Rapid communication

TNF- α gene polymorphisms in Type 1 (insulin-dependent) diabetes mellitus

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Summary. Type 1 (insulin-dependent) diabetes mellitus, like some other autoimmune diseases, is linked to certain alleles coded by genes in the HLA-D region. Sequence analysis of DQ β chains indicates that aspartic acid at codon 57 confers resistance to the development of Type 1 diabetes. However, a full explanation for the HLA-association of Type 1 diabetes, particularly the increased susceptibility of DR3/4 heterozygotes is still awaited.

The localisation of tumour necrosis factor genes on the short arm of chromosome 6 between HLA-B and the complement genes (Class III) prompted us to investigate a possible polymorphism of TNF- α at the genomic level in relation to Type 1 diabetes susceptibility. A diallelic TNF- α restriction fragment length polymorphism was found with NcoI and its segregation with HLA-haplotypes analysed in diabetic families. We describe here a strong linkage of TNF- α alleles with certain DR haplotypes. For example, the common extended haplotype HLA A1-B8-DR3 was almost exclusively

associated with the 5.5 kb TNF- α allele whereas Bw62-DR4 with the 10.5 kb allele. Thus both alleles segregate to diabetic patients.

DR matched haplotypes of affected family members differed significantly from those of the non-affected at the TNF alpha locus. All affected sibling pairs in 11 multiplex affected families were identical for TNF- α alleles, even if they were only haploidentical for HLA-B-DR haplotypes. In addition, heterozygosity for the TNF- α alleles was significantly more frequent in the patients.

This tight linkage of TNF- α alleles with some extended haplotypes could help to explain the HLA-association of Type 1 diabetes as well as some other autoimmune diseases.

Key words: Type 1 (insulin-dependent) diabetes, major histocompatibility complex, HLA-DR, extended haplotypes, tumour necrosis factor alpha, restriction fragment length polymorphism.

Type 1 (insulin-dependent) diabetes mellitus is generally considered to be a spontaneously occurring autoimmune disease linked to specific alleles of HLA-D region genes [1]. HLA-DR3 and/or 4 are the main disease associated specificities with DR3/4 heterozygotes conferring the highest risk. In addition, prospective family studies have demonstrated that HLA-identical and haplo-identical siblings of affected probands are at the highest risk of developing Type 1 diabetes compared with non-identical siblings [2].

Recent DNA studies of DR4 haplotypes have shown that the DR4-DQw8 (formerly DQw3.2 subtype) is more closely associated with Type 1 diabetes. This DQ β chain variation has also been studied at the nucleotide sequence level and it has been suggested that aspartic acid at the amino acid position 57 of the DQ β exon is critical for conferring resistance to the disease [3]. Serine, alanine and valine at position 57 of the DQ β

exon are found on haplotypes of predisposing DR-DQ alleles. Since this rule has exceptions in DR7-DQw2 of caucasoid Type 1 diabetes, in DR4-DQw4 and DR9-DQw9 of Japanese Type 1 diabetes haplotypes [3], the existence of other susceptibility genes in the major histocompatibility complex (MHC) is a distinct possibility.

This genetic region also harbours the genes for tumour necrosis factor (TNF- α and - β), which have been mapped between the HLA-B and complement genes [4]. The detection of TNF- α RFLP was shown to be linked to low levels of TNF production in murine autoimmune lupus nephritis [5], but no full description of TNF-RFLP has so far been described in man.

We studied TNF gene variation at the DNA level using a novel TNF restriction fragment length polymorphism (RFLP). One hundred and sixty-two haplotypes segregating in 43 informative families were studied with HLA-serology and TNF RFLP to see if genetic

Fig. 1. Autoradiograph of NcoI digested DNA hybridised with a TNF- α probe: two allelic fragment of 10.5 and 5.5 kb segregate in the family with the HLA-haplotypes (a, b, c and d): Lane 1 Father ab (a = B18-DR3-TNF 10.5; b = B44-DR4-TNF 5.5), lane 2 Mother cd (c = B8-DR7-TNF 5.5; d = B44-DR4-TNF 5.5), lane 3 sibling 2 bc, lane 4 sibling 3 ac. The diabetic proband is sibling 1 ac, blot not shown, identical to sibling 3. The homozygosity for the 5.5 kb fragments in the mother and sibling 2 is illustrated by the double intensity of the bands

variation of the TNF- α locus was associated with susceptibility to Type 1 diabetes as well as, or in conjunction with, HLA-B-DR variation.

