Linkage and association of the HLA gene complex with IDDM in 81 Danish families: strong linkage between DRβ1^{Lys71+} and IDDM

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Abstract

Many studies have shown an association of IDDM with polymorphisms in the HLA region on chromosome 6p21. Previously our case-control study in the Belgian population showed significant association between IDDM and certain HLA class II alleles, in particular Lys^{71+}, encoding DRB1 alleles. In the present study, 81 Danish multiplex IDDM families and 82 healthy Danish controls were examined for polymorphisms in the HLA-DRB genes and 54 of the 81 families for polymorphisms in HLA-B, -DQA1, -DQB1, -TNFA, and -TNFB genes.

The results confirm our previous studies in the Belgian population and show that DRB1$^{Lys71+/+}$ homozygotes have a relative risk (RR) of 103.5. Linkage between IDDM and DRB1 alleles that encode Lys^{71+} was shown by affected sib pair analysis which showed strong linkage ($p<1\times10^{-6}$). By family based association studies, the DRB1^{Lys71+} was identified as the allele which increased susceptibility to develop IDDM most in the HLA region (haplotype relative risk = 8.38). Haplotype analysis confirmed the increased risk contributed by DRB1^{Lys71+} alleles and in addition showed that DRB1^{Lys71-} provides protection against IDDM even in the presence of DQB1^{Asp57-}.

These results indicate that DRB1^{Lys71+} screening is a powerful test compared to full HLA typing to determine the risk for a random person to develop IDDM in the Danish population, with an even higher probability than shown previously for the Belgians.

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Key words: insulin dependent diabetes mellitus; HLA; amino acid polymorphism; linkage.

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Insulin dependent diabetes mellitus (IDDM) is characterised by the immunologically mediated destruction of the insulin producing islet β cells resulting in an absolute requirement for exogenous insulin administration. The aetiology of IDDM is complex, involving both genetic and environmental factors. A series of genetic components involved in IDDM susceptibility have been implicated, with a major susceptibility locus encoded by the HLA class II genes within the major histocompatibility complex (MHC) on chromosome 6p21.[1-6] Also, family studies showed that IDDM affected sib pairs have a non-random distribution of shared HLA haplotypes.[7-10]

Association studies between HLA class II polymorphisms and IDDM showed that alleles at the DR locus and at the DQ locus contributed to susceptibility or protection against IDDM. In particular, alleles DRB1*0401, DQB1*0302, Dqα^{Arg52+}, and DQβ^{Asp57-} associated positively, while DRB1*1500, *0701, Dqα^{Arg52-}, and DQβ^{Asp57+} alleles provided protection.[2 11-18]

Previously we were able to assign the highest susceptibility to the DRβ1^{Lys71+} alleles in the Belgian population, while the DQβ1^{Asp57-} allele had an additive effect to developing IDDM in DRβ1$^{Lys71+/+}$ subjects[19] (Lys71+ is encoded by DRB1*0301,2,3, *0401, *0409, *1303). Indeed 60.9% of IDDM patients carried at least one copy of DRB1^{Lys71+}, compared to about 19% of the control population and, even more significantly, approximately 38.6% of the IDDM patients were homozygous for DRB1^{Lys71+} alleles compared to 3.4% of the controls. It was suggested that the susceptibility provided by the DQα1$^{Arg52+/+}$ genotype and DR3 and DR4 subtypes could be explained by the presence of a lysine at position 71 of the DRβ1 chain.[19] Based on the three dimensional structure of the HLA-DR1 αβ heterodimer, lysine at position 71 has been shown to be located in the antigen binding cleft of the DRβ1 chain[20] and may therefore play an important role in antigen binding.

To confirm the results of these association studies in another population, to determine whether loci in the HLA region show strong linkage with IDDM, and to explore which allele in these linked loci confers the highest risk of developing IDDM, 81 Danish multiplex IDDM families and 82 healthy Danish controls were typed for the class II HLA-DRB genes and 54 of the 81 families for class I HLA-B, class II HLA-DQA1, -DQB1, and class III HLA-TNFA and -TNFB genes.

The results show that strong linkage of IDDM with the DRB1 locus is found and that Lys^{71+} encoded by this locus confers the highest haplotype relative risk (HRR = 8.38) for developing IDDM among the alleles studied in the HLA region.

Materials and methods

PATIENTS

Eighty-two families (388 subjects), in whom at least two people were affected with IDDM, were studied. The families, all of white Danish origin, were unrelated and the affected subjects were diagnosed as having IDDM according to the WHO criteria.

