragment was linked with the HLA-A1/B8 haplotype (3, 12, 27). In addition, loss of an *Eco*RI site in the 3′ untranslated region of the TNF-β gene does not segregate with either *Nco*I haplotype, providing four different alleles defined by RFLP typing (27, 30). Heterozygosity for the *Nco*I RFLP has been linked to autoimmunity-associated diseases, including type I diabetes mellitus and Graves' disease, rheumatoid arthritis and Sjögren's syndrome, and systemic lupus erythematosus, but not primary biliary cirrhosis or multiple sclerosis (2, 5, 16–18).

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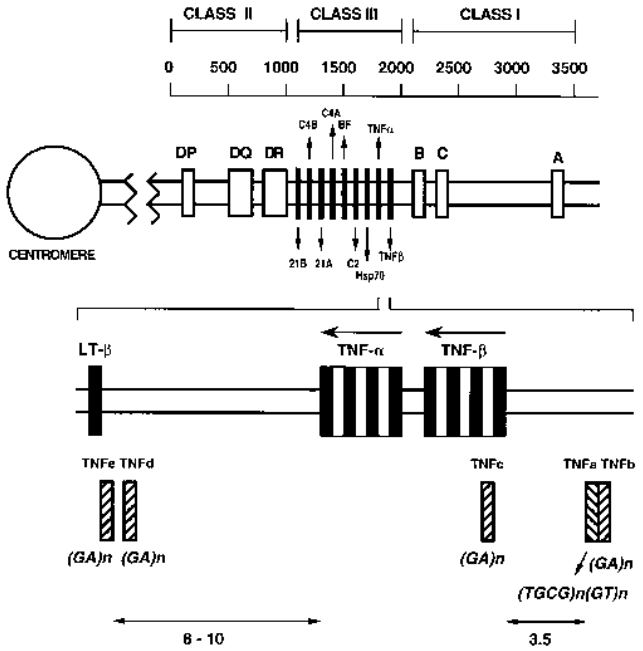

FIG. 1. Physical map of human MHC and locations of TNF microsatellites. Top to bottom, schematic relationship of class II, III, and I genes. Approximate distances are given in kilobases. 21B and 21A, 21-hydroxylase B and A, respectively; C4B, C4A, and C2, complement components; BF, properdin factor. LT-β, lymphotoxin-β; Hsp70, heat shock 70 proteins 2, 1, and HOM. The expanded scale reveals the relationship among the TNF microsatellites. Arrows above TNF-α and -β genes denote direction of transcription. Alternating black and white rectangles represent exons and introns comprising the TNF genes. Striped rectangles represent the five known microsatellites located relative to the TNF locus.

Finally, an adenine-for-guanine replacement at position −308 in the TNF-α promoter region gives rise to an additional *Nco*I biallelic polymorphism and was shown to be strongly associated with HLA-A1, B-*8, and DR3 alleles (43, 44).

In contrast, a cluster of five microsatellites associated with the TNF locus (Fig. 1) consists of multiple alleles and is considerably more polymorphic than that determined by *Nco*I/*Eco*RI RFLP (29, 42). A set of $(GT)_n$- and $(GA)_n$-containing microsatellites (TNFa and TNFb, respectively) are located approximately 3.5 kb telomeric to TNF-β (29). A second $(GA)_n$ microsatellite (TNFc) is located within the first intron of the TNF-β gene. A pair of linked $(GA)_n$ and $(GA)_n$-like microsatellites (TNFe and TNFd) are located approximately 8 to 10 kb centromeric to the TNF-α gene (29, 41). The genetic complexity of TNFa, -b, -c, -d, and -e has been reported to reflect a minimum number of 13, 7, 2, 6, and 3 alleles identified for each microsatellite, respectively (11, 29, 41). These polymorphic markers have the potential to define as many as 3,276 TNF haplotypes (not including the TNF-β *Nco*I RFLP) in random population studies and are a promising molecular genetic tool for studying possible associations between TNF-α and -β and disease (41).

