HLA-DQ beta-chain restriction fragment length polymorphism as a risk marker in Type 1 (insulin-dependent) diabetes mellitus: a Finnish family study

H. Reijonen^{1,3}, J. Ilonen^{1,3}, M. Knip², B. Michelsen⁵ and H. K. Åkerblom⁴

Departments of ¹ Medical Microbiology and ² Paediatrics, University of Oulu, ³ National Public Health Institute, Oulu,

⁴ The Children's Hospital, II Department of Paediatrics, University of Helsinki, Finland, and ⁵ Hagedorn Research Laboratory, Gentofte, Denmark

Summary. Finnish Type 1 (insulin-dependent) diabetic families were analysed for HLA-DQ beta-chain polymorphism using a short intron-specific probe. A simple hybridization pattern was obtained in which all fragments were associated significantly with Type 1 diabetes. The simultaneous presence of two different risk markers, the allelic 12-kilobase and 4-kilobase fragments were strongly associated with Type 1 diabetes since 50% of the patients had this combination compared with only 2% of the control subjects. The co-segregated 7.5/3.0 kilobase fragments, which were associated with HLA-DR2 and DRw6 were not detected among the

diabetic patients but were present in 48% of the control subjects. Our results provide further support for the location of susceptibility determining factors in the HLA-DQ gene area. The clear-cut, simple restriction fragment length polymorphism pattern obtained here, which bears a resemblance to a two allelic system, therefore makes this method applicable for estimating the risk of Type 1 diabetes at the population level.

Key words: Type 1 (insulin-dependent) diabetes, restriction fragment length polymorphism, HLA-DQ, prediction of Type 1 diabetes, genetics.

There is a strong relationship between HLA and Type 1 (insulin-dependent) diabetes mellitus, in that individuals having the HLA-DR3 and/or 4 antigen type run an increased risk of developing the disease. Determination of the Type 1 diabetes risk was previously based on serological and/or cellular HLA typing, but recent major breakthroughs in molecular biology have provided new tools for evaluation at the genomic level. Restriction fragment length polymorphism (RFLP) analysis in the HLA gene area has revealed several diabetes-associated polymorphisms [1, 2], suggesting that the susceptibility genes are located in the HLA-DQ region rather than the DR region. More recently it has been suggested that the amino acid at position 57 in the HLA-DQ beta-chain may play a critical role in determining predisposition to Type 1 diabetes. The presence of aspartic acid (Asp) in this position seems to provide protection against the development of the disease [3]. There are also results suggesting that Type 1 diabetes may be associated with an extended susceptibility haplotype [4, 5] or with a combination of DR/DQ alleles [6, 7] rather than with an isolated allele.

The RFLP method was used here in order to detect polymorphisms implicated as genetic elements predisposing subjects to Type 1 diabetes. Use of an HLA-DQ beta-chain gene intron-specific probe revealed a hybridization pattern simple enough to be employed in screening sur-

veys to identify subjects at risk of developing Type 1 diabetes.

Our results support the role of the BamHI-12-kilobase and 4-kb fragments as susceptibility conferring markers for the disease [1, 8] and show a striking difference between the diabetic patients and the control subjects in the frequency of 7.5/3.0-kb fragments reflecting a resistance to Type 1 diabetes. Furthermore, the trans-allelic genotypes indicate that the highest risk based on these markers is given by combination of two different RFLP risk alleles, 12/4 kb heterozygosity.

Subjects and methods

Subjects

A total of 51 diabetic families with one or more children with Type 1 diabetes diagnosed under the age of 16 were studied, including five with two and two with three diabetic children. Forty-two of the families had been included in a previous report [5]. Ten to 40 ml EDTA-blood samples were collected from the diabetic children and accompanying family members at routine visits to the diabetes clinic at the Department of Paediatrics, University of Oulu. A total of 34 families of our laboratory or hospital staff members were used as control subjects. Only unrelated members (parents) were considered as individual control subjects. None of them had a first-de-

gree relative with Type 1 diabetes. All the diabetic and control families were living in the Oulu area in Northern Finland. In addition, 50 unrelated blood donors with unknown HLA types were taken as control subjects. The study was approved by the Ethical Committee of the Medical Faculty, University of Oulu and informed consent was obtained from the blood donors.

