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Improved Risk Assessment for Insulin-Dependent Diabetes mellitus by Analysis of Amino Acids in HLA-DQ and DRB1 Loci

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Abstract
Polymorphisms in HLA class II genes have been shown to contribute to susceptibility or protection against insulin-dependent diabetes mellitus (IDDM). In the present study the role of HLA class II haplotypes and the role of $DQ\alpha^{Arg52}$, $DQ\beta^{Asp57}$ and of polymorphic amino acids, located in the antigen-binding groove and the CD4-binding domain of the DRβ1 chain, were studied in 210 unrelated Caucasian IDDM patients and 205 controls. The results showed that the genotype homozygous for $DR\beta1^{Lys71+}$, which is in linkage disequilibrium with $DQ\alpha1^{Arg52+}$ provided a major risk (relative risk, RR = 15.46) for IDDM and that combination of $DR\beta1^{Lys71+/+}$ with homozygosity for $DQ\beta1^{Asp57-/-}$ of the DQβ1 chain significantly increased the RR for developing IDDM (RR = 20.41). The $DQ\alpha1^{Arg52-}$-$DQ\beta1^{Asp57+}$ haplotype in cis or trans position conferred the highest protection against IDDM (RR = 0.08). Our findings confirm that protection against IDDM is provided by HLA-DQ loci but that susceptibility for IDDM is provided by both HLA-DRB1 and DQB1 loci. Our results also provide a new and more specific approach to determine the risk of any random Caucasian individual to develop IDDM. Indeed, increased susceptibility or protection against IDDM can be determined by the rapid and simple typing of $DR\beta1^{Lys71}$, $DQ\alpha1^{Arg52}$ and $DQ\beta1^{Asp57}$ in a random person.

Introduction

HLA class II alleles have been repeatedly found to play an important role in the development of insulin-dependent diabetes mellitus (IDDM). Alleles at the DR locus and at the DQ locus have been shown to contribute to susceptibility or protection against IDDM [1]. In particular, the DR4 and DR3 alleles associate positively, while DR2 alleles provide

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protection [2]. Previously we were able to assign the highest susceptibility to the DRB3*0200 encoded DR52b serologic specificity [3] and to DRB1*0401-DQB1*0302 encoding the DR4-DQ8 serologic specificity. For DQA1 the alleles encoding an arginine at position 52 ($DQ\alpha^{Arg52+}$) and for DQB1 the alleles encoding an aspartic acid at position 57 ($DQ\beta^{Asp57+}$) were found to show strong positive and negative association with IDDM susceptibility, respectively [1, 3–10]. Our previous study [3] also identified the high susceptibility conferred by the genotypes coding for $DQ\alpha^{Arg52+}/DQ\beta^{Asp57-}$ heterodimers.

More recently, Thorsby and Rønningen [11] reviewed the role of DQ haplotypes in cis or trans, in IDDM and in a study of haplotypes in IDDM families. Kockum et al. [12] concluded that in a high-risk population, HLA-DQ alleles provided protection, while DR alleles conferred susceptibility for IDDM. To study the role of haplotypes over the different HLA class II loci, we reexamined the typing results for 210 unrelated Caucasian IDDM patients and 205 controls for the DQ and DR alleles of our previous studies [3]. Recently the three-dimensional structure of the HLA-DR1αβ heterodimer has been determined by X-ray crystallography [13]. Based on the three-dimensional structure of the DR1αβ heterodimer, some residues have been shown to be located at the side of the antigen-binding groove of the DRβ chain [13]. We therefore examined specifically the role of $DQ\alpha^{Arg52}$, $DQ\beta^{Asp57}$ and of polymorphic amino acids localized in the antigen-binding and CD4-binding domain of DRβ1 alone and in combination with each other in individuals with IDDM and controls. The results show that individuals homozygous for Lys^{71+} in the DRβ1 chain, combined with the absence of an aspartic acid at position 57 in the DQβ1 chain, show a significantly increased risk for developing IDDM (relative risk, RR = 20.41).