Materials and methods

Families, patients and controls

Forty-six families of the Bart's-Windsor-Family Study [2] were studied for HLA-serology and TNF- α RFLP. The families recruited were those of probands in whom Type 1 diabetes had developed under the age of 21 and who had at least one unaffected sibling under that age. Additional probands without information on other haplotypes in the family were tested as well as 48 random healthy control subjects.

HLA-serology

HLA-serology was performed according to 9th International Histocompatibility Workshop definitions for the HLA A, B, C, DR, DRw52/53 and DQ antigens. In the genotyped families, haplotypes were divided into 2 groups: "affected" haplotypes found in the probands and members of the families with Type 1 diabetes and "non-affected" haplotypes detected in healthy relatives only.

RFLP studies

RFLP studies were performed according to standard procedures of the 10th International Histocompatibility Workshop Southern Blotting Protocol with some modifications. Briefly, DNA was prepared from peripheral blood, 10 μ g were digested with 30–40 units of 40 different restriction endonucleases, including NcoI, at 37°C (Bethesda Research Laboratories Bethesda, Md, USA), and electrophoresed on a 0.8% agarose gel in TAE-buffer (40 mmol/l Tris-acetate, 1 mmol/l EDTA) for 24 h at 45 V. Molecular weight markers were run in parallel on each gel, Hind III digested bacteriophage lambda DNA and Hae III cut phiX DNA. DNA was transferred onto "Biotrace membranes" using the alkaline method. Filters were prehybridised at 42°C and hybridised with a hexamer labelled genomic DNA-probe in the following mixture: 50% deionised formamide, 0.1% Denhardt's solution, 5 $\times$ SSPE, 1% SDS, 0.5% dextran sulphate and 100 μ g/ml salmon sperm DNA. The filters were washed down to 0.2 $\times$ SSPE, 0.1% SDS and exposed for 3–6 days with intensifying screens on Kodak-XAR-5 film at –80°C. The TNF- α probe used was a 2.9 kb EcoRI insert that had been subcloned in pAT153. This probe contains several introns and 4 exons of the human TNF- α gene [6].

Statistical analysis

Gene frequencies of the polymorphic fragments were determined by direct gene counting. RFLP data were correlated with HLA-serology. 2 $\times$ 2 tables were analysed by chi-square or Fisher's exact test where appropriate. Haplotype sharing analysis among sibling pairs affected by Type 1 diabetes was performed in eleven families [2].

Results

Forty different restriction enzymes were used on pooled samples of DNA from normal subjects and Type 1 diabetic patients. After probing the blots with the TNF- α probe, only one enzyme, NcoI, detected a polymorphism, namely two allelic bands of 5.5 and 10.5 kb (Fig. 1). These alleles were shown to segregate in families along with the HLA parental haplotypes. In total, 162 haplotypes were studied in 43 informative families. In 38 families all the four parental haplotypes could be assigned to TNF- α alleles, whereas in five families the informative segregation could only be established with 2 haplotypes, studying at least two members of the family. Three additional families were not informative for TNF- α segregation. As described below, and as shown on Table 1, analysis of these data revealed associations of TNF-alleles with certain DR specificities, as well as differences between affected and non-affected haplotypes bearing the same DR specificities.

TNF and HLA specificity

There are many examples of combinations of HLA Class I and Class II alleles which are frequently found together on a so called extended haplotype often including specific Class III markers. These extended haplotypes were associated with TNF alleles (Table 1). Thus, 25 out of 26 (96%) of B8+/DR3+ haplotypes had the 5.5 kb TNF- α band versus only 4/13 (31%) of the non-B8/DR3+ haplotypes ($p=0.000003$). Similarly, 8 of the 10 B44-DR4 haplotypes were associated with the 5.5 kb band, whereas 10 of the 12 Bw62-DR4 possessed the 10.5 kb band. These results show linkage disequilibrium between human TNF- α allelic variation and HLA B-DR extended haplotypes implying that if the genomic variation was functional, it would be reflected in HLA serotyping.