HLA-DR typing

Mononuclear cells were isolated from heparinized peripheral blood by Lymphoprep (Nyegard and Co A/S, Oslo, Norway) gradient centrifugation and B-lymphocytes were further enriched by depletion of T-cells with 2-aminoethylisothiuronium bromide (AET)-treated sheep erythrocytes and monocytes with plastic adherence. HLA-DR antigens were determined from B-lymphocytes using the standard two-stage microlymphocytotoxicity method and commercial tissue typing trays (Biotest AG, Frankfurt am Main, FRG).

The probe

The HLA-DQ specific intervening sequence 1 (IVS I) probe was constructed from a BamHI-3.7-kb fragment corresponding to the 154 basepair sequence of the first intron and the 12 basepairs of the second exon of the HLA-DQ beta-chain gene [8].

RFLP analysis

The leucocytes were collected from EDTA-blood samples by erythrocyte-haemolysis (155 mmol/l NH_4Cl , 10 mmol/l KHCO_3 , 0.1 mmol/l EDTA, pH 7.4). The cell pellet of leucocytes and erythrocyte ghosts was suspended in TE buffer (10 mmol/l TrisHCl, 1 mmol/l EDTA) and stored at -80°C . The cells were lysed at 37°C overnight (2 mmol/l Tris, 0.4 mmol/l EDTA, 2 mmol/l NaCl, 1% sodium dodecyl sulphate (SDS), 0.4% proteinase K) followed by phenol and chloroform extractions. DNA was precipitated from the aqueous phase with ethanol. The air-dried pellet was dissolved in an appropriate volume of TE buffer. BamHI was used to digest 10 μg of DNA according to the manufacturer's specifications (Boehringer Mannheim, FRG) and electrophoresed in 0.6% agarose gel at 40 V overnight. After an alkaline denaturation step the DNA was transferred to nylon filters (Hybond-N, Amersham, Bucks, UK) by the method of Southern [9], dried and UV-fixed for 3 min.

The purified IVS I probe was labelled with (α - ^{32}P)dCTP by nick translation to a specific activity of $3\text{--}5 \times 10^8$ cpm/ μg [10, 11]. Hybridizations were performed at 42°C overnight in the presence of 50% formamide and 10% dextran sulphate. After subsequent washings, 2×10 min, at room temperature in $2 \times$ standard sodium citrate (SSC), 0.1% SDS and 3×20 min at 55°C in $0.1 \times$ SSC, 0.1% SDS, the filters were exposed to an X-ray film for 3 days at -70°C with intensifying screens.

Statistical analysis

The results were evaluated statistically using the chi square test. The degree of linkage disequilibrium was estimated by calculating the delta (D) values [12]. Relative risks were calculated using the formula: $a/b \times d/c$ in which a and c are the numbers of marker positive patients and control subjects, and b and d are the numbers of marker negative patients and control subjects.

Results

Type 1 diabetes-associated restriction fragments

A very clear-cut RFLP pattern was seen. DQ-beta alleles were represented by single specific restriction fragments except the one characterized by 7.5-kb and 3-kb fragments which always segregated together. The 10-kb fragment, present in all the individuals, represents the non-polymorphic DX-beta allele. The frequencies of individuals carrying the various allelic HLA-DQ beta restriction fragments are given in Table 1. The distribution of all the major hybridization bands was significantly different between the two groups. The 12-kb fragment was the most strongly associated with Type 1 diabetes (92% vs 49%). Another risk marker was the 4-kb fragment (57% vs 25%). The other fragments were significantly less frequent among the diabetic patients. The most striking difference was found in the case of the co-segregated fragments 7.5/3.0 kb, which were completely absent among the diabetic patients whereas their frequency in the control subjects was 48%, suggesting a protective effect against Type 1 diabetes.

Table 1. Frequencies of individuals carrying BamHI restriction fragments detected after hybridization with the HLA-DQ-beta specific IVS I (intervening sequence 1) probe

	n	Fragment size (kilobase)					others
		12.0	7.5/3.0	4.0	3.7	3.2	
Diabetic patients	60	55 (92%)	0 (0%)	34 (57%)	2 (3%)	11 (18%)	4 (7%)
Control subjects ^a	118	58 (49%)	57 (48%)	30 (25%)	18 (15%)	40 (34%)	5 (4%)
Relative risk		11.4	0	3.8	0.19	0.44	
p-value <		1×10^{-6}	1×10^{-6}	8×10^{-5}	3×10^{-2}	0.048	NS