Materials and Methods

Patients

As previously described a population of 210 unrelated Caucasians with IDDM were studied [3]. Patients were diagnosed at the Paediatric and Adult Endocrinology Units of the University Hospital of Leuven according to the WHO criteria using clinical data, C peptide status and/or anti-islet autoantibodies. All patients were from Belgian origin. The mean age at onset of disease was 16.5 years (1–53 years). The control group of unrelated Caucasians from Belgian origin consisted of 205 blood donors. The control group did not have any personal or family history of IDDM, and the mean age at blood sampling was 40 years (28–52 years).

HLA Class II Typing with Sequence-Specific Oligonucleotides

HLA-DRB1, DRB3, DRB4, DRB5, DQA1 and DQB1 typing with sequence-specific oligonucleotides was performed on amplified DNA as described by Buyse et al. [14]. Briefly, the polymorphic second exons of the DRB and DQ genes were amplified from genomic DNA by the polymerase chain reaction, using specific genomic primers. The biotin-incorporated polymerase chain reaction products were hybridized at the appropriate temperatures for each locus to membrane-bound sequence-specific oligonucleotides. Positive signals were detected by chemiluminescence.

Analysis of the Role of DRβ1 Amino Acid Polymorphisms in Susceptibility or Protection against IDDM

Based on the three-dimensional structure model of Brown et al. [13], the amino acids of the antigen-binding side, which showed polymorphisms, as determined by the nucleotide composition of their codons, were examined. These included e.g. amino acid position 71, which can encode Lys, Arg, Ala and Glu, and position 9, including Trp, Glu, Lys and a few others. As a control, amino acids in nonfunctional positions were examined in the same fashion. The different alleles containing the particular amino acid were determined from the genomic sequence as published by Steven et al. [15].

Statistical Analysis

The significance of the differences in allele or genotype frequencies found was calculated by means of Fisher's exact test [16]. p values were corrected for multiple testing by the use of Bonferroni's method [17, 18]. RR (odds ratio) was calculated using the formula:

Table 1. Combination of $DQ\alpha^{Arg52}/DQ\alpha^{Arg52}$ genotypes (S = Arg^{52+}, P = Arg^{52-}) with the $DQ\beta^{Asp57}/DQ\beta^{Asp57}$ genotypes (S = Asp^{57-}, P = Asp^{57+}) in IDDM patients

DQα-DQβ genotypes	IDDM (n = 210)	Control (n = 205)	p value	RR	CL
SS-SS	0.467 (98)	0.044 (9)	$<10^{-7}$	19.1	9.1–36.1
SP-SS	0.257 (54)	0.141 (29)	0.020	2.10	1.3–3.4
SS-SP	0.114 (24)	0.063 (13)	n.s.		
SS-PP	0.014 (3)	0.029 (6)	n.t.		
PP-SS	0.029 (6)	0.088 (18)	n.s.		
Sp-SP	0.105 (22)	0.254 (52)	0.00045	0.34	0.2–0.6
PP-SP	0.014 (3)	0.146 (30)	1.8×10^{-6}	0.08	0.03–0.28
SP-PP	0.000 (0)	0.151 (31)	$<10^{-7}$	0.01[a]	
PP-PP	0.000 (0)	0.083 (17)	3.6×10^{-5}	0.02[a]	

Results are expressed as frequencies, with numbers of individuals studied in parentheses.

p value of Fisher's exact test [16] with correction for multiple comparison. n.s. = Not significant at p = 0.05; CL = 95% confidence limit; n.t. = not tested since there were fewer than 10 individuals.

[a] RR calculated according to Haldane (see Materials and Methods).

[number of patients with the specific allele (A)/number of patients without this allele (B)]/[number of controls with the specific allele (C)/number of controls without this allele (D)] [19]. When one element of the equation was zero, the RR was calculated by the method of Haldane: [(2A + 1) (2D + 1)]/[(2B + 1)(2C + 1)]. Only p values and RR were calculated for those alleles or genotypes which were observed more than 10 times in the total (patient and control) population.