In the course of our own studies, we recognized a difference involving the characterization of the previously published flanking sequence immediately centromeric to the TNFa $(GT)_n$ repeat (20, 29). The two sequences, TGCGTGCATGC GTGCG and TGCGTGCG, result in an 8-base disparity that fundamentally affects the calculation of TNFa allelic size when performing PCR-based microsatellite genetic analyses. Further, the pattern of the TNFa polymorphic repertoire and the frequencies of individual TNFa microsatellite alleles diverged significantly from genetic data previously published (23, 29). A variable 8-bp "deletion" occurring just upstream to the TNFa dinucleotide repeat was detected by Honchel et al. (19) in a population of patients with colorectal cancer and in normal individuals. This polymorphism may correspond to the 8-bp difference observed in earlier studies (20, 29) and suggests a greater degree of complexity surrounding the TNFa microsatellite than previously appreciated. To investigate further, we performed extensive subcloning and sequence analyses of the region surrounding the TNFa microsatellite from a subset of 38 healthy subjects (76 alleles sequenced). We now report the presence of an imperfect variable tetranucleotide repeat consisting of $(TGCG)_{1\text{-}5}$ juxtaposed upstream from the $(GT)_n$ repeat. This compound tetra-, dinucleotide microsatellite contributes a greater degree of genetic polymorphism than previously appreciated. The characterization of the PCR-generated compound TNFa (cpdTNFa) microsatellite and the allelic frequencies from a population of 52 individuals (104 alleles screened) is presented.

MATERIALS AND METHODS

Study population. Fifty-two unrelated, healthy, Caucasian volunteers of North American descent were randomly selected. The population comprised 26 males and 26 females. Allelic frequencies for the compound TNFa microsatellite were derived from the 52 normal controls (104 alleles studied). In a subset of 38 randomly chosen controls, the entire PCR-generated compound TNFa microsatellite was sequenced (76 alleles sequenced).

DNA and oligonucleotide preparation. Peripheral blood mononuclear cells were isolated from whole blood by density gradient centrifugation, and high-molecular-weight DNA was extracted from peripheral blood mononuclear cells by the sodium dodecyl sulfate-proteinase K method, purified by phenol-chloroform extraction, precipitated in absolute ethanol, and resuspended in 10:1 TE (10 mmol of Tris-HCl per liter and 1 mmol of EDTA per liter) buffer as previously described (25, 33).

Oligonucleotide primers and probes were synthesized by the phosphoramidite method on an automated 391 DNA synthesizer (Applied Biosystems, Foster City, Calif.), and 5′ trityl-retained oligonucleotides were purified by chromatography using Nensorb Prep cartridges (Dupont, Boston, Mass.) and then concentrated by lyophilization under vacuum. The primer sequence used in PCR to amplify the TNFa microsatellite was derived from the work of Nedospasov et al. (29) and defines the variable number tandem repeat framed by 55- and 15-bp conserved sequences. TNFa positive strand is 5′-GCCTCTAGATTTCATCCA GCCACA-3′; TNFa negative strand is 5′-CCTCTCTCCCCTGCAACACACA-3′.

Amplification and detection of the TNFa microsatellite region. All PCRs were carried out against 1 μg of DNA template in 100 μl adjusted to final concentrations of 10 μM KCl, 1.5 mM $MgCl_2$, 0.001% gelatin, 200 μM deoxynucleoside triphosphates, and 200 nM each primer to which 0.8 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.) was added. Temperature cycling was carried out in a DNA Thermal Cycler (Perkin-Elmer Cetus) as follows: an initial denaturation for 7 min at 94°C and then 1 min at 94°C and 3 min at 72°C for 35 cycles. Subsequent to gene amplification and prior to sample analysis, a 1-μl aliquot of the PCR product was added to 0.5 μl of ROX fluorophore-labeled oligonucleotide internal molecular weight standards (Applied Biosystems)–0.5 μl of loading buffer supplied by the manufacturer (ABI)–2 μl of formamide. For microsatellite polymorphic characterization of the cpdTNFa, the 4-μl mixture containing the PCR amplicon was loaded onto 24-well, high-resolution denaturant polyacrylamide gels, subjected to electrophoresis at a constant power of 30 W for 4 h, and laser scanned and analyzed by GenesScanner 373 (Applied Biosystems). Amplified fragments were identified by fluorescent label and sized according to polynucleotide length.