^a unrelated healthy members of control families and non-HLA-typed blood donors

Table 2. The frequencies of individuals with specific combinations of HLA-DQ beta restriction fragments

	n	12.4	12/12	4/4	12/x	4/x	x/x
Diabetic patients	60	30 (50%)	12 (20%)	2 (3%)	13 (22%)	2 (3%)	1 (2%)
Control subjects ^a	118	2 (2%)	8 (7%)	5 (4%)	45 (38%)	22 (19%)	35 (30%)
Relative risk		38.3	3.4	0.78	0.45	0.15	0.04
p-value <		1×10^{-6}	1.7×10^{-2}	NS	0.04	9.5×10^{-3}	2.7×10^{-5}

^a unrelated healthy members of control families and non-HLA-typed blood donors

x = any fragment other than 12 kb or 4 kb

Table 3. HLA-DQ protective (prot) markers in Type 1 (insulin-dependent) diabetic and control genotypes

	<i>n</i>	non-prot/non-prot	prot/non-prot	prot/prot
Diabetic patients	60	44 (73%)	15 (25%)	1 (2%)
Control subjects ^a	118	16 (13.5%)	67 (56.5%)	35 (30%)

Relative risk associated with non-prot homozygosity = 17.5
($p < 1 \times 10^{-6}$)

^a Unrelated healthy members of control families and non-HLA-typed blood donors

Analysis of the simultaneous presence of restriction fragments in the diabetic patients by comparison with the control subjects (Table 2) shows that the relative risk attached to 12/4-kb heterozygosity exceeds by far the risk for genotypes with only the 12-kb or 4-kb fragment. The frequency of patients with only the 12-kb fragment was

also increased among the diabetic patients, whereas apparent 4-kb homozygosity was similar among the diabetic patients and control subjects. Accordingly, the 4-kb fragment seems only to potentiate the effect of the 12-kb fragment in conferring susceptibility to Type 1 diabetes. The distribution of genotypes of reduced frequency, including 7.5/3.0 kb, 3.7 kb and 3.2 kb (Table 3) as protective markers, confirms the efficiency of the protection. Only one combination of two protective restriction fragments was observed among the diabetic patients.

The study of families allowed the analysis of haplotypic segregation and combinations. The frequencies of the various restriction fragments in the diabetic and control haplotypes are indicated in Table 4. All the haplotypes found in the patients were designated as diabetic haplotypes, while the control haplotypes are taken as being both those of the control families ($n = 81$) and those found only in non-affected members in the diabetic families ($n = 56$). There was no difference between the

Table 4. Frequencies of restriction fragments in Type 1 (insulin-dependent) diabetic and non-diabetic haplotypes

	<i>n</i>	Fragment size (kilobase)					
		12.0	7.5/3.0	4.0	3.7	3.2	Others
Diabetic haplotypes	92	52 (57%)	0 (0%)	24 (26%)	2 (2%)	10 (11%)	4 (4%)
Control ^a haplotypes	137	32 (23%)	45 (33%)	19 (14%)	12 (9%)	23 (17%)	5 (4%)
<i>p</i> value <		1×10^{-6}	1×10^{-6}	3.2×10^{-2}	5.5×10^{-2}	0.23	NS

^a Haplotypes of control families and non-diabetic haplotypes of diabetic families

Table 5a, b. HLA-DQ genotypes associated with HLA-DR antigens. The delta value shows the difference between the observed and expected haplotype frequencies

a. Type 1 (insulin-dependent) diabetic patients

Alleles			Haplotypes (DQB RFLP/DR)				Delta value
DQB RFLP	DR	Expected DQ	+ / +	+ / -	- / +	- / -	
12 kb	DR4	DQw8	41	11	1	39	0.19 ^c
12 kb	DRw8	DQw4	6	46	1	39	0.022
12 kb	DR9	DQw9	4	48	0	40	0.021
4 kb	DR3	DQw2	22	1	1	68	0.16 ^c
4 kb	DR7	DQw2	1	22	1	68	0.006
7.5/3 kb	DR2	DQw6	0	0	1	91	0
7.5/3 kb	DRw6	DQw6	0	0	2	90	0
3.2 kb	DR1	DQw5	8	2	3	79	0.07 ^c
3.7 kb	DR5	DQw7	0	2	0	90	0
3.7 kb	DR4	DQw7	0	2	40	50	0
3.7 kb	DRw8	DQw7	1	1	6	84	0.009 ^a