For the DRB association study the 82 IDDM probands were selected from the families. For the DRB1 linkage studies the sibs, affected and unaffected, together with the parents of the original 82 IDDM probands were studied. In one family incorrect segregation of all HLA markers was observed and this family was excluded from the study. The remaining 81 multiplex families comprised a total of 382 subjects, 173 IDDM patients and 52 healthy sibs. For the DRB1 haplotype relative risk (HRR) study, the families with affected or missing parents were excluded.

In the linkage and HRR study for the other HLA loci, only 54 of the 81 multiplex families were typed.

The control group of unrelated white subjects of Danish origin comprised 82 people and was used in the DRB1 case-control study. The control group did not have any personal or family history of IDDM or other endocrinopathies.

GENOTYPING

The genotypes of 81 Danish multiplex IDDM families and 82 Danish controls were determined for HLA-DRB1, DRB3, DRB4, and DRB5 by Inno lipa DRB (Innogenetics NV). In short, the highly polymorphic second exon of the DRB genes was amplified from genomic DNA by the polymerase chain reaction (PCR), using specific genomic primers. The biotin incorporated PCR products were hybridised at the appropriate temperatures to membrane bound sequence specific oligonucleotides (SSOs). Positive signals were detected by a non-radioactive colourimetric method (Innogenetics NV). Fifty-four of the 81 families (251 subjects) were typed for TNFA, TNFB, HLA-B, DQA1, and DQB1 genes. For the typing of the TNFA microsatellite polymorphism, primers were used as described by Jongeneel *et al*[21] and non-radioactive detection was performed. Briefly, one biotin labelled primer was used in the PCR amplification, the amplified DNA was separated on a polyacrylamide sequencing gel and transferred to nylon membrane, and alleles were detected by chemiluminescence. The *Nco*I restriction fragment length polymorphism in the first intron of TNFB was detected by PCR-RFLP analysis.[6] For HLA-B typing, cells were incubated with HLA class I Dynabeads. Bead attached cells were incubated with rabbit complement containing 3.5% acridine orange/ethidium bromide in a typing plate and were read by fluorescence microscopy.[6] As described by Rønningen *et al*,[22 23] the DQA1 and DQB1 genotypes were determined by amplification of the second exon of each gene using slot-blot analysis with end labelled sequence specific oligonucleotide probes.

ANALYSIS OF THE AMINO ACID POLYMORPHISMS

Based on the three dimensional structural model of Brown *et al*[20] the polymorphic amino acids located in the antigen binding site of the DRβ1 chain were determined from the nucleotide composition and were examined further. These included, for example, amino acid position 71, which can encode Lys, Arg, Ala, Glu, and position 9, including Trp, Glu, Lys, and a few others. The different alleles in the DRβ1, DQα1, and DQβ1 chains containing a particular amino acid were determined from the nucleotide sequence as published by Marsh and Bodmer.[24]

STATISTICAL METHODS

Relative risk analysis

Relative risk (odds ratio) was calculated using the method of Woolf[25]: (number of patients with the specific allele/number of patients without this allele)/(number of controls with the specific allele/number of controls without this allele). The level of significance in allele or genotype frequencies was assessed by Fisher's exact test[26] and p values were corrected for multiple comparisons by Bonferoni's correction.[27 28] Only p values and relative risks (RR) were calculated for those alleles or genotypes which were observed more than 10 times in the total population (patients and controls).

Affected sib pair analysis

Linkage analysis on affected sib pairs was performed by the ESPA (extended affected sib pair analysis) computer program.[29] In sib pair analysis, which is a parameter free method to evaluate linkage between a trait and a marker, the frequencies of affected sib pairs (ASPs) that share 0, 1, and 2 alleles identical by descent were compared with the expected values of 0.25, 0.5, and 0.25, respectively. If there is no linkage between the marker and the proposed disease gene, these frequencies should approximate the expected values. A significant deviation with an excess of shared alleles indicates linkage between the marker and the disease gene. Secondly, the contribution of HLA to the familial clustering of IDDM can also be evaluated by measuring the proportion of affected sib pairs that share no alleles or haplotypes identical by descent (IBD).[30]

Haplotype relative risk analysis

Haplotype relative risk is a reliable alternative method to RR for calculating the risk of disease in the presence of a particular genotype. For HRR, probands and their parents are genotyped. The "case" alleles are the alleles which are transmitted to the affected probands, the "control" alleles are the non-transmitted alleles.[31 32] For instance, if the parents have the genotypes $DRB1^{Lys71+/-}$ and $DRB1^{Lys71+/-}$ and the affected proband has the genotype $DRB1^{Lys71+/+}$, the control will be $DRB1^{Lys71-/-}$. The measurement of the HRR is different from the RR. HRR is based on the number of transmitted and non-transmitted alleles to the prob-

