Rapid HLA-DQB1 Genotyping for Four Alleles in the Assessment of Risk for IDDM in the Finnish Population

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OBJECTIVE — To study the effectiveness of MHC genotyping in the assessment of risk for IDDM based on the identification of alleles that are significantly associated with risk for IDDM (DQB1*0302 and *0201) and protection from it (DQB1*0602/*0603 and *0301).

RESEARCH DESIGN AND METHODS — A long series of 649 index cases of IDDM, together with their healthy siblings and 756 healthy blood donors, was collected in Finland. The samples were analyzed using a large-scale assay procedure that was developed for rapid screening purposes. The method utilizes time-resolved fluorometry to detect the hybridization of lanthanide-labeled allele-specific oligonucleotide probes with amplified gene product.

RESULTS — A total of 61.9% of IDDM index cases had high risk (DQB1*0201/*0302) or moderate risk (DQB1*0302/x [x meaning DQB1*0302 or a nondefined allele]) genotypes compared with 14.3% of the reference population. In patients and control subjects, the frequencies of low risk genotypes were 28.0 and 22.1%, respectively, and those of decreased risk genotypes, 10.0 and 63.6%. The relative risk of a *0201/*0302 genotype was 53.5 (31.1–92.8) compared with the decreased risk genotypes (63.6% of controls). The graded risk estimation was equally efficient in assessing the risk of IDDM in siblings of child with IDDM.

CONCLUSION — The near-automatic typing procedure developed is attractive for large-scale screening projects, such as diabetes prevention and intervention trials.

IDDM is a polygenic autoimmune disease. The most important genes that influence human susceptibility to IDDM are located within the major histocompatibility complex (MHC), the HLA region on the short arm of the human sixth chromosome. The exact number of HLA gene loci that affect an individual's susceptibility to IDDM, as well as their localization, is still unknown, but the HLA-DQ locus contains the strongest known risk markers and protective alleles, probably reflecting the relevance of HLA-DQ's role in the pathogenesis of IDDM (1).

The prediction of individual genetic risk is of considerable interest, particularly with respect to diabetes prevention and intervention trials and in research projects that aim at the identification of environmental factors triggering or enhancing β-cell damage (2,3). In the growing number of intervention trials, subjects with high risk are recruited to undergo experimental therapies or diets (4).

We have previously suggested a simple risk-grading system based on the definition of genotypes that encode either high or intermediate risk or that show protection (genotypes that are very rare in patients) (5). Here, we wanted to test the practicality and effectiveness of this strategy in large cohorts of patients and controls. Toward this end, we developed a rapid, nearly automated typing system (6). The data obtained confirm and extend our risk-grading strategy, and they demonstrate the utility of this new genotyping procedure.

RESEARCH DESIGN AND METHODS

Blood samples from subjects with IDDM and from their family members were collected in the Childhood Diabetes in Finland (DiMe) study (7). The study included 801 families of children <15 years of age who were diagnosed with IDDM between September 1986 and April 1989. These families represent the vast majority of all new IDDM cases presented in Finland during this period. Signs of an acute infection were registered at diagnosis, and a blood sample was taken for the measurement of blood glucose, capillary blood gases, HbA_1 or HbA_{1c}, and serum C-peptide concentrations before the initiation of insulin treatment.

The collection of venous blood samples for DNA extraction was added to the study protocol later, but samples from 697 families were obtained. PCR-based HLA-DQB1 typing was successfully carried out in 649 IDDM index cases and in 662 siblings, 56 of whom were diagnosed to have IDDM before or after the DiMe study index case. A total of 756 DNA samples from healthy blood donors from different parts of the country (Helsinki, Joensuu, Kuopio, Rovaniemi, Turku, and Vaasa) were analyzed in parallel.

The HLA-DQB1 typing method has been described in detail previously (6). In brief, 158 bp of the second exon of the DQB1 gene from each DNA sample were amplified by the polymerase chain reaction (PCR) using a primer pair with a biotinylated 3′ primer. The amplification product was bound to streptavidin-coated microtitration plates and dena-

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DiMe, Diabetes in Finland; MHC, major histocompatibility complex; PCR, polymerase chain reaction; RR, relative risk.