Cloning and sequencing TNFa microsatellite alleles. The amplified product was separated from minor contaminating bands and excess PCR primers by subjecting the amplicon to filtration centrifugation using ultrafree-MC filter units, 30,000-molecular-weight cutoff (Millipore, Bedford, Mass.). The purified, amplified TNFa microsatellite sequences were directly ligated into pCR II plasmid (Invitrogen, San Diego, Calif.). Subsequent to ligation and bacterial transformation into INV a F^1 cells (Invitrogen), the transformants were selectively expanded in kanamycin-containing agar and productive clones were selected by extinction of the β-galactosidase marker. TNFa insert-containing plasmids were extracted from bacterial transformants by the plasmid miniprep technique (33), and the TNFa segments were directly sequenced from the purified plasmid by the dideoxy chain termination method (34). Sequencing primers span either the T7 promoter or the SP6 promoter plasmid regions. The other reagents used for the DNA sequencing reactions were supplied with the USB Sequencing Kit (United

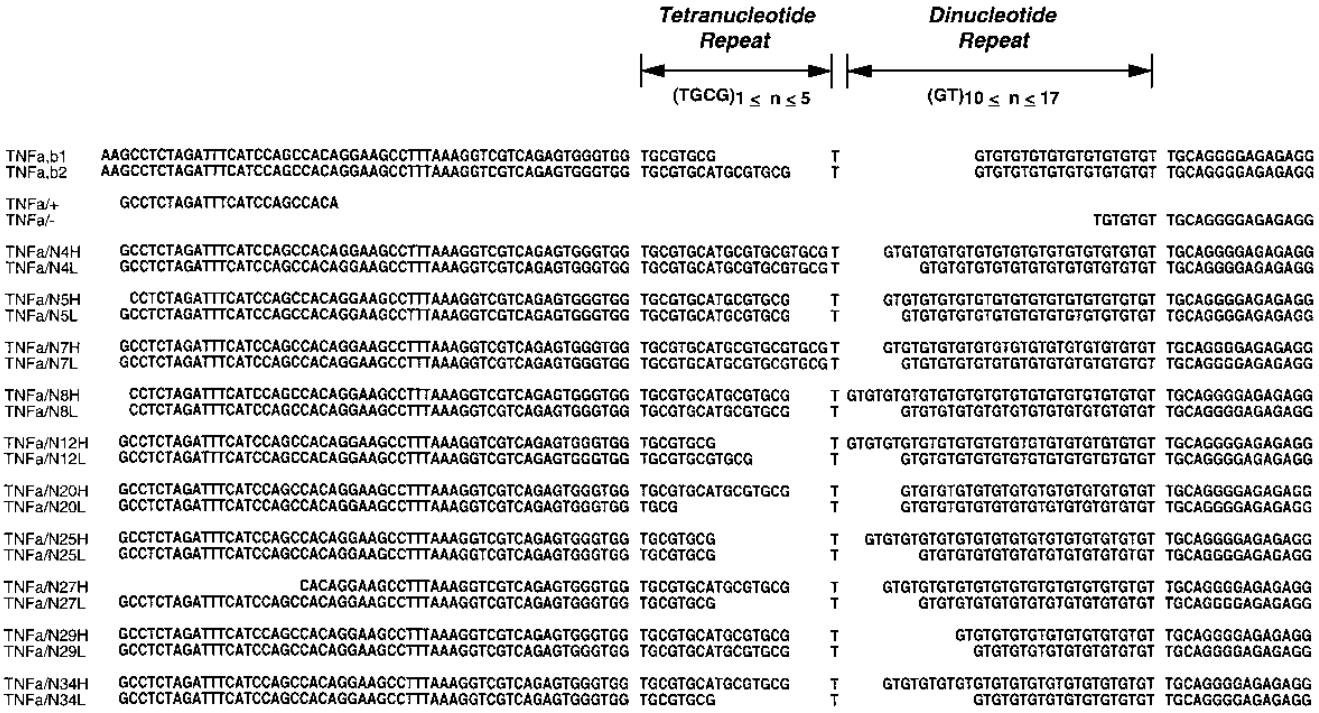

FIG. 2. Compound TNFa microsatellite sequence data. DNA sequences from a representative subset of 10 of 38 unrelated healthy individuals examined (TNFa/N4, 5, 7, 8, 12, 20, 25, 27, 29, and 34) have been aligned to emphasize the variable TGCG tetranucleotide and GT dinucleotide tandem repeats. All 10 representative individuals were heterozygous with regard to the compound TNFa microsatellite. H and L added after each TNFa microsatellite allele, e.g., TNFa/N4H and TNFa/N4L, reflect the higher- and lower-molecular-weight compound microsatellite, respectively. TNFa,b1 and TNFa,b2 display sequence data originally reported by Iris et al. (20) and Nedospasov et al. (29), respectively. TNFa/+ and TNFa/− are the positive and negative deoxyoligonucleotide primer sequences, respectively, used in PCR to frame the cpdTNFa microsatellite region. The TGCG tetranucleotide varied from 1 to 5 repeats and the GT dinucleotide varied from 10 to 17 repeats, producing a compound microsatellite that varied from 28 to 52 bp in length.

States Biochemical Corp., Cleveland, Ohio). At least three separate clones were fully sequenced to obtain a consensus genetic sequence for each TNFa microsatellite allele. Consensus sequences were assembled with a base designation threshold of ≥66%.

Statistical analysis. Sequence data were collected with a Gel Reader (IBI, Eastman Kodak Company, Rochester, N.Y.) light scanner interfaced with a IIci Macintosh computer by using MacVector software (Scientific Imaging Systems, Eastman Kodak Company, New Haven, Conn.). Distribution of alleles among healthy volunteers was analyzed by Hardy-Weinberg equilibrium. Fisher's exact test was used in all comparisons of TNFa alleles.