Table 1 Distribution of DRB alleles in Danish IDDM patients and healthy controls

Alleles	*IDDM (n=164)*		*Controls (n=164)*		*p**	*RR (CL)*
	No	*Fr*	*No*	*Fr*		
DRBI						
0101	8	0.049	16	0.098	NS	
0103	0	0.000	1	0.006		
0301	54	0.330	15	0.092	1.4×10^{-8}	4.88 (2.6–8.7)
0400	4	0.025	15	0.092		
0401	70	0.427	9	0.055	$<10^{-8}$	12.83 (6.1–24.8)
0701	3	0.018	26	0.159	6×10^{-5}	0.1 (0.04–0.33)
0801	3	0.018	2	0.012		
0802	0	0.000	1	0.006		
0804	0	0.000	1	0.006		
0901	1	0.006	0	0.000		
1101	2	0.012	12	0.073	NS	
1103	1	0.006	2	0.012		
1201	2	0.012	4	0.024		
1301	1	0.006	12	0.073	0.02	0.08 (0.02–0.5)
1302	12	0.073	14	0.085		
1303	0	0.000	2	0.012		
1401	0	0.000	1	0.006		
1500	1	0.006	30	0.183	$<10^{-8}$	0.03 (0.01–0.17)
1600	2	0.012	1	0.006		
DRB3						
0101	44	0.268	23	0.140	0.010	2.27 (1.3–3.9)
0201	16	0.098	26	0.159	NS	
0301	14	0.085	15	0.091		
—	90	0.549	100	0.610		
DRB4						
0101	78	0.476	49	0.299	0.0014	2.13 (1.35–3.3)
—	86	0.524	115	0.701	0.0017	0.47 (0.30–0.74)
DRB5						
0101	1	0.006	28	0.171	8×10^{-8}	0.03 (0.01–0.19)
0102	0	0.000	1	0.006		
0201	2	0.012	1	0.006		
—	161	0.982	134	0.817	8×10^{-8}	12.0 (3.6–30.4)

The following alleles were not observed in either non-insulin dependent diabetes mellitus or control populations: 0102, 0302, 0406, 0409, 0410, 0411, 0412, 0803, 0805, 1001, 1104, 1105, 1106, 1202, 1304, 1305, 1402, 1403, 1404, 1405, 1406, 1407, and 1408. The DRB1*0400 group includes all DRB1*04 alleles except for DRB1*0401. The DRB1*1101 and DRB1*1301 groups contain the DRB1*1101, *1102 and DRB1*1301, *1305 alleles respectively.
Fr = frequency, No = number of chromosomes, NS = not significant.
* p value of Fisher's exact test[26] with correction for multiple comparison, CL: 95% confidence limits of RR.

Table 2 Role of $DRB1^{Lys71+}$ and increased relative risk for Danish IDDM patients

	IDDM		*Controls*		*p**	*RR (CL)*
	No	*Fr*	*No*	*Fr*		
Alleles	*(n=164)*		*(n=164)*			
$DRB1^{Lys71+}$	124	0.756	26	0.159	$<10^{-8}$	17.3 (9.8–29)
$DRB1^{Lys71-}$	40	0.244	138	0.841	$<10^{-8}$	0.06 (0.04–0.11)
Genotypes	*(n=82)*		*(n=82)*			
$DRB1^{Lys71+/+}$	46	0.561	1	0.012	$<10^{-8}$	103.5 (16–300)
$DRB1^{Lys71-/-}$	4	0.049	57	0.695	$<10^{-8}$	0.02 (0.01–0.07)
$DRB1^{Lys71+/-}$	32	0.390	24	0.293	NS	

Fr = frequency, No = number of chromosomes, n = number of subjects.
* p value of Fisher's exact test[26] with correction for multiple comparison.
CL: 95% confidence limits of RR.

Table 3 Sib pair analysis for different loci in IDDM families

	Not shared (%)	*Shared (%)*	*Non-informative*	*Total p*
In 81 IDDM families				
DRB1	44 (29.4)	105.8 (70.6)	32.2	$<1 \times 10^{-6}$
In 54 IDDM families				
DRB1	32 (31.7)	69 (68.3)	23	<0.00011
TNFA	26 (28)	67 (72)	31	<0.000011
TNFB	16 (31.4)	35 (68.6)	73	<0.0039
HLA-B	26 (28.9)	64 (71.1)	18	<0.000032
DQA1	30 (30)	70 (70)	24	<0.000032
DQB1	32 (33)	65 (67)	27	<0.00040
Haplo	34 (30.9)	76 (69.1)	14	<0.000032

and (first affected child) while the RR is based on the number of case and control alleles. In the haplotype analysis, instead of alleles, transmitted and non-transmitted haplotypes to the affected probands were considered.