Table 1—*The frequencies of IDDM patients (DiMe probands) and control subjects positive for studied HLA-DQB1 alleles*

DQB1* allele	IDDM patients	Control subjects	*P*	RR (95% CI)
0201	281 (43.3)	179 (23.7)	<0.0001	2.46 (1.95–3.11)
0301	89 (13.7)	161 (21.3)	0.0003	0.59 (0.44–0.79)
0302	468 (72.3)	168 (22.2)	<0.0001	9.04 (7.04–11.62)
0602–0603	44 (6.8)	321 (42.5)	<0.0001	0.10 (0.07–0.14)
Totals	649	756		

Data are *n* (%).

tured with NaOH. After washing, bound DNA was analyzed using two different hybridization mixtures: one containing a europium (Eu)-labeled internal reporter probe for DQB1*0602 and *0603 alleles (*0602–*0603) plus a terbium (Tb)-labeled consensus probe. The second hybridization mixture included Tb-, samarium (Sm)-, and Eu-labeled probes specific for DQB1*0201, *0301, and *0302 alleles, respectively. For measurement of probe hybridization, microtitration plates were analyzed by time-resolved fluorescence (Delfia Research Fluorometer, Wallac Oy, Turku, Finland). The different emission wavelengths and delay times were used to distinguish the signals of each lanthanide label.

In five probands and one control subject, it was apparent that three alleles were detected. All of these samples were positive for both *0301 and *0302, simultaneously. This may reflect the presence of a DQB1*0304 allele recognized by both DQB1*0301- and *0302-specific probes. These cases were not included in our analyses.

We used a χ^2 test with continuity correction for our statistical analysis. Estimates of relative risk (RR) or odds ratios were calculated according to the formula $(a \times d)/(b \times c)$, in which a and b are the numbers of patients who were positive and negative for the marker, respectively, and c and d, the respective numbers for control subjects.

Blood glucose concentrations and capillary blood gases were measured using routine laboratory methods. Diabetic ketoacidosis at diagnosis was defined as a capillary blood pH of <7.30. The various hospitals participating in the study used standard methods to determine HbA_1 or HbA_{1c} results. To make the glycosylated hemoglobin results comparable, they were expressed as a standard deviation (SD) based on the reference range in nondiabetic subjects at each hospital. Serum C-peptide concentrations were analyzed radioimmunologically with antiserum K6 using commercial reagents (Novo Research, Bagsvaerd, Denmark). The detection limit was 0.02 nmol/l.

RESULTS — The frequencies of the four defined DQB1 alleles in IDDM index cases and control subjects are shown in Table 1. The earlier observations (5) of alleles associated with strong (DQB1*0302) and weak (DQB1*0201) susceptibility to disease as well as strong (DQB1*0602–*0603) and weak (DQB1*0301) protection were confirmed.

The distribution of genotypes defined by the detection of these alleles is shown in Table 2. A different strength for each allele in risk and protection can be seen. The highest risk was associated with heterozygosity for DQB1*0201 and *0302, but an increased risk was also detected with DQB1*0302 alone, provided the absence of protective alleles. The allele associated with a strongly decreased risk, DQB1*0602–*0603, is still protective in a genotype with the weak risk marker DQB1*0201 (RR = 0.13 for DQB1*0201/*0602–*0603), whereas the combination of *0602–*0603 with the strong susceptibility allele, DQB1*0302, codes equivocal risk (RR = 0.87 for DQB1*0302/*0602–*0603). In contrast, the protection mediated by the DQB1*0301 allele appeared to be passive in effect, since both DQB1*0301/*0302 and DQB1*0201/*0301 genotypes fell, like the DQB1*0201/x genotype, into the group of equivocal risk genotypes. Similar patterns were detected in the comparison of the IDDM index cases and their unaffected siblings (Table 3). However, risk ratios were lower because of the enrichment of risk alleles within families.