b. Control subjects^a

Alleles			Haplotypes (DQB RFLP/DR)				Delta value
DQB RFLP	DR	Expected DQ	+ / +	+ / -	- / +	- / -	
12 kb	DR4	DQw8	14	16	1	101	0.09 ^c
12 kb	DRw8	DQw4	6	24	2	100	0.04 ^b
12 kb	DR9	DQw9	5	25	0	102	0.04 ^c
4 kb	DR3	DQw2	7	8	1	116	0.05 ^c
4 kb	DR7	DQw2	4	11	4	113	0.02 ^b
7.5/3 kb	DR2	DQw6	22	22	5	83	0.1 ^c
7.5/3 kb	DRw6	DQw6	14	30	3	85	0.07 ^c
3.2 kb	DR1	DQw5	17	5	2	108	0.11 ^c
3.7 kb	DR5	DQw7	7	6	1	118	0.04 ^c
3.7 kb	DR4	DQw7	1	12	14	105	0.003
3.7 kb	DRw8	DQw7	1	2	7	112	0.0002 ^a

Significances are marked as follows ^a $p < 0.05$; ^b $p < 0.01$; ^c $p < 0.001$; ^a haplotypes of control families and non-diabetic haplotypes of diabetic families

two subgroups of control haplotypes in the frequency of RFLP fragments. The 12-kb and 4-kb fragments comprised 83% of the diabetic haplotypes, while the fragments with reduced frequency were 7.5/3.0 kb (0%), 3.7 kb (2%) and 3.2 kb (11%). The few exceptional hybridization patterns found in the diabetic patients (designated as others in Table 4) need further characterisation.

HLA-DQ RFLP genotypes in association with HLA-DR-antigens

The RFLP bands in the family material were connected with segregating HLA-DR haplotypes (Table 5). Delta values for linkage disequilibrium were calculated for combinations of various allelic fragments with DR antigens. Highly significant associations were found for the major RFLP alleles. In 79% of the 12-kb positive diabetic patients the haplotype was positive for DR4, the remaining 12-kb positive patients being DRw8 or DR9. In the control subjects the relationship between the 12-kb fragment and the DR-antigen types was much more heterogeneous, and only 47% of the 12-kb positive control subjects were positive for DR4. The 4-kb fragment was associated with HLA-DR3 in 96% of the diabetic haplotypes and in 47% of the control haplotypes.

Among the protective markers, the combination of 7.5/3.0-kb fragments was associated mostly with DR2, but sometimes also with DRw6. The 3.2-kb fragment was related preferentially to DR1, with a few exceptions, e.g. the only DR2 positive diabetic haplotype in this material was represented by the 3.2-kb fragment (data not shown).

All except one of the 42 DR4 positive diabetic haplotypes were associated with the 12-kb fragment, and 93% of the DR4 control haplotypes were also 12-kb positive, only one out of 15 being represented by the 3.7-kb fragment.

Discussion

Several reports have revealed differences in corresponding HLA-DQ polymorphic fragments between Type 1 diabetic patients and healthy control subjects [1, 2, 13–16]. Owerbach et al. were the first to report an association between the DR4 related BamHI 12-kb fragment and Type 1 diabetes [1], while the allelic 3.7-kb fragment was found to be rare among diabetic subjects. These two fragments correlate with the presence of the DQw8 and DQw7 alleles. This split between DR4 positive diabetic and control haplotypes has been confirmed in several other reports among them that of Michelsen and Lernmark, who used the short, intron-specific IVS I probe [8]. This was constructed from a cloned 3.7-kb fragment and had the particular advantage of generating a simple hybridization pattern with only seven polymorphic bands and one or two hybridization signals per segregating haplotype.

The results of our DNA analyses of the BamHI-di-
gests performed with this IVS I probe were in agreement

with a previous report [8], supporting the role of the 12-kb and 4-kb fragments as risk markers for diabetes susceptibility. The remaining RFLP alleles (7.5/3.0, 3.7, 3.2 kb) were found to be inversely associated with diabetes, being of reduced frequency among diabetic patients. Compared with HLA-DR typing, which leaves a group of neutral antigen types without any association with Type 1 diabetes, the "IVS I-typing" serves better to delineate the frequency of risk markers in the background population.