Results

CONFIRMATION OF THE ASSOCIATION WITH DRB1

In the present study our results in a Danish population identified almost the same susceptible or protective alleles as in the Belgian population[2 19] (table 1), except for DRB3*0101 which was only associated with Danish IDDM and for DRB1*1101 that, in spite of the low frequency in Danish IDDM patients, did not show significant negative association with IDDM, as found in the Belgian population.

The relative risk (RR) for DRB1*0401 was increased in the Danish population (RR = 12.8) compared with the Belgians (RR = 5) and was almost the same for DRB1*0301 in the two populations. Protection by DRB1*1500 remained unchanged while protection by DRB1*0701 was increased in the Danish population (table 1). When we examined the presence or absence of specific amino acids, the highest susceptibility to developing IDDM was provided by $DRB1^{Lys71+}$ ($p<10^{-8}$, RR = 17.3) which was higher than in the Belgian population. This susceptibility for IDDM was very significantly increased for the homozygous $DRB1^{Lys71+/+}$ genotypes (RR = 103.5, with 95% of confidence limits of 16–300). Indeed, 56.1% of the IDDM patients carried $DRB1^{Lys71+/+}$ compared to only 1.2% of the controls (table 2).

AFFECTED SIB PAIR ANALYSIS

An extended affected sib pair analysis (ESPA) between IDDM and DRB1 in 81 multiplex families is summarised in table 3. A total of 91 sib pairs were analysed. One hundred and forty-nine parent/sib pair combinations were informative, one pair was partially informative since allele information from one parent needed to be reconstructed, and 32 parent/sib pair combinations were uninformative as the parent was homozygous for DRB1. In 70.6% of the informative cases, allele sharing was observed, which deviates clearly from the expected allele sharing of 50% ($p<1 \times 10^{-6}$).

Markers DQB1, DQA1, TNFA, TNFB, and HLA-B were analysed in 54 of the 81 multiplex families, consisting of 62 sib pairs by ESPA (table 3). Maximal segregation information was gathered by haplotyping all markers tested. Again, significant deviations from the expected allele sharing distribution assuming no linkage were observed for all loci, indicating close linkage of the HLA complex with IDDM in the Danish population.

FAMILIAL CLUSTERING OWING TO HLA

The degree of familial clustering of a disease (λ_s) can be estimated from the risk for sibs of patients divided by the population risk.[30] For IDDM, λ_s is estimated to be about 15 in the white population.[30] The λ_s for HLA ($\lambda_{s\text{-HLA}}$) to IDDM can easily be estimated by the ratio of the expected proportion of affected sib pairs sharing zero alleles identical by descent, which is 0.25, and the observed proportion. Using the HLA haplotypes, 49 sib pairs were completely

*Table 4 Haplotype relative risk (HRR) of alleles for TNFA, TNFB, HLA-DQA1, DBQ1, and DRB1*0401 significantly associated in Danish IDDM families*

Alleles	Transmitted alleles, IDDM		Non-transmitted allles, controls		p*	HRR (CL)
	No	Fr	No	Fr		
TNFA	n=106		n=106			
2	54	0.509	34	0.321	0.032	2.18 (1.25–3.74)
TNFB	n=108		n=108			
10.5	53	0.491	70	0.648	0.027	0.25 (0.3–0.9)
5.5	55	0.509	38	0.352	0.027	1.91 (1.11–3.26)
DQA1						
0201	1	0.009	13	0.120	0.0046	0.07 (0.02–0.45)
0301	43	0.398	19	0.176	0.0016	3.10 (1.65–5.65)
DBQ1						
0302	43	0.398	18	0.167	0.0009	3.31 (1.74–6.1)
DRB1						
0401	46	0.426	15	0.139	2×10^{-5}	5.48 (2.76–10.33)

Only those alleles showing significant association with IDDM are shown. For comparison, the DRB1*0401 results for the same families are given.
Fr = frequency, No = number of chromosomes.
* p value of Fisher's exact test with correction for multiple testing.