TNF RFLP and diabetes

When haplotypes from diabetic probands were compared with haplotypes from non-affected individuals, overall there was little difference in frequency of TNF alleles: 44.6% had the 5.5 kb allele in contrast to 34.3% in the non-affected (Table 1). However, when patient's haplotypes and non-affected haplotypes were matched for DR there were some striking differences. Both DR2

Type 1 diabetic haplotypes had the 5.5 kb band, whereas this was present in only one out of 15 of the DR2 healthy individuals ($p=0.02$). This difference cannot be explained by DR2 subtype variation since all DR2 haplotypes were DQ1.2 and none were DQ1.AZH using DQB RFLP or oligonucleotide hybridisation analysis (data not shown). Thirty of the 39 DR4 affected haplotypes had the 10.5 kb band whereas this was present in 9 of 18 non-affected DR4s ($p=0.04$). These results suggest that TNF alleles are differentially distributed in the Type 1 diabetic patients, somewhat independently of their expected HLA-DR associations referred to above. In order to clarify this issue, haplotype sharing analysis among sibling pairs affected by Type 1 diabetes was done.

Sibling pair analysis

This was performed in eleven multiplex affected families. Interestingly, all 11 pairs of affected siblings were identical for TNF alleles (in eight families, at least one parent was heterozygous for TNF alleles). This was different for HLA markers since only 3 sibling pairs were identical, 4 haplo-identical and 1 was non-identical for HLA haplotypes.

Heterozygosity

One additional aspect of the data deserves comment. As shown on Table 2 when Type 1 diabetic patients were compared with random control subjects there was a marked difference in the heterozygosity of TNF alleles in the two groups. Compared with the control sample, the Type 1 diabetic patient group contained a significantly higher number of heterozygotes and less homozygotes of the 10.5 kb band.

Discussion

Several studies have shown that extended haplotypes comprising HLA-A, B, C, DR, DQ and complement alleles are involved in susceptibility to Type 1 diabetes [8]. A recent report finds C4 allotypes independently associated with Type 1 diabetes from DQ alleles and suggests a susceptibility gene between HLA-B and C4, pointing to the TNF-region [7].

We give here, for the first time, full description of variation at the human TNF locus. TNF- α RFLPs show clearly 2 alleles in control subjects and patients. Both alleles segregate in families along with the HLA haplotypes. The TNF alleles that we have identified are associated with some DR specificities and there are strong indications from the data that TNF alleles are non-randomly associated with Type 1 diabetes. Although TNF alleles are closely linked to some extended haplotypes,

Table 1. TNF alleles in 162 haplotypes of 43 informative diabetic families: comparison of affected and non-affected haplotypes matched for DR-specificities

DR	Affected		Non-affected		Total	
	10.5 kb	5.5 kb	10.5 kb	5.5 kb	10.5 kb	5.5 kb
DR1	4	3	8	1	12	4
DR2	0	2	14	1	14	3
DR3+ /B8+	0	19	1	6	1	25
DR3+ /B8-	9	3	0	1	9	4
DR4	30	9	9	9	39	18
DR5	0	2	4	3	4	5
DRw6	2	0	3	1	5	1
DR7	5	2	5	1	10	3
DRw8	0	0	2	1	2	1
DR9	1	1	-	-	1	1
ALL	51	41	46	24	97	65
DR4 {	DR4+ /					
	B44+	2	6	0	2	8
	DR4+ /					
Bw62+	10	1	0	1	10	2

p -values as determined by Fisher's exact test: DR1: $p=0.1923$, DR2: $p=0.0206$, DR3B8+: $p=0.2692$, DR3B8-: $p=0.3077$, DR3B8+ /DR3B8-: $p=0.00003$, DR4: $p=0.04367$, DR5: $p=0.2777$, DRw6: $p=0.67$, DR7: $p=0.5629$, All: $p=0.12277$. DR4-B44/DR4-Bw62: $p=0.0048$ affected DR4-B44/affected DR4-Bw62: $p=0.006$.

"Affected" = haplotypes found in Type 1 (insulin-dependent) diabetic patients in the families, "non-affected" = haplotypes found in healthy family members

Table 2. TNF RFLP in 79 Type I (insulin-dependent) diabetic patients

	10.5 kb	10.5/5.5 kb	5.5 kb
Type 1 diabetic patients:	19 (24%)	48 (61%)	12 (15%)
Control subjects:	21 (44%)	19 (40%)	8 (16.7%)

$\chi^2 = 6.26$, $p = 0.044$

like B8-DR3, B44-DR4 and Bw62-DR4, haplotypes matched for DR specificities differ between those of probands and of normal family members. The sibling pair analysis shows that the effect of TNF is not simply due to linkage disequilibrium with DR or DQ markers characteristic of Type 1 diabetes since all the affected sibling pairs in the multiplex families shared both alleles with the proband even if only haplo-identical or non-identical for HLA. Furthermore, Type 1 diabetic patients differ significantly from random control subjects in the heterozygosity of TNF alleles and the homozygosity of the 10.5 kb band regardless of HLA typing. This could also explain the highest risk in DR3/DR4 heterozygotes.