When the genotypes in children with IDDM were analyzed separately, the DQB1*0201/x genotype was found more often in boys than in girls. A total of 56 male index cases (15.6%) had this genotype compared with 28 female index cases (9.7%; $P = 0.034$). The proportion of patients with the DQB1*0201/x genotype was especially low among those diagnosed from April to June. The difference was significant ($P = 0.025$) when these 3 months were compared with others, but the heterogeneity of the whole distribution did not reach statistical significance.

A simplified risk-grading scheme with four categories was created by combining genotypes that had decreased among IDDM patients (DQB1*0602–

Table 2—*Defined HLA-DQB1 genotypes in IDDM patients (DiMe index cases) and control subjects*

DQB1* genotype	IDDM patients	Control subjects	*P*	RR (95% CI)
0201/0302	159 (24.5)	22 (2.9)	<0.0001	10.8 (6.70–17.65)
0302/x	243 (37.4)	86 (11.4)	<0.0001	4.66 (3.51–6.20)
0301/0302	36 (5.5)	20 (2.6)	0.0084	2.16 (1.20–3.92)
0201/0301	33 (5.1)	23 (3.0)	NS	1.71 (0.96–3.04)
0201/x	83 (12.8)	84 (11.1)	NS	1.17 (0.84–1.64)
0302/0602–0603	30 (4.6)	40 (5.3)	NS	0.87 (0.52–1.47)
x/x	34 (5.2)	124 (16.4)	<0.0001	0.28 (0.19–0.43)
0301/x	17 (2.6)	76 (10.1)	<0.0001	0.24 (0.14–0.42)
0201/0602–0603	6 (0.9)	50 (6.6)	<0.0001	0.13 (0.05–0.32)
0301/0602–0603	4 (0.6)	42 (5.6)	<0.0001	0.11 (0.04–0.31)
0602–0603/x	4 (0.6)	189 (25.0)	<0.0001	0.02 (0.01–0.05)
Total	649	756		

Data are *n* (%).

Table 3—*Defined HLA-DQB1 genotypes in IDDM patients (DiMe index cases) and unaffected siblings*

DQB1* genotype	IDDM patients	Unaffected siblings	P	RR (95% CI)
0201/0302	159 (24.5)	51 (8.4)	<0.0001	3.53 (2.49–5.02)
0302/x	243 (37.4)	135 (22.2)	<0.0001	2.09 (1.62–2.68)
0301/0302	36 (5.5)	34 (5.6)	NS	0.99 (0.59–1.64)
0201/0301	33 (5.1)	24 (4.0)	NS	1.30 (0.74–2.30)
0201/x	84 (12.9)	77 (12.7)	NS	1.01 (0.71–1.42)
0302/0602–0603	30 (4.6)	41 (6.8)	NS	0.67 (0.40–1.11)
x/x	34 (5.2)	79 (13.0)	<0.0001	0.37 (0.24–0.57)
0301/x	18 (2.8)	45 (7.4)	<0.0003	0.36 (0.20–0.64)
0201/0602–0603	6 (0.9)	28 (4.6)	0.0001	0.19 (0.07–0.49)
0301/0602–0603	4 (0.6)	20 (3.3)	0.0011	0.18 (0.07–0.57)
0602–0603/x	5 (0.8)	72 (11.9)	<0.0001	0.06 (0.02–0.15)
Totals	649	607		

Data are *n* (%).

*0603/x, DQB1*0301/*0602-*0603, DQB1*0201/*0602–*0603, DQB1*0301/x, and DQB1x/x), as well as those with equivalent or low risk (DQB1*0301/*0302, DQB1*0201/*0301, DQB1*0201/x, and DQB1*0302/*0602–*0603). The genotypes DQB1*0302/x and DQB1*0201/*0302 were included in the categories with moderate and high risk. A comparison of IDDM patients and control subjects is shown in Table 4. This classification was used in further analyses.

