*Rapid communications***HLA-DQB 1 codon 57 and genetic susceptibility to Type 1 (insulin-dependent) diabetes mellitus in French children**I. Vallet-Colom¹, C. Lévy-Marchal², D. Zarrouk¹, J. Tichet³, R. Krishnamoorthy¹, P. Czernichow² and J. Elion¹¹ Laboratoire de Biochimie Génétique, INSERM U 120 and ² Service d'Endocrinologie Pédiatrique, Hôpital Robert Debré, Paris, and³ Institut Régional pour la Santé, Tours, France

Summary. Variations in the incidence rate of Type 1 (insulin-dependent) diabetes mellitus might relate to ethnic-specific genetic backgrounds. HLA-DQB 1 alleles were typed in 75 French diabetic children and 85 matched control children. Enzymatically-amplified DQB 1 exon 2 was hybridized with oligoprobes specific for the six most common alleles. Alleles coding for an Asp residue at position 57 in the DQ β chain are strongly negatively associated with Type 1 diabetes in the French population. Nevertheless, one of the diabetic children was an Asp 57 homozygote (DQB 1.2/3.1). Among alleles coding for a residue other than Asp at position 57, alleles 3.2 and 2 (Ala 57) are positively associated with

diabetes but not allele 1.1 (Val 57) which is less frequent in diabetic children than in control children. Heterozygosity for 2/3.2 alleles is the genotype most strongly associated with diabetes (Odds ratio = 52.9). Large comparative population studies will be necessary to determine whether the frequency of DQB 1 alleles positively associated with Type 1 diabetes (2 and 3.2) in a given ethnicity is related to its incidence rate in the same population.

Key words: Type 1 (insulin-dependent) diabetes, HLA-DQB 1 gene, genetic susceptibility, incidence rate, France.

The incidence rate of Type 1 (insulin-dependent) diabetes in Europe varies from North to South. France has one of the lowest values [1], i.e. about six times lower than that reported in Finland [2]. HLA-DR 3 and DR 4 antigens have been identified as susceptibility markers for the disease in caucasian populations. DR and DQ loci are in strong linkage disequilibrium and several studies have suggested that only certain subsets of DR 3 and DR 4 are increased in diabetic patients, namely those linked to DQB 2 and 3.2 alleles [3]. In North-American caucasians, Todd et al. [4] found that most haplotypes positively associated with Type 1 diabetes were related to genes encoding an amino acid other than aspartic acid at position 57 in the DQ β chain (Asp 57). They postulated that these non-Asp alleles (NA) of the DQB 1 gene were high risk markers for Type 1 diabetes as more than 90% of the patients were NA homozygotes [5]. Frequency of Asp (A) and NA alleles in various populations and its eventual relationship to the corresponding incidence of the disease are still poorly documented. In this study, we determined this frequency in a population of French diabetic children in comparison to a matched group of control children.

Subjects and methods*Subjects*

We studied 75 diabetic children randomly selected at the Diabetic Clinic of the Hôpital Robert Debré (Paris, France). Mean age was 12.2 ± 4.6 years and mean duration of the disease 2.9 ± 3.4 years. Nine of the children were studied within the first year of the disease. Diagnosis was ascertained by the occurrence of a ketotic or ketoacidotic episode. All patients were insulin-treated and of European caucasian extraction. The control group was made up of 85 school children recruited through systematic examinations at the Institut Régional pour la Santé (Tours, France). Ages ranged 7–15 years, none of the control group had symptoms of diabetes.

Methods

Genomic DNA was prepared from peripheral blood leucocytes by the guanidium chloride procedure [6]. DNA was digested with Bst EII (Boehringer, Mannheim, FRG) to specifically destroy the interfering DQB 2 sequence [7]. Exon 2 of the HLA-DQB 1 gene was amplified by the polymerase chain reaction (PCR) [8]. The reaction mixture (100 μ l, final volume) contained 1 μ g of template DNA, 50 pmol of each primer, 200 μ mol/l of each dNTP (Pharmacia, Up-

Fig. 1. Distribution of HLA-DQB 1 allele frequencies. Frequencies are expressed in percentage in French diabetic children ■ ($n = 150$ chromosomes) and in control children □ ($n = 170$). Alleles are divided in three subsets. The two first are according to the presence (A) or the absence (NA) of an aspartic acid residue at position 57 in the corresponding DQ β polypeptide chain. The third subset corresponds to a small number of unidentified alleles (NI)