RESULTS

Preliminary characterization of the highly polymorphic TNFa microsatellite among a cohort of healthy volunteers produced a pattern and frequencies of microsatellite alleles that did not coincide with previously reported data. Previously, we used a detection system based on direct incorporation of [^{32}P]dATP during PCR which was comparable to the method used originally by others to characterize the TNFa microsatellite (data not shown and reference 29). In the studies reported here, a fluorophore-labeled primer was incorporated during PCR. Subsequently, the amplicon was subjected to denaturant acrylamide gel electrophoresis and microsatellite analysis was performed by a laser detection system. The differences were reproducible and independent of methodology. In seeking to understand these differences, the published human TNFa microsatellite sequence data was carefully reviewed. Nedospasov et al. reported a 16-mer, TGCGTGCATGCGTGCG, juxtaposed centromeric to the GT tandem variable repeat (29). The sequence submitted by Iris et al. (20) disclosed an octamer, TGCGTGCG, in the corresponding flanking position next to the GT repeat. Reductive analysis of this set of sequence data yields the octanucleotide TGCGTGC(A/G). We, therefore, speculated that the antecedent 16-mer of the work of Nedospasov et al. represented an imperfect TGCG tetranucleotide tandem repeat element and that the octamer of the work of Iris et al. represented a double TGCG tetranucleotide repeat. Finally, it was postulated that the TNFa microsatellite consisted of two adjacent variable tandem repeat elements, a tetranucleotide TGCG repeat and a dinucleotide GT repeat.

Sequence data was generated from 38 unrelated individuals (76 alleles sequenced). Of a total of 7,448 bases sequenced, there were 16 base mismatches among clones of individual alleles or 99.7% agreement. Figure 2 displays the sequence alignment data from a representative subset of 10 individuals. A TGCG element exists as a tetranucleotide tandem variable repeat and exhibits at least five polymorphisms, presenting as a single-, two-, three-, four-, or five-repeat element. The second G of the second TGCG repeat element was degenerate and was more frequently replaced with an A. Individuals were homozygous (n = 17 [61%]) or heterozygous (n = 11 [39%]) for the TGCG tetranucleotide microsatellite allele. Further, although the number of alleles screened was limited, there was no linkage disequilibrium between a specific TGCG tandem repeat and a specific GT tandem repeat and both microsatellites appeared to vary independently.