Table 5 Frequency of transmitted (case) and non-transmitted DRB1 alleles (control) to affected children and haplotype relative risk (HRR) in Danish IDDM families

Alleles	Transmitted alleles, IDDM (n=150)		Non-transmitted alleles, controls (n=150)		p*	HRR (CL)
	No	Fr	No	Fr		
DRB1						
0101	8	0.053	19	0.127	NS	
0103	0	0.000	1	0.007		
0301	47	0.313	22	0.147	0.0040	2.65 (1.5–4.6)
0400	4	0.027	8	0.053		
0401	64	0.427	19	0.127	$<10^{-8}$	5.13 (2.8–8.9)
0701	3	0.020	18	0.120	0.0045	0.15 (0.06–0.51)
0801	3	0.020	3	0.020		
0901	1	0.007	1	0.007		
1101	2	0.013	12	0.080	NS	
1103	1	0.007	0	0.000		
1201	2	0.013	5	0.033		
1301	1	0.007	6	0.040	NS	
1302	11	0.073	12	0.080		
1303	0	0.000	1	0.007		
1401	0	0.000	4	0.027		
1500	1	0.007	18	0.120	2×10^{-4}	0.05 (0.02–0.31)
1600	2	0.013	1	0.007		

The following alleles were not observed in IDDM families: 0102, 0302, 0406, 0409, 0410, 0411, 0412, 0802, 0803, 0804, 0805, 1001, 1104, 1105, 1106, 1202, 1304, 1305, 1402, 1403, 1404, 1405, 1406, 1407, and 1408.
Fr = frequency, No = number of chromosomes, NS = not significant.
* p value of Fisher's exact test with correction for multiple testing, CL = 95% confidence limits of HRR.

Table 6 Effect of HLA class II amino acids in increased and decreased HRR for IDDM in Danish IDDM families

	Transmitted alleles, IDDM		Non-transmitted alleles, controls		p*	HRR (CL)
	No	Fr	No	Fr		
Alleles	(n=108)		(n=108)			
$DRB1^{Lys71+}$	81	0.750	34	0.314	$<10^{-8}$	6.53 (3.5–11.5)
$DRB1^{Lys71-}$	34	0.314	81	0.750	$<10^{-8}$	0.15 (0.09–0.28)
$DQB1^{Asp57-}$	93	0.861	73	0.676	0.0019	2.97 (1.5–5.7)
$DQB1^{Asp57+}$	15	0.139	35	0.324	0.0019	0.34 (0.18–0.67)
$DQA1^{Arg52+}$	90	0.833	56	0.519	1×10^{-6}	4.64 (2.5–8.5)
$DQA1^{Arg52-}$	18	0.167	52	0.481	1×10^{-6}	0.22 (0.12–0.41)
	(n=150)		(n=150)			
$DRB1^{Lys71+}$	111	0.740	39	0.260	$<10^{-8}$	8.38 (4.9–13.8)
$DRB1^{Lys71-}$	39	0.260	111	0.740	$<10^{-8}$	0.12 (0.07–0.20)

informative (both parents heterozygous), of which five shared zero haplotypes IBD. This leads to an $\lambda_{s\text{-HLA}}$ of 2.45. From this value it can be derived that the contribution of the HLA region to the familial clustering of IDDM will be about 33%. Assuming a simple multiplicative model, this would mean that other familial determinants are implicated in IDDM with a combined λ_s of about 6.

HIGH RISK ALLELES FOR IDDM

HRR was determined for the alleles of TNFA, TNFB, HLA-B, HLA-DQA1, DQB1, and DRB1 which were significantly associated with IDDM (tables 4, 5, and 6). Despite the strong linkage between TNFA and IDDM, statistical analysis of the TNFA genotype in 53 IDDM families showed that only allele 2 with a p value of 0.032 was associated with IDDM (HRR = 2.18). For TNFB, allele 5.5 showed positive association with IDDM with a p value of 0.027 (HRR = 1.91) (table 4). For the HLA-B gene, the frequency of alleles 15 (62) and 7 increased and decreased respectively in IDDM patients but did not show significant differences (not shown). In the DQ loci, DQA1*0301 and DQB1*0302 were identified as susceptible alleles with a HRR of 3.10 and 3.31 respectively, while DQA1*0201 was protective (table 4). These analyses showed that the DQ alleles are more strongly associated with IDDM susceptibility than the TNF alleles. However, analysis of the DRB1 alleles showed even stronger association between the DRB1*0401 allele and IDDM ($p<10^{-8}$, HRR = 5.12); allele 0301 also showed susceptibility (HRR = 2.65) (table 5). The strongest protection was offered by the DRB1*1500 allele (p = 0.0002, HRR = 0.05). These association studies based on the families show that HLA-DRB1*0401 and *1500 alleles are more strongly associated with IDDM than alleles in the TNF, HLA-B, and HLA-DQ loci.