Results

Combinations of $DQ\alpha^{Arg52}/DQ\alpha^{Arg52}$ with $DQ\beta^{Asp57}/DQ\beta^{Asp57}$ Genotypes in IDDM

As previously shown [3], analysis of HLA-DQ genotypes revealed (table 1) that the combination of $DQ\alpha^{Arg52+}/DQ\alpha^{Arg52+}$ (DQαSS; S = Arg^{52+}) with $DQ\beta^{Asp57-}/DQ\beta^{Asp57-}$ (DQβSS; S = Asp^{57-}) genotypes, in which all of the DQαβ heterodimers presented on the cell surface are DQαS-DQβS, confer a very significant risk to develop IDDM ($p < 10^{-7}$, RR = 19.1 with 95% confidence limit of 9.1–36.1). In addition, we show here that combination of DQαPP (P = Arg^{52-}) or SP with DQβPP (P = Asp^{57+}) genotypes on the other hand was extremely protective (RR = 0.02 and 0.01, respectively); indeed no IDDM patient was found with this combination.

Association of Amino Acid Polymorphisms on the DRβ1 Chain with IDDM

Analysis of the polymorphic amino acids in the functional domains of the DRβ1 chain showed (table 2) that those alleles encoding $DR\beta1^{Glu9+}$, $DR\beta1^{Gln70+}$, $DR\beta1^{Lys71+}$ and $DR\beta1^{Thr140+}$ increased significantly the risk to develop IDDM (in all cases $p < 10^{-8}$), while those alleles encoding $DR\beta1^{Arg71+}$ and $DR\beta1^{Trp9+}$ had a significant negative association with IDDM (for both $p < 10^{-8}$). According to the three-dimensional structure of DRβ1, residues 9, 70 and 71 are located in the antigen-binding groove of the DRβ1 chain, and residue 140 is located in the CD4-binding domain of the DRβ1 chain [13]. HLA-DRα chains are not polymorphic in structure except for minor variations in the cytoplasmic

Table 2. Effect of amino acid polymorphisms in the DRβ1 molecule on IDDM risk

Alleles	IDDM (n = 420)	Control (n = 140)	p value	RR	CL
$DR\beta1^{Lys71+}$	256	79	$<10^{-8}$	6.54	4.76–8.89
$DR\beta1^{Gln70+}$	330	198	$<10^{-8}$	3.93	2.89–5.29
$DR\beta1^{Glu9+}$	340	229	$<10^{-8}$	3.36	2.45–4.56
$DR\beta1^{Thr140+}$	293	194	$<10^{-8}$	2.57	1.93–3.40
$DR\beta1^{Arg71+}$	99	177	$<10^{-8}$	0.41	0.30–0.55
$DR\beta1^{Trp9+}$	70	172	$<10^{-8}$	0.28	0.20–0.38
$DR\beta1^{Asp10+}$	194	205	n.s.		
$DR\beta1^{Phe31+}$	379	363	n.s.		

$DR\beta1^{Thr140+}$: position in the CD4-binding domain; $DR\beta1^{Asp10+}$ and $DR\beta1^{Phe31+}$: nonfunctional position; n = number of chromosomes. p value of Fisher's exact test [16] with correction for multiple comparison. CL = 95% confidence limits of RR.

portion of the molecule [20]. It would therefore appear that the $DR\beta1^{Glu9+}$, $DR\beta1^{Gln70+}$, $DR\beta1^{Lys71+}$ and $DR\beta1^{Thr140+}$ residues contribute to the susceptibility and that $DR\beta1^{Arg71+}$ and $DR\beta1^{Trp9+}$ are associated with a protective effect in the DRαβ heterodimers for developing IDDM. As a control, the polymorphic positions for residues $DR\beta1^{Asp10+}$ and $DR\beta1^{Phe31+}$ located in a nonfunctional domain of the DRβ1 chain were tested and did not show any significant association with IDDM (table 2).