The arrangement of a TGCG tetranucleotide repeat as proposed results in a single T just downstream of the terminal tetranucleotide and just upstream from the GT dinucleotide repeat (Fig. 2). An alternative proposal is to group the tetranucleotide as a GTGC repeat element, permitting the terminal G of the TGCG element to be coupled with the lone T

TABLE 1. Characterization of DNA samples with regard to cpdTNFa microsatellite polymorphism

cpdTNFa microsatellite size (bp)	Allele occurrence	Frequency
24	0	0.000
26	0	0.000
28	1	0.010
30	19	0.183
32	3	0.029
34	1	0.010
36	7	0.067
38	23	0.221
40	9	0.087
42	0	0.000
44	5	0.048
46	18	0.173
48	15	0.144
50	0	0.000
52	3	0.029
54	0	0.000

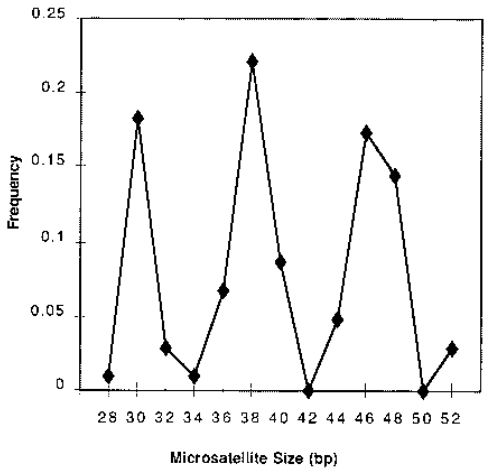

FIG. 3. Frequencies of the cpdTNFa microsatellites. The repertoire of the compound tetra-, dinucleotide TNFa microsatellite was characterized according to overall length in base pairs and plotted against frequency derived from a population of 52 unrelated, healthy individuals. Eleven microsatellite alleles were detected, cpdTNFa.28 to cpdTNFa.52, spanning 28 to 52 bp. Potential cpdTNFa.42 and cpdTNFa.50 were not found within the population screened. The pattern of frequencies was heterogeneous with peak frequencies at cpdTNFa.30, cpdTNFa.38, and cpdTNFa.46,48, producing an equally spaced triphasic graph.

to form an additional GT dinucleotide repeat. This strategy would produce an (A/G) degeneracy in the first purine of the third tetranucleotide sequence. Interestingly, except for this degeneracy, all G's are in phase with the adjacent GT repeat element. Conceptually, then, the entire G/TGCG region may be considered as an extended run of imperfect GT repeats. However, the region was found to vary by 4 base units only. This observation presents a more compelling argument that the genetic polymorphism within this region is a product of a tetranucleotide microsatellite allelic variation. We have arranged the tetranucleotide repeat as a TGCG element for historical reasons, because the original published sequences for TNFa differed by an 8-base TGCGTGCG insertion/deletion. In addition, arranging the tetranucleotide as a GTGC repeat element not infrequently creates a lone A that when paired with the adjacent downstream T does not result in a dinucleotide repeat. Therefore, in certain alleles an extra GT would be created, but in others an isolated AT would be created. To eliminate this inconsistency, the tetranucleotide repeat region was organized as a TGCG element.

DNA samples from 52 unrelated healthy volunteers were characterized with regard to the PCR-generated cpdTNFa microsatellite polymorphism (Table 1). For routine analyses of large populations, it is more practical to treat the cpdTNFa microsatellite as an aggregate representing the sum of the dinucleotide and the tetranucleotide repeats. Although the potential range is from 24 to 54 bp, the actual cpdTNFa detected ranged in size from 28 to 52 bp, and of 16 potential polymorphisms, 11 were identified within the population examined. Six individuals (11.5%) were homozygous for the cpdTNFa microsatellite allele, equivalent to the expected number of homozygotes by chance alone (a 1/11 chance of homozygosity among 66 possible diplotypes). Interestingly, five (83%) of the homozygous individuals were female. There was considerable heterogeneity among the frequencies of cpd TNFa microsatellites; however, the cpdTNFa microsatellite alleles appeared to segregate into three distinct size ranges with peak frequencies at 30, 38, and 46 bp at exactly 8-bp intervals (Fig. 3).