The greatest risk is associated with the presence of both the 12-kb and the 4-kb fragment. The homozygosity for the 12-kb restriction fragment also contributed to an increased diabetes risk while the presence of two 4-kb fragments did not. This indicates the superior effect of the 12-kb fragment over the 4-kb fragment as a determinant of susceptibility. Dominance of DR4 over DR3 as a risk marker has also been seen in several surveys of subjects with Type 1 diabetes in Northern Europe [17, 18], but the situation may be different in other populations [19, 20]. It is also remarkable that none of the control subjects apparently homozygous for the 12-kb fragment was positive for DR4 (data not shown). They were all DRw8 positive, which is in positive linkage disequilibrium with DQw4 [21], also defined by the BamHI-12-kb restriction fragment [18, 22, 23], making DQw4 indistinguishable from DQw8 in this respect. Heterozygosity for HLA-DR4/DRw8 and more specifically the DR4, DQw8/DRw8, DQw4 combination has, on the other hand, been demonstrated to be associated with Type 1 diabetes [17, 18]. In addition to DQw8 and DQw4, the DR9-associated DQw9 is defined by the same hybridization pattern, the BamHI-12 kb fragment [23]. DR9 is a major antigen associated with Type 1 diabetes in oriental populations [24, 25]. Both DQw4 and DQw9 are Asp 57 positive [3, 26], and the finding of these antigens in diabetic patients is contradictory to the hypothesis of the primary protective role of this amino acid [3]. Although both the DRw8 and DR9 haplotypes were found among 12-kb fragment positive haplotypes in our series, DR4-positive ones were clearly over-represented among the diabetic haplotypes. Whether Asp 57 positivity of the DQ beta-chain creates differences in the strength of the 12-kb fragment as a Type 1 diabetes risk marker and whether diabetic patients possessing the 12-kb fragment are positive for Asp 57 at all despite the common DR/DQ associations are points that remain to be clarified by analysis with Asp 57-specific oligonucleotide probes.

Three major allelic fragments were clearly associated with protection against the disease, the differences between these alleles in the strength of the protection afforded are still conspicuous. The combination of the 7.5/3.0-kb fragments had the most striking effect, as it was totally absent among the diabetic patients in spite of the fact that 48% of the control subjects were positive. The 7.5/3.0 kb combination was associated with DR2 and DRw6, probably representing the Asp 57 positive DQw6 [27].

The frequency of DQw7 representing the 3.7-kb fragment is somewhat lower in Finnish control subjects posi-

tive for DR4 (7%) than in other Caucasian populations [28–31]. All the published series, including this one, are still very small, however. Our results, nevertheless, indicate that dissection of the DQ genes may reveal specific genetic susceptibility in this population which has the highest incidence of childhood diabetes in the world [32].

Only one of the diabetic patients had a double dose of protective markers, while the proportion of the control subjects negative for these protective markers was 13.5%. This frequency was similar to that reported by Morel et al. [33] for asp 57 positivity, which confers resistance to Type 1 diabetes. The frequency of control subjects at risk is far too high for the test to be used for screening disease susceptibility, however. In this respect the combination of two different susceptibility RFLP alleles seems more promising, since only 2% of the control subjects were positive. On the other hand, although the proportion of diabetic patients detected by this method decreased to 50%, this frequency is still higher than the 36% observed for DR3/4.

In summary, the RFLP method allows the detection of a considerable number of new markers of Type 1 diabetes in the HLA complex. A very simple hybridization pattern was obtained here with several restriction fragments significantly associated with the disease. The true genetic basis for these restriction fragments needs further characterization by sequence-specific oligonucleotide probing. A combination of the two techniques could point to suitable population screening methods.

Acknowledgements. We thank Prof. Å. Lernmark for his constructive criticism and support during the work, and we also wish to thank Ms. A. Jaakkola for her expert technical assistance and the diabetic and control families for their cooperation. This research was supported by grants from the Emil Aaltonen Foundation, the Foundation for Diabetes Research in Finland, the Alma och K. A. Snellman Foundation, Oulu, Finland, and the Association of Finnish Life Insurance Companies.