Combination of $DQ\alpha^{Arg52}$ and $DQ\beta^{Asp57}$ Alleles and Genotypes with DRβ1 Amino Acid Polymorphisms in IDDM Provides Higher Susceptibility or Protection

Analysis of the combined genotypes for HLA-DR and -DQ revealed that the RR for the combined genotypes $DR\beta1^{Lys71+/+}$-$DQ\alpha^{Arg52+/+}$-$DQ\beta^{Asp57-/-}$ was higher (RR = 20.41) than for other amino acids alone (table 3). To determine whether the polymorphisms in the DQα, DQβ and DRβ1 chain were required together for this high risk, the different combinations were tested separately. This revealed that only the $DR\beta1^{Lys71+/+}$-$DQ\beta^{Asp57-/-}$ and $DQ\alpha^{Arg52+/+}$-$DQ\beta^{Asp57-/-}$ combinations provided a similar high risk (table 3). $DQ\alpha^{Arg52+}$ is however in linkage disequilibrium with $DR\beta1^{Lys71+}$ (table 4). Since $DR\beta1^{Lys71+/+}$ by itself provides a risk of 15.46, it would appear that the $DQ\alpha^{Arg52+}$ allele contributes only to the risk by being in linkage disequilibrium with $DR\beta1^{Lys71+}$. The combination of $DR\beta1^{Lys71+/+}$ with $DQ\beta^{Asp57-/-}$ therefore identified the highest-risk genotype.

The alleles containing $DR\beta1^{Trp9+}$, $DR\beta1^{Lys71-}$, $DQ\alpha^{Arg52-}$ and $DQ\beta^{Asp57+}$ provided significant protection on their own against IDDM (table 5). When the respective genotypes for each of these alleles were examined, an even higher protection for the different loci was observed (RR = 0.04–0.15). To determine whether the combination of these alleles would increase the protection against IDDM, the different haplotypes including $DR\beta1^{Trp9+}$, $DR\beta1^{Lys71-}$, $DQ\alpha^{Arg52-}$ and $DQ\beta^{Asp57+}$ in cis or in trans in the protein chains were examined. The highest protection (RR = 0.08) was obtained for the $DQ\alpha^{Arg52-}$-$DQ\beta^{Asp57+}$ combination with or without the

Table 3. Effect of $DQ\alpha1^{Arg52+}$, $DQ\beta1^{Asp57-}$ and $DR\beta1^{Lys71+}$ on the RR for IDDM

	IDDM	Controls	p value	RR	CL
Alleles	(n = 420)	(n = 410)			
$DR\beta1^{Lys71+}$	256	79	$<10^{-8}$	6.54	4.76–8.89
$DQ\alpha1^{Arg52+}$	325	167	$<10^{-8}$	4.98	3.67–6.69
$DQ\beta1^{Asp57-}$	359	208	$<10^{-8}$	5.72	4.08–7.91
Genotypes	(N = 210)	(N = 205)			
$DR\beta1^{Lys71+/+}$	81	8	$<10^{-8}$	15.46	7.10–30.13
$DQ\alpha1^{Arg52+/+}$	127	28	$<10^{-8}$	9.67	5.89–15.36
$DQ\beta1^{Asp57-/-}$	158	56	$<10^{-8}$	8.08	5.17–12.35
Combined genotypes DRβ1-DQα1-DQβ1					
$Lys^{71+/+}$-$Arg^{52+/+}$	84	8	$<10^{-8}$	16.42	7.54–30.96
$Lys^{71+/+}$------$Asp^{57-/-}$	80	6	$<10^{-8}$	20.41	8.48–42.26
------$Arg^{52+/+}$-$Asp^{57-/-}$	98	9	$<10^{-8}$	19.06	9.08–36.12
$Lys^{71+/+}$-$Arg^{52+/+}$-$Asp^{57-/-}$	80	6	$<10^{-8}$	20.41	8.48–42.26

p value of Fisher's exact test [16] with correction for multiple comparison. N = Number of individuals studied; n = number of chromosomes; CL = 95% confidence limit.