IN THE HLA REGION, $DRB1^{Lys71+}$ CONFERS THE HIGHEST RISK OF DEVELOPING IDDM

Analysis of amino acid polymorphisms encoded by DQ loci (DQA1 and DQB1 genes) showed that $DQ\beta1^{Asp57-}$ and $DQ\beta1^{Asp57+}$, with a HRR of 2.97 and 0.34, were susceptible and protective respectively (both with a p value of 0.0019). On the other hand, $DQ\alpha1^{Arg52+}$ (HRR = 4.64) and $DQ\alpha1^{Arg52-}$ (HRR = 0.22) showed stronger positive and negative association ($p = 1 \times 10^{-6}$). However, the strongest association was found with those alleles encoding $DR\beta1^{Lys71+}$, located in the functional domain of the DRβ1 chain, with a HRR of 6.53 ($p<10^{-8}$) in the 54 families studied (table 6). Furthermore, in the full data set (81 families), the HRR for $DR\beta1^{Lys71+}$ increased to 8.38 (table 6).

HAPLOTYPE ANALYSIS

Haplotype analysis of amino acid polymorphisms encoded by the DRB1, DQA1, and DQB1 loci in IDDM families showed (table 7) that the $DR\beta1^{Lys71-}$-$DQ\alpha1^{Arg52+}$-$DQ\beta1^{Asp57-}$ haplotype, in spite of carrying two susceptibility alleles $DQ\alpha1^{Arg52+}$ and $DQ\beta1^{Asp57-}$, was not significantly associated with IDDM susceptibility. The number of cases (transmitted) compared to controls (non-transmitted) was even decreased in IDDM. When in this haplotype $DR\beta1^{Lys71-}$ was replaced by $DR\beta1^{Lys71+}$, the most susceptible haplotype $DR\beta1^{Lys71+}$-$DQ\alpha1^{Arg52+}$-$DQ\beta1^{Asp57-}$ for IDDM was obtained ($p<10^{-8}$, HRR = 7.57), indicating the major susceptibility effect of $DR\beta1^{Lys71+}$. When

Table 7 Haplotype analysis of HLA class II amino acids and their role in the susceptibility for and protection against IDDM in Danish IDDM families

Haplotypes	*Transmitted haplo, IDDM*		*Non-transmitted haplo, controls*		*p**	*HRR (CL)*
	No	*Fr*	*No*	*Fr*		
DRB1-DQA-DqB1	(n=180)		(n=180)			
Lys^{71-}-Arg^{52+}-Asp^{57-}	10	0.055	13	0.072	NS	
Lys^{71-}-Arg^{52+}-Asp^{57+}	6	0.033	27	0.15	6.7×10^{-4}	0.20 (0.09–0.5)
Lys^{71+}-Arg^{52+}-Asp^{57-}	131	0.728	47	0.261	$<10^{-8}$	7.57 (4.7–11.9)
Lys^{71+}-Arg^{52+}-Asp^{57+}	7	0.039	9	0.050	NS	
Lys^{71+}-Arg^{52-}-Asp^{57-}	0	0	0	0		
Lys^{71+}-Arg^{52-}-Asp^{57+}	0	0	0	0		
Lys^{71-}-Arg^{52-}-Asp^{57-}	23	0.128	59	0.328	3.4×10^{-5}	0.30 (0.18–0.52)
Lys^{71-}-Arg^{52-}-Asp^{57+}	3	0.017	25	0.139	5.4×10^{-5}	0.11 (0.04–0.35)
Lys^{71-}----------ASP^{57-}	34	0.189	72	0.04	6.5×10^{-5}	0.35 (0.22–0.57)
Lys^{71-}----------ASP^{57+}	9	0.05	52	0.289	$<10^{-8}$	0.13 (0.07–0.28)
Lys^{71+}----------ASP^{57+}	7	0.039	9	0.05	NS	
Lys^{71+}----------ASP^{57-}	131	0.728	47	0.261	$<10^{-8}$	7.57 (4.7–11.9)
Lys^{71-}-Arg^{52+}-Asp^{57-}	10	0.055	13	0.072	0.004*	
Lys^{71+}-Arg^{52+}-Asp^{57-}	131	0.728	47	0.261		
Lys^{71-}----------ASP^{57-}	34	0.189	72	0.04	$<10^{-8}$*	
Lys^{71+}----------ASP^{57-}	131	0.728	47	0.261		
Lys^{71-}-Arg^{52+}-Asp^{57-}	10	0.055	13	0.072	NS	
Lys^{71-}-Arg^{52-}-Asp^{57-}	23	0.128	59	0.328		