psala, Sweden), 4 mmol/l $MgCl_2$, 10 mmol/l Tris, 50 mmol/l KCl, 10% dimethylsulfoxide and 2.5 U of Taq polymerase (Cetus Corporation, Emeryville, Calif., USA). It was subjected to 30 repeated cycles of 30 sec at 95 °C, 40 sec at 56 °C and 30 sec at 72 °C in a DNA Thermal Cycler (Perkin Elmer-Cetus, Norwalk, Conn., USA). Six duplicate Zeta-Probe membranes (Bio-Rad, Richmond, Calif., USA) were slot-blotted with 4 μ l of the PCR-product. The 5 allele-specific probes (ASO) described by Todd et al. [4] were used to identify DQB 1 alleles 1.1, 2, 3.1, 3.2 and 3.3. Allele 1.2 was detected with the ASO probe 5'-GGCGGCCTGATGCCGAG-3' (amino-acids 54–59). Probes were ^{32}P -labelled by the T4-polynucleotide kinase procedure (Amersham, Amersham, UK). Hybridization was carried out as in [4]. The final wash for probe 1.2 was at 60 °C. Samples reacting with a single probe were tested for homozygosity by digesting the PCR-products with the restriction enzymes Hae III and Sma I. Primers and probes were prepared on a model 381A DNA-Synthesizer (Applied Biosystems, Foster City, Calif., USA) by the β cyanoethyl-phosphoramidite method and purified by electrophoresis on a 20% acrylamide gel.

Statistical analysis

Chi-square test was used to compare allele frequencies, and odds ratios calculated to express the relative risk conferred by the different markers in the two populations.

Results

HLA-DQB 1 allele frequencies

Figure 1 shows a histogram of the HLA-DQB 1 allele frequencies determined on 150 chromosomes in the diabetic group and 170 control chromosomes. Clearly, whereas A and NA allele frequencies are about equal in the control

children, NA alleles are enriched in diabetic children as they are present on 92% of the chromosomes ($\chi^2 = 66.6$, $p < 0.001$). This increased frequency is not equally due to all the NA alleles; only alleles 3.2 and 2, both corresponding to an Ala residue at position 57 in the DQ β chain, are positively associated with Type 1 diabetes ($\chi^2 = 74.5$ and 39.3 , $p < 0.001$; Odds ratios (OR) = 11.0 and 2.5, respectively). On the contrary, allele 1.1 (Val 57) is less frequent in diabetic children than in control children ($\chi^2 = 23$, $p < 0.001$; OR = 0.5). The presence of A alleles is negatively associated with diabetes ($\chi^2 = 29.3$, $p < 0.001$; OR = 0.1). Interestingly, unidentified alleles are more common in the control group (8.0% vs 1.3%).

Distribution of HLA-DQB 1 allelic combinations

Distribution of the various DQB 1 allelic combinations in diabetic and control groups is reported in Table 1. Although A/A homozygosity is negatively associated with Type 1 diabetes ($\chi^2 = 18.5$, $p < 0.001$, OR = 0.04), one individual in the diabetic group was bearing such an allelic combination, namely 1.2/3.1. NA/NA genotypes, as a whole, are significantly associated with the disease ($\chi^2 = 47.8$, $p < 0.001$; OR = 13.2). Homozygosity for the 3.2 (NA) allele was not observed in the control children, but six times in the diabetic children. Among the NA/NA combinations, 2/3.2 heterozygosity is the genotype most strongly associated with diabetes (OR = 52.9). However,

Table 1. Distribution of individual HLA-DQB 1 allelic combinations. NA and A alleles are those coding respectively for a non-Asp or an Asp residue at position 57 in the corresponding DQ β chain. NI are individuals for which at least one allele could not be identified with the ASO probes used in this study

Allelic combinations	Diabetic group ($n = 75$)		Control group ($n = 85$)		Odds ratio
	<i>n</i>	Frequency	<i>n</i>	Frequency	
NA/NA	65	0.86	28	0.33	13.2
DQB 1.1/1.1	2	0.02	9	0.11	
DQB 1.1/2	7	0.09	8	0.09	
DQB 1.1/3.2	8	0.11	0		
DQB 2/2	13	0.17	10	0.12	
DQB 2/3.2	29	0.39	1	0.01	52.9
DQB 3.2/3.2	6	0.08	0		
NA/A	8	0.11	29	0.34	0.23
DQB 1.1/1.2	1	0.01	6	0.07	
DQB 1.1/3.1	0		4	0.05	
DQB 2/1.2	2	0.03	9	0.10	
DQB 2/3.1	2	0.03	2	0.02	
DQB 2/3.3	0		2	0.02	
DQB 3.2/1.2	2	0.03	3	0.03	
DQB 3.2/3.1	1	0.01	3	0.03	
A/A	1	0.01	21	0.24	0.04
DQB 1.2/1.2	0		7	0.08	
DQB 1.2/3.1	1	0.01	6	0.07	
DQB 3.1/3.1	0		7	0.08	
DQB 3.3/3.3	0		1	0.01	
NI	1	0.01	7	0.08	0.15

as expected from the observed allele frequencies, the NA/NA 1.1/1.1 genotype is much less frequent in the diabetic group (OR = 0.23). Finally, A/NA heterozygosity is negatively associated with Type 1 diabetes ($\chi^2 = 12.3$, $p < 0.001$; OR = 0.23).