addition of $DR\beta1^{Lys71-}$. From table 4 it would appear that $DR\beta1^{Trp9+}$ and $DR\beta1^{Lys71-}$ are in linkage disequilibrium with $DQ\alpha^{Arg52-}$ and that $DR\beta1^{Trp9-}$ is also in linkage disequilibrium with $DQ\beta^{Asp57-}$. Also $DQ\alpha^{Arg52-}$ in the heterozygotes or the homozygotes gives better protection than $DR\beta1^{Lys71-}$ or $DR\beta1^{Trp9+}$ in either combination. It would therefore appear that the haplotype $DQ\alpha^{Arg52-}$-$DQ\beta^{Asp57+}$ with a relative risk of 0.08, without considering the DRβ1 allele, is the best measure of the role of class II alleles in protection against type I diabetes.

Discussion

IDDM is the direct consequence of the destruction of insulin-secreting β cells. In the immune system, combination of HLA class II α and β chains forms the shape of the antigen-binding groove of the αβ heterodimer. In this fashion, HLA-αβ heterodimers could play a

Table 4. Frequency of the combined $DQ\alpha^{Arg52}$, $DQ\beta^{Asp57}$, $DR\beta1^{Trp9}$ and $DR\beta1^{Lys71}$ genotypes in 415 individuals

DRβ1-DQ	IDDM (n = 210)	Controls (n = 205)	Total (n = 415)
$Trp^{9+/+}$-$Arg^{52+/+}$	0	0	0
$Trp^{9+/+}$-$Arg^{52-/-}$	6	33	39
$Trp^{9-/-}$-$Arg^{52+/+}$	127	28	155
$Trp^{9-/-}$-$Arg^{52-/-}$	1	5	6
$Lys^{71+/+}$-$Arg^{52+/+}$	84	7	91
$Lys^{71+/+}$-$Arg^{52-/-}$	0	0	0
$Lys^{71-/-}$-$Arg^{52+/+}$	7	6	13
$Lys^{71-/-}$-$Arg^{52-/-}$	9	65	74
$Lys^{71+/+}$-$Asp^{57+/+}$	1	0	1
$Lys^{71+/+}$-$Asp^{57-/-}$	80	6	86
$Lys^{71-/-}$-$Asp^{57+/+}$	2	50	52
$Lys^{71-/-}$-$Asp^{57-/-}$	14	23	37
$Trp^{9+/+}$-$Asp^{57+/+}$	5	0	5
$Trp^{9+/+}$-$Asp^{57-/-}$	12	5	17
$Trp^{9-/-}$-$Asp^{57+/+}$	16	3	19
$Trp^{9-/-}$-$Asp^{57-/-}$	14	112	126

n = Number of individuals studied.

Table 5. Effect of $DQ\alpha1^{Arg52-}$, $DQ\beta1^{Asp57+}$, $DR\beta1^{Lys71-}$ and $DR\beta1^{Trp9+}$ on the relative protection against IDDM

	IDDM	Controls	p value	RR	CL
Alleles	(n = 420)	(n = 410)			
$DR\beta1^{Lys71-}$	164	331	$<10^{-8}$	0.15	0.11–0.21
$DR\beta1^{Trp9+}$	70	172	$<10^{-8}$	0.28	0.20–0.38
$DQ\alpha1^{Arg52-}$	95	244	$<10^{-8}$	0.20	0.15–0.27
$DQ\beta1^{Asp57+}$	61	202	$<10^{-8}$	0.17	0.13–0.25
Genotypes	(N = 210)	(N = 205)			
$DR\beta1^{Lys71-/-}$	19	127	$<10^{-8}$	0.06	0.04–0.11
$DR\beta1^{Trp9+/+}$	6	33	2×10^{-6}	0.15	0.07–0.38
$DQ\alpha1^{Arg52-/-}$	9	65	$<10^{-8}$	0.10	0.05–0.20
$DQ\beta1^{Asp57+/+}$	3	54	$<10^{-8}$	0.04	0.02–0.13
Haplotypes (cis or trans) DRβ1-DQα1-DQβ1					
Lys^{71-}-Arg^{52-}-Asp^{57+}	25	126	$<10^{-8}$	0.08	0.05–0.14
Lys^{71-}-Arg^{52-}	87	168	$<10^{-8}$	0.16	0.10–0.25
Lys^{71-}-------Asp^{57+}	48	145	$<10^{-8}$	0.12	0.08–0.19
-------Arg^{52-}-Asp^{57+}	25	130	$<10^{-8}$	0.08	0.05–0.13
Trp^{9+}-Arg^{52-}-Asp^{57+}	17	97	$<10^{-8}$	0.10	0.06–0.18
Trp^{9+}-Arg^{52-}	53	138	$<10^{-8}$	0.17	0.11–0.26
Trp^{9+}-------Asp^{57+}	18	98	$<10^{-8}$	0.10	0.06–0.18
-------Arg^{52-}-Asp^{57+}	25	130	$<10^{-8}$	0.08	0.05–0.13