References

- Owerbach D, Lernmark Å, Platz P, Ryder LP, Rask L, Peterson PA, Ludvigsson J (1983) HLA-D region beta-chain DNA endonuclease fragments differ between HLA-DR identical healthy and insulin-dependent diabetic individuals. *Nature* 303: 815–817
- Cohen-Haguenaier O, Robbins E, Massart C, Busson M, Deschamps I, Hors J, Lalouel J-M, Dausset J, Cohen D (1985) A systematic study of HLA class II-beta DNA restriction fragments in insulin-dependent diabetes mellitus. *Proc Natl Acad Sci USA* 82: 3335–3339
- Todd JA, Bell JI, McDevitt HO (1987) HLA-DQ beta-gene contributes to susceptibility and resistance to insulin-dependent diabetes mellitus. *Nature* 329: 599–604
- Deschamps I, Marcelli-Barge A, Lallemand N, Poirier JC, Bochu V, Prevost P, Busson A, Masset M, Lestrade H, Hors J (1988) Study of cis and trans interactions between extended HLA-haplotypes in insulin-dependent diabetes. *Tissue Antigens* 31: 259–269
- Ilonen J, Surcel H-M, Partanen J, Koskimies S, Knip M, Käär M-L (1988) Extended HLA haplotypes in patients with insulin-dependent diabetes mellitus in Northern Finland. *Tissue Antigens* 32: 139–144
- Monos DS, Mickelson E, Hansen JA, Baker L, Zmijewski CM, Kamoun M (1988) Analysis of DR and DQ gene products of the DR4 haplotype in patients with IDDM: possible involvement of more than one locus. *Hum Immunol* 23: 289–299
- Sheehy MJ, Scharf SJ, Rowe JR, de Gimenez MH, Meske LM, 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
- Michelsen B, Lernmark Å (1987) Molecular cloning of a polymorphic DNA endonuclease fragment associates insulin-dependent diabetes mellitus with HLA-DQ. *J Clin Invest* 79: 1144–1152
- Southern EM (1975) Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J Mol Biol* 98: 503–517
- Maniatis T, Fritsch EF, Sambrook J (eds) (1982) *Molecular cloning*. Cold Spring Harbor, New York
- Ausubell F, Brent R, Kingston R, Moore D, Smith J, Seidman JG, Struhl K (eds) (1987) *Current protocols in molecular biology*. Greene Publ Assoc, New York
- Mattiaz PL, Ihde D, Piazza A, Ceppellini R, Bodmer WF (1970) New approaches to the population genetic and segregation analysis of the HLA system. In: Terasaki PI (ed) *Histocompatibility testing*. Munksgaard, Copenhagen, pp 193–205
- Böhme J, Carlson B, Wallin J, Möller E, Persson B, Peterson PA, Rask L (1986) Only one DQ beta restriction fragment pattern of each DR specificity is associated with insulin-dependent diabetes. *J Immunol* 137: 941–947
- Nepom BS, Palmer J, Kim SJ, Hansen JA, Holbeck SL, Nepom GT (1986) Specific genomic markers for the HLA-DQ subregion discriminate between DR4+ insulin-dependent diabetes mellitus and DR4+ seropositive juvenile rheumatoid arthritis. *J Exp Med* 164: 345–350
- Henson V, Maclaren N, Riley W, Wakeland EK (1987) Polymorphism of DQ beta genes in HLA-DR4 haplotypes from healthy and diabetic individuals. *Immunogenetics* 25: 152–160
- Carrier CM, Mollen N, Rothman WC, de Cordoba SR, Rey-Campos J, Ginsberg-Fellner F, Carpenter C, Rubinstein P (1989) Definition of IDDM-associated HLA DQ and DX RFLPs by segregation analysis of multiplex sibships. *Hum Immunol* 24: 51–63
- Svejgaard A, Jakobsen BK, Platz P, Ryder LP, Nerup J, Cristy M, Borch-Johnsen K, Parving H-H, Deckert T, Molstedt-Pedersen L, Kuhl C, Buschard K, Green A (1986) HLA associations in insulin-dependent diabetes: search for heterogeneity in different groups of patients from a homogeneous population. *Tissue Antigens* 28: 237–244
- Rönningen KJ, Markussen G, Iwe T, Thorsby E (1989) An increased risk of insulin-dependent diabetes mellitus (IDDM) among HLA-DR4, DQw8/DRw8, DQw4 heterozygotes. *Hum Immunol* 24: 165–173
- Vela M, Adorno D, Longo A, Papola F, Maccarone D, Centis D, Raponi MP, Candela A, Campea L, Orsini M, Ferrara GB (1987) HLA typing data on 100 type I (insulin-dependent) diabetic patients from central Italy show a negative association with DR5 rather than with DR2. *Clin Immunol Immunopathol* 45: 143–146
- Thivolet C, Beaufriere B, Betuel H, Gebuhrer L, Chatelain P, Durand A, Tourniaire J, Francois R (1988) Islet cell and insulin autoantibodies in subjects at high risk for development of Type 1 (insulin-dependent) diabetes mellitus: the Lyon family study. *Diabetologia* 31: 741–746
- Trucco M, Ball E (1989) RFLP analysis of DQ-beta chain gene workshop report In: Dupont B (ed) *Immunobiology of HLA*, Vol 1. Springer, New York, pp 860–867
- Bushell A, Wood KJ, Morris PJ (1988) DQ Wa gives rise to a DQ beta restriction fragment pattern indistinguishable from that of the DQw3.2 sub-type of DQw3. *Tissue Antigens* 32: 41–45
- Aparicio JMR, Wakisaka A, Takada A, Matsuura N, Aizawa M (1988) HLA-DQ system and insulin-dependent diabetes melli-