*Significant difference between two haplotypes.
Haplo = haplotypes, No = number of haplotypes.

only $DR\beta1^{Lys71}$ and $DQ\beta1^{Asp57}$ were considered, the haplotype $DR\beta1^{Lys71-}$-$DQ\beta1^{Asp57-}$ was significantly protective ($p=6.5\times10^{-5}$, HRR=0.35) although this haplotype carried the susceptibility allele $DQ\beta1^{Asp57-}$. When the haplotype carried the susceptibility allele $DR\beta1^{Lys71+}$ and the protective allele $DQ\beta1^{Asp57+}$, no significant protection was provided, reinforcing the protective effect of $DR\beta1^{Lys71-}$. The protection was increased by the $DR\beta1^{Lys71-}$-$DQ\beta1^{Asp57+}$ haplotype ($p<10^{-8}$, HRR=0.13), showing an additive effect of $DQ\beta1^{Asp57+}$ to $DR\beta1^{Lys71-}$ in the protection. Statistical analysis also showed significant difference between $DR\beta1^{Lys71-}$-$DQ\alpha1^{Arg52+}$-$DQ\beta1^{Asp57-}$ and $DR\beta1^{Lys71+}$-$DQ\alpha1^{Arg52+}$-$DQ\beta1^{Asp57-}$ haplotypes ($p=0.004$) and also between $DR\beta1^{Lys71-}$-$DQ\beta1^{Asp57-}$ and $DR\beta1^{Lys71+}$-$DQ\beta1^{Asp57-}$ haplotypes ($p<10^{-8}$), confirming the significantly increased susceptibility conferred by $DR\beta1^{Lys71+}$.

Discussion

To confirm our previous studies[19] in another population, to determine whether particular loci in the HLA region are more strongly linked to IDDM than other loci, and to identify the alleles or genotypes providing the strongest risk, we performed four types of studies in a Danish population: association studies based on two independent groups (patients and controls), linkage analysis on affected sib pairs, association studies based on the families, and haplotype analysis. One of the advantages of using families for association studies is that disease samples and appropriate controls are provided from the same homogeneous population.

Since the development of IDDM is probably the result of an interaction of genetic components and environmental factors, other advantages of family based association studies could be that people who inherit disease associated or non-associated parental alleles share more environmental factors than two unrelated people, whether patients or controls.

As shown by Knapp *et al*,[33] in general HRR calculated by using non-transmitted parental alleles to affected children as controls never exceeds the RR calculated on the same patient using independent controls. This was true for our two different types of association studies on the same Danish patients (RR in table 1 and HRR in table 5 for DRB1).

Previously we identified $DRB1^{Lys71+}$ as an important susceptibility factor to developing IDDM in a Belgian population. In the present study, a highly significant association between $DRB1^{Lys71+}$ and IDDM was also found in the Danish population (RR=17.3). The risk provided by $DRB1^{Lys71+}$ was even higher in the Danish population compared to Belgians; the frequency of $DRB1^{Lys71+}$ was 0.756 in Danish IDDM patients compared to 0.609 in Belgian IDDM patients. Also the frequency of $DRB1^{Lys71+}$ in the Danish control population (0.159) was lower than in Belgian controls (0.193). The risk for IDDM is even more dramatically increased in subjects carrying two copies of $DRB1^{Lys71+}$ with a RR=103.5, compared to a RR=15.46 in the Belgian IDDM population. These results therefore clearly confirm our previous study[19] that $DRB1^{Lys71+/+}$ is a major contributor to IDDM susceptibility. The fact that the RR for Danish IDDM patients is higher than for Belgians may be because of the difference in the two populations examined, families versus sporadic cases. Nevertheless these results confirm the importance of the $DRB1^{Lys71+}$ allele. Homozygous $DRB1^{Lys71-}$ provided the highest protection, while heterozygotes for $DRB1^{Lys71+/-}$ were only slightly increased in the patient group (table 2). On the other hand, in our previous study on a large number of Belgians, 110/210 IDDM patients carried $DRB1^{Lys71+/-}$ compared to 70/205 controls, which was significantly different ($p=0.00036$, RR=2.12). This may suggest that $DRB1^{Lys71+}$ acts in an intermediate mode.