DISCUSSION

Susceptibility to more than 50 diseases has been associated with expression of specific MHC antigens (7). Putative susceptibility genes for several autoimmune disorders and for immunodeficiency were mapped to the MHC class III locus (1, 13, 14, 26, 36). Many proteins encoded by class III genes are involved in immunologic recognition and interactions among lymphocytes, antigen-presenting cells, and target antigens. Some code for proteins of the classical and alternative complement pathways, e.g., C4A, C4B, C2, and factor Bf. Genes that code for products involved in inflammation and immune regulation include the heat shock protein-70 gene complex (Hsp70), TNF-α and TNF-β, and perhaps, the recently recognized member of the TNF locus, lymphotoxin-β (8).

TNF-α promotes cytotoxicity and is found in high concentrations in regions of acute inflammatory reactions (9, 15). TNF-α activates leukocytes, promoting endothelial adhesion and inflammation (6, 40). Mice expressing a human TNF-α transgene modified in the 3′ negative regulatory region, causing high TNF-α constitutive production, spontaneously develop a chronic inflammatory disorder which is prevented by a monoclonal antibody to TNF-α (24). TNF-α can mediate oligodendrocyte damage and destruction of the myelin sheath in vitro, and immune-mediated demyelination in experimental autoimmune encephalomyelitis can be neutralized by treatment with anti-TNF antibody (38, 39). In the central nervous system, astrocytes have the capacity to secrete TNF-α, and it has been proposed that production of TNF-α by astrocytes may play a pivotal role in augmenting intracerebral immune responses and inflammatory demyelination (10). TNF-α and TNF-β were prominently detected associated with astrocytes and foamy macrophages and in endothelial cells in acute lesions of multiple sclerosis (37). Collectively, these observations suggest that TNF gene locus-encoded products may play a fundamental role in the immunopathogenesis of several autoimmune disorders.

The possibility that variable levels of cytokine induction and expression may be related to certain genetic polymorphisms has led to speculation that TNF-α and TNF-β genetic variations may be linked to disease activity. For example, in individuals with systemic lupus erythematosus, DR3 and DR4 an-

tigen expression is associated with high-level mononuclear cell TNF-α secretion in response to lipopolysaccharide stimulation (4, 21). Cytokine production was inhibited by anti-MHC class II antibodies (35). Moreover, patients expressing DR3 often coexpressed the A1 and B8 antigens, thus suggesting an extended MHC-disease association. Disease-specific extended MHC haplotypes and levels of TNF-α and TNF-β expression were also linked to insulin-dependent diabetes mellitus (31). It has been proposed that variable levels of production of TNF-α and TNF-β are related to TNF *Nco*I polymorphisms located in the promoter region 5′ of the TNF-α initiation site and in the first intron of TNF-β (21, 27, 31).

The wealth of informative microsatellites clustered around the TNF locus has raised expectations that these polymorphic genetic markers may be useful in searching for susceptibility genes in certain autoimmune disorders. Indeed, in using the published TNF microsatellite profiles, a genetic association between multiple sclerosis and TNFa, TNFb, and TNFc was not found (32). However, the data presented here suggests a need to expand the nature of the TNFa microsatellite so that its full complexity is appreciated. The normal population frequency statistics presented here diverge significantly from data which failed to account for the compound nature of TNFa (32). A reanalysis of possible linkage between autoimmune disease and the cpdTNFa is warranted in lieu of the more complete characterization of this microsatellite.

The full scope of the complexity of cpdTNFa in association with TNFb is not known, nor is it known whether one can distinguish variable patterns of TNF-α or TNF-β secretion based on these genetic polymorphisms. Because of the very close proximity of all three variable tandem repeats, it may be more informative to assess genetic linkage and cytokine production based on the combined contribution of all three tandem repeats. These compelling issues are the source of ongoing investigations.

It is reasonable to assume that microsatellites may exist in a more complex compound nature elsewhere throughout the human genome. Information concerning the genetic structural organization of the human genome is accumulating exponentially. Newly identified microsatellites, however, are rarely characterized with regard to adjacent potential polymorphic structures since much of the sequence data relies on single source material. It is not clear what degree of impact the presence of a compound microsatellite may have on genetic analysis. A careful, preliminary search for polymorphisms flanking the designated microsatellite appears justified.

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