HLA-DQB1*0201/0302 is associated with severe retinopathy in patients with IDDM

D. Agardh¹, L.K. Gaur², E. Agardh³, M. Landin-Olsson¹, C.-D. Agardh¹, Å. Lernmark⁴

¹ Diabetes and Endocrinology Unit, Department of Internal Medicine, University Hospital, Lund, Sweden

² Department of Immunogenetics, Puget Sound Blood Center and Program, Seattle, Washington, USA

³ Department of Ophthalmology, University Hospital, Lund, Sweden

⁴ Department of Medicine, R.H. Williams Laboratory, University of Washington, Department of Medicine, Seattle, Washington, USA

Summary Some insulin-dependent diabetic (IDDM) patients develop severe forms of retinopathy. Putative risk factors such as hypertension, poor metabolic control, nephropathy and growth hormone levels do not fully explain the progress of retinopathy in these patients. It has been discussed whether there is a genetic marker, since some diabetic patients without any known predisposing risk factors develop severe retinopathy and others do not. In the present study, HLA-DR and DQ were compared in two patient groups with IDDM. One group consisted of patients with early-onset diabetes, with severe non-proliferative or proliferative retinopathy; the other group had no or only mild signs of retinopathy. High resolution HLA typing was carried out by polymerase chain reaction (PCR) and hybridization with allele specific probes. Alleles on the DR3-DQ2 haplotype, DRB1*0301, DQA1*0501 and DQB1*0201, were

more frequent in patients with severe retinopathy. A difference was seen when combining certain alleles in the genotypes of DQA1*03/0501 ($p > 0.05$) and DQB1*0201/0302 ($p < 0.01$). The findings of the present study suggest that DQB1*0201/0302 is the strongest genetic marker for severe retinopathy and DRB1*0301/0401 only has a secondary influence when combined with this genotype. It seems as if IDDM patients who are positive for the genotype DR3-DQ2/DR4-DQ8 (DRB1*0301-DQA1*0501-DQB1*0201/DRB1*0401 -DQA1*03-DQB1*0302) are at greater risk of developing severe retinopathy. [Diabetologia (1996) 39: 1313–1317]

Keywords Severe retinopathy, insulin-dependent diabetes mellitus, HLA-DR, HLA-DQ, allele, haplotype, genotype.

Diabetic retinopathy is a common complication of diabetes mellitus. Patients with insulin-dependent diabetes mellitus (IDDM) start to develop signs of retinopathy after 5–10 years disease duration and after 15 years nearly 100 % have retinopathy of some kind [1]. Some patients in their twenties develop a particularly severe sight-threatening retinopathy [2, 3] which requires prompt laser treatment [4]. Early onset of diabetes

has been found to be associated with proliferative retinopathy [5] and some [6, 7] but not all studies [8] have suggested increased growth hormone and insulin-like growth factor 1 (IGF I) levels to accelerate the progress of severe retinopathy. Other putative risk factors such as poor metabolic control, hypertension and nephropathy have failed to fully explain the development of severe retinopathy. Furthermore, about 25 % of the patients develop severe retinopathy despite no sign of nephropathy [9]. Since no single predisposing risk factor has been identified, it has been suggested that the development of severe retinopathy is due to genetic factors, but different results have been obtained [10–13].

The HLA complex, located on the short arm of chromosome 6, is the strongest genetic marker for IDDM. HLA-DR4-DQ8 (DRB1*04-DQA1*0301-

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Corresponding author: M. Landin-Olsson MD, PhD, Diabetes and Endocrinology Unit, Department of Internal Medicine, University Hospital, S-211 85 Lund, Sweden

Abbreviations: IDDM, Insulin-dependent diabetes mellitus; NIDDM, non-insulin-dependent diabetes mellitus; HLA, human leucocyte antigen; PCR, polymerase chain reaction.

DQB1*0302) and DR3-DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) have been shown to be significantly associated with IDDM [14, 15]. In the present study we have used state-of-the-art high resolution HLA genotyping of DRB1, DQA1, and DQB1 alleles to compare different subtypes in order to evaluate the genetic influence on the development of severe diabetic retinopathy in IDDM. To that end, we compared one group with severe non-proliferative or proliferative retinopathy, with another group which despite long diabetes duration had no or only mild signs of retinopathy.