- tus in Japanese: does it contribute to the development of IDDM as it does in Caucasians? *Immunogenetics* 28: 240–246
24. Bertrams J, Baur MP (1984) Disease reports, insulin-dependent diabetes mellitus. In: Albert ED, Baur MP, Mayr WR (eds) *Histocompatibility testing 1984. Report on the Ninth International Histocompatibility Workshop and Conference*. Springer, Berlin Heidelberg New York, pp 348–358
 25. Kida K, Mimura G, Kobayashi T, Nakamura K, Sonoda S, Inouye H, Tsuji K (1989) Immunogenetic heterogeneity in Type 1 (insulin-dependent) diabetes among Japanese – HLA antigens and organ-specific autoantibodies. *Diabetologia* 32: 34–39
 26. Gregersen PK, Shen M, Song Q, Merryman P, Degar S, Seki T, Maccari J, Goldberg D, Murphy H, Schwenzer J, Wang CY, Winchester RJ, Nepom GT, Silver J (1986) Molecular diversity of HLA-DR4 haplotypes. *Proc Natl Acad Sci USA* 83: 2642–2646
 27. Horn GT, Bugawan TL, Long CM, Manos MM, Ehrlich HA (1988) Sequence analysis of HLA class II genes from insulin-dependent diabetic individuals. *Hum Immunol* 21: 249–263
 28. Schreuder GM, Tilanus MGJ, Bontrop RE, Bruining GJ, Giphart MJ, van Rood JJ, de Vries RRP (1986) HLA-DQ polymorphism associated with resistance to type I diabetes detected with monoclonal antibodies, isoelectric point differences, and restriction fragment length polymorphism. *J Exp Med* 164: 938–943
 29. Sansom DM, Bidwell JL, Maddison PJ, Campion G, Klouda PT, Bradley BA (1987) HLA DQ alpha and DQ beta restriction fragment length polymorphism associated with Felty's Syndrome and DR4-positive rheumatoid arthritis. *Hum Immunol* 19: 269–278
 30. Wallin J, Carlsson B, Ström H, Möller E (1988) A DR4-associated DR-DQ haplotype is significantly associated with rheumatoid arthritis. *Arthritis Rheum* 31: 72–79
 31. Bignon JD, Ferec C, Barrier J, Pennec Y, Verlingue C, Cheneau ML, Lucas V, Muller JY, Saleun JP (1988) HLA class II genes polymorphism in DR4 giant cell arteritis patients. *Tissue Antigens* 32: 254–258
 32. Åkerblom HK, Reunanen A (1985) The epidemiology of insulin-dependent diabetes mellitus (IDDM) in Finland and in Northern Finland. *Diabetes Care* 8 [Suppl 1]: 10–16
 33. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M (1988) Aspartic acid at position 57 of the HLA-DQ beta chain protects against type I diabetes: a family study. *Proc Natl Acad Sci USA* 85: 8111–8115

Received: 25 September 1989
and in revised form: 10 January 1990

Dr. H. Reijonen
University of Oulu
Department of Medical Microbiology
Kajaanintie 46 E
SF-90220 Oulu
Finland