The 65 index cases who presented with IDDM despite HLA-DQB1 genotypes conferring a decreased risk were characterized by an increased frequency of infections (26.2 vs. 14.8% in the other cases; $P = 0.034$) and ketoacidosis at diagnosis (34.5 vs. 20.2%; $P = 0.02$), while there was no significant difference in age (8.0 ± 4.0 vs. 8.5 ± 3.8 years) between the two groups. In addition, the former group had higher blood glucose concentrations (median 22.4 vs. 20.0 mmol/l; $P = 0.05$) and GHb levels (14.7 ± 7.0 vs. 12.7 ± 5.9%; $P = 0.03$) at diagnosis. The serum C-peptide concentrations were similar in both groups at clinical presentation (median 0.17 vs. 0.16 nmol/l).

A high genetic risk was associated with an early age at diagnosis. A total of 51 of 147 (34.7%) index cases that were diagnosed before 5 years of age had a DQB1*0201/*0302 genotype, compared with 108 of 502 (21.5%) cases that were diagnosed later in life ($P = 0.0016$). Siblings of DiMe index cases who had been diagnosed with IDDM before or after their index cases had genotypes associated with higher risk more often than did index cases from single-case families (Table 5). The frequency of the high-risk genotype was 37.5% in these siblings compared to 24.0% in the simplex cases ($P = 0.036$). The genotype frequencies in index cases of multiplex families, however, did not differ significantly from either simplex cases or diabetic siblings.

The genotype effect in second cases in multiplex families, as well as the effect of the genotype of the proband in the family on the risk in initially healthy children, is shown in Table 6. A total of 56 second cases were recorded among 606 genotyped children. A graded genotype-dependent risk was clearly seen in second cases: 29.4% of children with the high risk genotype became diabetic, compared with only 1.3% of those with a decreased risk ($P < 0.0001$). A similar gradient was seen within each family group according to the genotype of the first IDDM case. Second affected cases were more common in families in which the proband had either a high- or moderate-risk genotype (together 45/374, 12.0%) than in those in which the proband had a low- or decreased-risk genotype (11/219, 5.0%; $P = 0.0076$).

CONCLUSIONS — Our results demonstrate that the genotyping of only four alleles of the HLA-DQB1 locus can be used in the efficient grading of disease risk and the screening for susceptibility to IDDM in the Finnish population. The observations from the second affected cases confirm the applicability of the risk gradation in families with more than one diabetic child. Siblings of probands who did not have high- or moderate-risk genotypes also had a lower risk of developing IDDM, consistent with the lower frequency of DQB1 susceptibility genes in these families. If DQ genes were only in linkage disequilibrium with strong susceptibility genes, one would expect to see a similar frequency of secondary IDDM cases in all families. The development of IDDM in cases with low-risk genotypes might have accumulated the effects of minor susceptibility genes, rare environmental factors, or random elements that are associated with the development of immune system (8).

The significance of some key amino acids in the HLA-DQ heterodimer, such as aspartic acid at position 57 (Asp57) in the β-chain and arginine at position 52 in the α-chain (Arg52), has been strongly emphasized (9–11). The contribution of these specific amino acid positions, however, may be due to the fact that risk-associated heterodimers (DQA1*0301–DQB1*0302 and DQA1*0501–DQB1*0201) are positive for Arg52 and negative for Asp57, whereas the protective DQA1*0102–DQB1*0602 molecule is Arg52 negative and Asp57 positive (1).

In practice, the definition of key amino acids can be laborious, since different alleles can code for specific residues

Table 4—*The various risk genotypes found among IDDM index cases and control subjects*

DQB1* genotype	IDDM patients	Control subjects	RR*	RR† (95% CI)
High risk	159 (24.5)	22 (2.9)	10.8	53.5 (31.1–92.8)
Moderate risk	243 (37.4)	86 (11.4)	4.66	20.9 (14.4–30.4)
Low risk	182 (28.0)	167 (22.1)	1.37	8.1 (5.7–11.4)
Decreased risk	65 (10.0)	481 (63.6)	0.064	1.0
Totals	649	756		

Data are *n* (%). *RR calculated against all others. †RR calculated against those with a decreased risk.