Our data are consistent with the notion that susceptibility to diabetes is due to a combination of factors within the MHC, one of which is DQ β [3]. Another could be TNF- α , or a gene closely linked to it. TNF alleles described by RFLP analysis may reflect DNA sequence variation in the introns or TNF flanking regions, although some of these variations may not necessarily

imply differences in functionally relevant regions. The polymorphisms described should be linked to any such differences and may reflect distinct regulatory functions. Since TNF production by stimulated monocytes differs between individuals [8], this difference may be controlled by such genetic variation. We have no information at present on the nature of any allelic variation in TNF at the sequence level. In the light of our data it would seem worth obtaining this information to firmly establish the role of TNF polymorphism in disease susceptibility.

There are several reasons for suspecting involvement of TNF in the development of Type 1 diabetes. TNF- α or β in conjunction with interferon (IFN)- γ elicits Class II molecule expression on human pancreatic islets that are normally MHC Class II negative and cannot be stimulated to express Class II by IFN- γ alone [9]. Since TNFs and IFN- γ can protect cells from viral infection, it was suggested that one possibility is that in response to a viral infection of the B cells, local release of IFN- γ and TNF could be one of the mechanisms for Class II expression of the B cell in the diabetic pancreas [9].

However, it has to be emphasised that the cytokine combination *in vitro* also elicits Class II expression on glucagon and somatostatin cells, which are unaffected in the insulinitis process in human diabetes. One is tempted to speculate that, like in the experimental systemic lupus erythematosus model [5], low concentration of TNF- α in response to local viral infections could also have a pathological role in predisposed individuals. It remains to be established whether TNF- α polymorphism especially at the heterozygous state could reflect a differential functional release of the cytokine or a form of a molecule with a less active biological activity [10]. TNF alleles in combination with DR-DQ alleles could therefore confer susceptibility at the functional level.

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References

1. Bosi E, Todd I, Pujol-Borrell R, Bottazzo GF (1987) Mechanisms of autoimmunity: relevance to the pathogenesis of Type 1 insulin-dependent diabetes mellitus. *Diabetes Metab Rev* 3: 893-924
2. Tarn A, Thomas JM, Dean BM, Ingram D, Schwarz G, Bottazzo GF, Gale EAM (1988) Predicting insulin-dependent diabetes. *Lancet* i: 845-850
3. Todd JA, Acha-Orbea H, Bell JI, et al. (1988) A molecular basis for MHC Class II associated autoimmunity. *Science* 240: 1003-1009
4. Inoko H, Trowsdale J (1987) Linkage of TNF genes to the HLA-B locus. *Nucleic Acids Res* 15: 8957-8962
5. Jacob CO, McDevitt HO (1988) Tumour necrosis factor- α in murine autoimmune "lupus" nephritis. *Nature* 331: 356-358
6. Shirai T, Yamguchi H, Ito H, Todd CW, Wallace RB (1985) Cloning and expression in *Escherichia coli* of the gene for human tumour necrosis factor. *Nature* 313: 803-806
7. Thomsen M, Molvig J, Zerbib A, et al. (1988) The susceptibility to insulin-dependent diabetes mellitus is associated with C4 allotypes independently of the association with HLA-DQ alleles in HLA-DR3,4 heterozygotes. *Immunogenetics* 28: 320-327
8. Molvig J, Baek L, Christensen P, et al. (1988) Endotoxin stimulated human monocyte secretion of interleukin-1, tumour necrosis factor α and prostaglandin E2 show stable interindividual differences. *Scand J Immunol* 27: 705-716
9. Pujol-Borrell R, Todd I, Doshi I, Bottazzo GF, Sutton R, Gray D, Adolf GR, Feldmann M (1987) HLA Class II induction in human islets by interferon- γ plus tumour necrosis factor or lymphotoxin. *Nature* 326: 304-306
10. Kriegler M, Perez C, DeFay K, Albert I, Lu SD (1988) A novel form of TNF/cachectin is a cell surface cytotoxic transmembrane protein: Ramifications for the complex physiology of TNF. *Cell* 53: 45-53

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