Alleles encoding $DQ\alpha^{Arg52+}$ and the DQB^{Asp57-} alleles have been shown to contribute to susceptibility to IDDM.[2 11–18] Previously we were able to show that the sus-

ceptibility found with DQα1^{Arg52+} could be explained by DRB1^{Lys71+} which is in linkage disequilibrium with DQα1^{Arg52+}. We also showed an additive effect on the increased risk when DQβ1^{Asp57-} segregated together with DRB1^{Lys71+}. In the 82 Danish IDDM probands, only four original probands did not carry any copy of DRB1^{Lys71+}. We observed that three out of four Lys negative probands carried the DQβ1$^{Asp57-/-}$ genotype and that one was heterozygous for DQβ1^{Asp57-}. By examining the whole patient population, we identified nine DRB1$^{Lys71-/-}$ patients, six of whom carried two copies of DQβ1^{Asp57-} and three of nine who carried one copy of the DQβ1^{Asp57-} allele. This again suggests that the role of DRB1^{Lys71+} and DQβ1^{Asp57-} may not be simply additive or mutually exclusive. On these nine DRB1$^{Lys71-/-}$ patients, no other correlations were found with the other studied HLA-B, DQA1, TNFA, and TNFB loci.

In order to avoid the classical problems observed in case-control association studies, we evaluated the contribution of the HLA complex genes to IDDM by sib pair analysis and family based association studies in Danish families.

By using the affected sib pair analysis, we showed linkage between HLA loci and IDDM. For all loci tested, and also for the haplotypes, significantly more sharing of alleles was observed versus non-sharing between sibs (table 3).

In the sib pair analysis, we observed that five out of 49 completely informative sib pairs did not share any alleles identical by descent. Based on formulae derived by Risch,[30] we estimated the familial clustering coefficient owing to HLA ($\lambda_{s\text{-}HLA}$) to be 2.45. This is lower than found in other large studies[3 30 34] where values above 3 were found. However, for HLA this λ is probably an underestimate since it does not take into account situations where affected sibs receive one susceptibility factor from a different parent, when both parents are heterozygous for this factor. In those cases both sibs are counted as having inherited zero alleles identical by descent, while both might have received a susceptibility factor, but from a different parent. In the present situation, inspecting the five sib pairs who do not share any haplotypes, in three out of five both parents are DRB1^{Lys71+} heterozygous, and both children receive one DRB1^{Lys71+} from a different parent (Lys71+ is encoded by DRB1*0301, 2, 3, *0401, *0409, *1303). In one case, one parent is heterozygous for DRB1, but both alleles carry DRB1^{Lys71+}. Only in the fifth family, where only one parent is a carrier of the DRB1^{Lys71+} allele, one of the two affected children did not inherit the DRB1^{Lys71+} allele. By not being able to take into account these segregation patterns, $\lambda_{s\text{-}HLA}$ might underestimate the risk contribution of HLA to the familial clustering of IDDM. Similar observations can be made when DQA^{Arg52+} or DQB^{Asp57-} are considered.

In order to be able to confirm that DRB1^{Lys71+} was the major risk factor in HLA for IDDM, we determined HRRs for all significantly associated alleles in all loci. In the TNF loci, the highest HRR was given by the allele TNFA*2 (HRR=2.18) while HRRs of DQA1*0301 (HRR=3.10) and of DQB1*0302 (HRR=3.31) were higher than TNF (table 4). When the DRB1 alleles were analysed (table 5), DRB1*0401 was identified as the susceptible allele with a higher HRR than those found at the TNF and the DQ loci. To determine the most important risk factors, amino acid polymorphisms in DQ and DRB1 loci were studied and haplotype analysis was performed. The highest HRR was given by the presence of lysine at position 71 in the DRB1 chain (tables 4, 5, and 6). When the number of probands studied increased from 54 to 81, the HRR for DRB1*0401 remained almost unchanged but it increased for DRB1^{Lys71+} (tables 4, 5, and 6).

The haplotype analysis further confirmed the predominant role of DRβ1^{Lys71+} encoded alleles over DQα1^{Arg52+} and DQβ1^{Asp57-} in providing susceptibility and the additive effects of DRβ1^{Lys71-} and DQβ1^{Asp57+} in protection (table 7). Moreover, confirming our previous results,[19] the role of DQα1^{Arg52+} could be fully explained by linkage disequilibrium with DRβ1^{Lys71+}.

In conclusion, our results confirm our previous studies in the Belgian population[19] and provide evidence for the protective function of DRβ1^{Lys71-}. Strong linkage between IDDM and DRB1 loci which encode Lys^{71+} is shown and DRB1^{Lys71+} is further identified as the highest risk allele for IDDM among the alleles of the HLA region linked to IDDM.

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