Table 5—*HLA-DQB1 risk genotypes in index cases of simplex and multiplex families and in siblings with IDDM who were diagnosed before or after the index case*

DQB1* genotype	Single case probands	Sibpair probands	IDDM siblings
High risk	143 (24.0)	16 (31.3)	21 (37.5)*
Moderate risk	223 (37.1)	20 (41.7)	22 (39.3)
Low risk	173 (28.8)	9 (18.8)	10 (18.2)
Decreased risk	61 (10.1)	4 (8.3)	3 (5.5)
Totals	600	49	56

Data are *n* (%). *$P < 0.036$ compared to single case probands.

and since there are several alternatives for a non-Asp residue. Non-Asp57 homozygosity is a rather sensitive marker for IDDM susceptibility: 73% of white patients in the 11th international HLA workshop were homozygous, but 26% of the control subjects also had this genotype, demonstrating the poor specificity of this marker (12). Similar data have also been presented in smaller series in which 18–47% of the control subjects were homozygous for non-Asp57, having a sensitivity of 61–89% (5,13–21).

In the 11th HLA workshop series, the combination of subjects with genotypes that are able to code for four or two possible susceptibility molecules would detect 82% of patients but also 27% of control subjects (12). The corresponding numbers in a large Belgian study were 84 and 27% (18). The specificity of the assay increases if a distinction is made between the genotypes encoding two susceptibility heterodimers, those with SS,SS,SP,SP and SS,SS,PS,PS genotypes. The first letter in each molecule indicates the risk conferred by an α-chain and the latter β-chain mediated risk (S, susceptibility; P, protective). The β-chain seems to be more important in which SS,SS,PS,PS genotypes were increased, but SS,SS,SP,SP genotypes usually have similar frequencies in diabetic and control subjects (10,12,18). This differentiation procedure reduces the number of healthy control subjects with risk genotypes to 16–18% but still identifies 69–70% of the patients (12,18). These figures are in fact close to those obtained by our single-locus typing procedure, where 14% of control subjects had high or moderate IDDM risk and where those two risk groups covered 62% of the patients studied.

The linkage disequilibrium between α- and β-chain alleles, especially in a homogeneous population, is very strong, and additional information obtained by typing for the α-chain appears to be of limited significance. The most obvious example of valuable information is the distinction between DR3 and DR7 haplotypes with a similar DQB1*0201 β-chain but different α-chains with either DQA1*0501 ($Arg52^+$) DQA1*0201 ($Arg52^-$). To some extent, this would increase the sensitivity of our assay, but at the cost of an additional PCR reaction, which is not a trivial cost in large screening programs. The genotypes with DQB1*0201 alone without DQB1*0302 were relatively scarce among our IDDM patients, and anyway, those with the DQB1*0201/*0302 genotype would belong to the moderate risk group if the DQB1*0201 haplotype represented DQA1*0201–DQB1*0201 instead of DQA1*0501–DQB1*0201.

The proportion of DQB1*0201/*0302 heterozygotes (24.5%) was lower than expected from our previous study (5) and from several other studies in which the percentage has varied from 39 to 45% (16,18–20,22,23), but it is in accordance with the low number of DR3/DR4 heterozygotes reported earlier from the DiMe study (24) and is similar to the frequencies reported by Baisch et al. (14) and Gutierrez-Lopez et al. (17). These differences might reflect a sampling effect because the study population was recruited within a relatively short period of time, introducing bias from environmental accelerators and/or inhibitors of disease (e.g., infections) (25,26). Subjects