Discussion

We have typed the alleles of the HLA-DQB 1 locus in a population of French diabetic children ($n = 75$) and in a matched control group ($n = 85$). Our results are in general agreement with the observation of Todd et al. [4] that alleles coding for an Asp residue at position 57 in the DQ β chain (A alleles) are strongly negatively associated with Type 1 diabetes in caucasian populations. Nevertheless, one of our diabetic children was an A/A homozygote (DQB 1.2/3.1). Indeed, the proposed protective effect of Asp 57 is not absolute as diabetic patients from Japanese and Chinese origins have now been found to be A/A homozygotes [9, 10]. More importantly, we find that among NA alleles, only those coding for Ala at position 57 (alleles 2 and 3.2) are positively associated with diabetes, whereas allele 1.1 (Val 57) appears to be protective. As Val and Ala are both neutral aminoacids, the hypothesis of a direct implication of residue 57 in the pathogenesis of Type 1 diabetes should be regarded with caution. Some allelic combinations involve two alleles which individually exhibit an opposite association with the disease. Among these, A/NA heterozygosity is associated with a protective effect (OR = 0.23), but not the combination of allele 1.1 (Val 57) with an other NA allele.

With the ASO probes used in this study, only the six most frequent DQB 1 alleles can be identified. Interestingly, unidentified alleles are much more common in control children than in diabetic children suggesting that allelic diversity may be restricted in this latter group.

Whether frequency of the DQB 1 alleles positively associated with Type 1 diabetes (2 and 3.2) in a given ethnicity is related to the incidence rate of the disease in this population remains to be clarified. From our data and the only three surveys published to date [5, 9, 10], this seems not to be the case: frequencies of NA/NA homozygotes in Japanese, Chinese, North-American and French normal subjects do not correlate to the known incidence figures in these countries. Large population-based studies are thus necessary to answer this question related to the genetic background of Type 1 diabetes.

Acknowledgements. This work was supported in part by two "Contrats de Recherche Clinique" from the Assistance Publique-Hôpitaux de Paris to P.C. and J.E.

References

1. Levy-Marchal C, Papoz L, de Beaufort C, Doutreix J, Froment V, Voirin J, Czernichow P (1989) Incidence rate of Type 1 juvenile insulin-dependent diabetes in France in 1988. *Diabetologia* 32: 510A (Abstract)
2. Diabetes Epidemiology Research International Group (1988) Geographic pattern of childhood insulin-dependent diabetes mellitus. *Diabetes* 37: 1113–1119
3. Sheehy MJ, Scharf SJ, Rowe JR, Neme de Gimenez MH, Meske LH, Erlich HA, Nepom BS (1989) A diabetes susceptible HLA haplotype is best defined by a combination of HLA-DR and DQ alleles. *J Clin Invest* 83: 830–835
4. Todd JA, Bell JI, Mac Devitt HO (1987) HLA-DQ β gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329: 599–604
5. Morel PA, Dorman JJ, Todd JA, Mac Devitt HO, Trucco M (1988) Aspartic acid at position 57 of the HLA-DQ β chain protects against Type 1 diabetes: a family study. *Proc Natl Acad Sci USA* 85: 8111–8115
6. Jeanpierre M (1987) A rapid method for the purification of DNA from blood. *Nucleic Acid Res* 15: 9611
7. Gu XF, Elion J, Ouagued M, Clauser E, Assan R, Krishnamoorthy R (1988) A simple strategy to specifically amplify the HLA-DQ β gene region with genomic DNA as template. *FEBS Lett* 236: 23–26
8. Mullis K, Faloona F, Scharf S, Saiki G, Horn G, Erlich H (1986) Specific enzymatic amplification of DNA in vitro: the polymerase chain reaction. *Cold Spring Harbor Sym. Quant Biol* 51: 263–273
9. Yamagata K, Nakajima H, Hanafusa T, Noguchi T, Miyazaki A, Miyagawa J, Sada M, Amemiya H, Tanaka T, Kono N, Tarui S (1989) Aspartic acid at position 57 of DQ β chain does not protect against Type 1 (insulin-dependent) diabetes mellitus in Japanese patients. *Diabetologia* 32: 762–764
10. Bao MZ, Wang JX, Dorman JS, Trucco M (1989) HLA-DQ β non-Asp 57 allele and incidence of diabetes in China and the USA. *Lancet* II: 497 (Letter)

Received: 13 November 1989

Dr. J. Elion
Laboratoire de Biochimie Génétique
Hôpital Robert Debré
48 Boulevard Sérurier
F-75935 Paris Cedex 19
France