p value of Fisher's exact test [16] with correction for multiple comparison. N = Number of individuals studied; n = number of chromosomes; CL = 95% confidence limit.

critical role in the predisposition for IDDM. The DQαSS/DQβSS genotype with an RR of 19.1 was carried by 47% of the IDDM patients, while only 4% of the normal population had this combined genotype. These results confirm the hypothesis that IDDM susceptibility associates quantitatively with the nature of the cell-surface-expressed DQαβ heterodimers.

The $DR\beta1^{Lys71+/+}$ is a major contributor to IDDM susceptibility. Lys^{71+} is in linkage disequilibrium with $DQ\alpha1^{Arg52+}$ and is found in the DRB1*0401 and the 0300 group, and is also linked in the DRB3 and DRB4 subtypes, respectively, to the DRB4*0101 and DRB3*0200, *0101 alleles. Some of these high-susceptibility haplotypes were identified previously [3]. This means that the susceptibility provided by the $DQ\alpha1^{Arg52+/+}$ genotype and DR3 and DR4 subtypes can be explained by the presence of a Lys at position 71 of the antigen-binding domain.

Also, the susceptibility found with $DQ\alpha^{Arg52+}$ and $DQ\beta^{Asp57-}$ appears to be due mainly to the Lys^{71+} in the DRβ1 locus. The susceptibility or RR is not altered when the DQα alleles carrying the Arg^{52+} are not taken into account. However, the Asp^{57-} allele of DQβ1 increases the RR found with the $DR\beta1^{Lys71+/+}$ genotype alone to 20.41, suggesting an additive and as previously shown [3] independent role of these $DQ\beta1^{Asp57-}$ alleles. The most susceptible genotype therefore can be defined by $DR\beta1^{Lys71+/+}$ combined with $DQ\beta1^{Asp57-/-}$. This contrasts with the findings of Kockum et al. [12] who did not

however consider the role of the amino acids in DRβ1.

The combined DQα-DQβ genotypes show that the presence of one protective allele (Arg^{52-}, Asp^{57+}) at both loci provides significant protection against IDDM (table 1). This protection increases as the number of protective alleles increases further to a maximum of 4. In DRβ1, tryptophan at position 9 and non-lysine amino acids at position 71 also provide protection in heterozygotes and homozygotes (table 5), but Trp^{9+} and Lys^{71-} in DRβ1 are in strong linkage disequilibrium with $DQα^{Arg52-}$ and are not as protective as the $DQα^{Arg52-}$ alleles.

It has been previously shown that the presence of Asp^{57+} in the DQβ chain induces salt bridge formation with arginine in the DQα chain at position 79, thereby altering the shape of the antigen-binding cleft of the DQαβ heterodimer [21]. Presumably a similar mechanism may be achieved in the DRβ1 antigen-binding region as a consequence of the amino acids which compose it. How the configuration of the antigen-binding domains affects the susceptibility or protection for IDDM remains to be determined. Our results nevertheless provide a new and more specific approach to test for IDDM susceptibility. Indeed the typing for the presence of $DRβ1^{Lys71}$, $DQβ1^{Asp57}$ and of $DQα1^{Arg52}$ is a rapid and simple assay to determine the increased or decreased susceptibility of a random person for IDDM.

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