Table 6—*The risk of IDDM in siblings according to the genotype of the proband*

DQB1* genotype proband	Sibling	Progression to IDDM
High risk	High risk	16/48 (33.3)*
	Moderate risk	4/32 (12.5)
	Low risk	1/36 (2.8)
	Decreased risk	0/42 (0)
	Total	21/158 (13.3)†
Moderate risk	High risk	2/11 (18.2)
	Moderate risk	17/99 (17.2)‡
	Low risk	4/45 (8.9)
	Decreased risk	1/63 (1.6)
	Total	24/218 (11.0)§
Low risk	High risk	1/7 (14.3)
	Moderate risk	1/10 (10.0)
	Low risk	5/80 (6.3)
	Decreased risk	0/60 (0)
	Total	7/157 (4.9)
Decreased risk	High risk	1/2 (50.0)
	Moderate risk	0/4 (0)
	Low risk	1/6 (16.7)
	Decreased risk	2/61 (3.3)
	Total	4/73 (5.5)
Total	High risk	20/68 (29.4)‖
	Moderate risk	22/145 (15.2)¶
	Low risk	11/167 (6.6)#
	Decreased risk	3/226 (1.3)
	Total	56/606 (9.2)

Data are *n* (%). *$P = 0.0001$ vs. decreased risk; $P = 0.0015$ vs. low risk. †$P = 0.011$ vs. low risk total; $P = 0.012$ vs. decreased risk total. ‡$P = 0.0048$ vs. decreased risk. §$P = 0.05$ vs. low risk total. ‖$P = 0.0001$ vs. decreased risk; $P = 0.0001$ vs. low risk; $P = 0.043$ vs. moderate risk. ¶$P = 0.0001$ vs. decreased risk; $P = 0.023$ vs. low risk. #$P = 0.012$ vs. decreased risk.

with different genotypes might differ in their susceptibility to disease-modifying agents like enteroviruses or mumps (27, 28). The higher proportion of DQB1*0201/x genotype in boys, who are known to be more susceptible to complications associated with enterovirus infections (29), is in accord with this hypothesis as well as the tendency toward a seasonal pattern of new patients with this genotype. Lower numbers of IDDM diagnoses during summer months have also been detected in HLA-DR3–positive subjects in earlier studies (30–32). A recent virological study of the DiMe series emphasized the importance of enterovirus infections (33). However, these infections were found increased not only during the time period preceding clinical diagnosis but also years before, such that no clear-cut seasonal or yearly associations can be expected.

We confirmed our earlier finding of a higher proportion of children with high-risk genotype among those with IDDM who were diagnosed at an early age (32). The increased percentage of patients with DR3/4 genotype among children diagnosed at <5 years of age did not reach statistical significance ($0.05 < P < 0.1$) in an earlier report based on the DiMe series (24). Thus, our present data demonstrate the superiority of the DQB1-based method in this respect also. The increased number of high-risk genotypes in younger-onset patients has also been detected in several other studies including young adults (34–36).

Our procedure using four sequence-specific oligonucleotide probes does not define only four alleles but combines others, such as DQB1*0602 and DQB1*0603. Both of these alleles are strongly protective, and a distinction would not be of added value. DQB1*0304 is a recently described allele that in our typing scheme gives a positive reaction for both DQB1*0301 and DQB1*0302. Its frequency is low in whites of northern European descent (37). The few samples excluded from our study that show positivity for three alleles might still represent this allele.

Our observation of more severe metabolic decompensation at diagnosis in a small minority of children with the protective HLA-DQB1 genotypes may be explained by their increased frequency of acute infections at clinical presentation. However, whether this feature is related to the DQB1 genotype remains an issue for future studies.

Genetic typing provides a means to identify susceptible individuals for studies of diabetes prediction and prevention, and it aids in the estimation of individual risk, especially when used in association with immunological risk markers. It is reasonable for a screening system to be most effective in predicting the onset of disease in the young so that this group can take advantage of preventive treatments as the results of risk-prediction studies rapidly accumulate. Our oligonucleotide choice is attractive in populations of northern European descent, but would be much less ideal in other populations, e.g., southern Europe (38). Thus, each screening protocol must be carefully tested for the population under study. The described procedure may also be used as the first step in the MHC typing of large series that focuses on specific gene combinations. Our increasing knowledge of MHC makes a "full-house" typing strategy a less and less practical strategy. The ultimate value of effective and affordable strategies for mass screening of diabetes risk markers will be determined by the development of effective disease prevention and intervention strategies.

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