Subjects, materials and methods

Subjects. Fifty-six patients with IDDM were studied. Patient characteristics are shown in Table 1. In the severe retinopathy group, patient characteristics represent values at the diagnosis of severe retinopathy and in the control group at the time of sampling for HLA analysis. In the severe retinopathy group, HbA_{1c} levels 1 year before the diagnosis ($10.3 \pm 2.3\%$) did not differ from HbA_{1c} levels at diagnosis.

The patients in the retinopathy group had severe or very severe non-proliferative retinopathy ($n = 24$) according to the definitions by the Early Treatment Diabetic Retinopathy System (ETDRS) [4], i.e. at least one of the following criteria: 1) multiple haemorrhages in four quadrants, 2) venous beading, 3) intraretinal microvascular abnormalities. Out of these 24 patients, 22 developed proliferative retinopathy within 2 years despite panretinal photocoagulation, resulting in extended preretinal or vitreous haemorrhages in 10 patients. Another 4 patients had proliferative retinopathy when photocoagulation was instituted, in 2 the proliferative vessels caused vitreous haemorrhages. Thus, at the time of retinopathy diagnosis 24 of 28 patients had severe non-proliferative retinopathy and 4 had proliferative retinopathy. Despite panretinal photocoagulation, there was a progression in 22 of 24 with severe non-proliferative retinopathy and in 2 of 4 with proliferative retinopathy. Another group ($n = 28$) selected for comparison, consisted of patients who, despite a very long diabetes duration, had no or minimal background retinopathy, i.e. single microaneurysms and/or haemorrhages.

Analytical techniques. Venous blood samples were collected in tubes containing EDTA. The erythrocytes were lysated by ammonium-chloride and the leukocytes were washed with phosphate-buffered saline. The pellet was stored at -80°C . DNA was isolated from the leukocytes by incubation overnight with lysis buffer containing proteinase K (Promega, Laboratory and Research Products, Eastman Chemical Company, New Haven, Conn., USA). DNA was precipitated with a saturated sodium-chloride solution [16].

High resolution HLA typing was carried out by polymerase chain reaction (PCR) [17] and hybridization with allele specific probes. The DNA was mixed with specific amplification primers and the amplified DNA was electrophoresed in 3% agarose gels stained with ethidium bromide to control amplification before hybridization. Details have been described elsewhere [18]. Nylon membranes (Schleider and Schuell, Keene, N.H., USA) were prepared as described [18], prehybridized for 30 min and then hybridized for 1 h at 53°C . Stringency washing was performed at 55°C for 15 min and the DIG-labelled (digoxigenin-AP) PCR products were detected by alkaline phosphatase

Table 1. Characteristics of IDDM patients with (Ret. +) and without (Ret. -) severe retinopathy

Variable	Ret. + $n = 28$	Ret. - $n = 28$	<i>p</i> -value
Age (years)	24.9 ± 3.9	44.6 ± 12.8	< 0.0001
Onset of diabetes (years)	8.4 ± 4.6	14.5 ± 9.7	< 0.02
Diabetes duration (years)	17.0 ± 5.1	30.1 ± 9.1	< 0.001
Insulin dosage (IU/kg)	0.90 ± 0.34	0.57 ± 0.13	< 0.001
HbA _{1c} (%)	9.9 ± 2.2	8.0 ± 1.6	< 0.001
Urinary albumin (mg/l)	$63.5 (< 5-390)$	$9 (< 5-98)$	< 0.009
Serum creatinine ($\mu\text{mol/l}$)	68.8 ± 10.8	66.5 ± 13.5	NS
SBP (mm Hg)	124.0 ± 11.1	123.1 ± 14.1	NS
DBP (mm Hg)	80.0 ± 10.0	75.4 ± 7.5	NS
Antihypertensive treatment (<i>n</i>)	2	3	NS
BMI (kg/m^2)	23.2 ± 3.1	23.3 ± 3.1	NS

Values are given as means $\pm$ SD, except for urinary albumin which is given as median (range). NS, Not significant; SBP, systolic blood pressure; DBP, diastolic blood pressure

(Boehringer Mannheim, Mannheim, Germany). Hybridized DNA was visualized by 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium salt (Boehringer Mannheim).

Statistical methods

Frequencies were compared by the chi-square test and *p*-values less than 0.05 were considered significant. When comparing frequencies of HLA alleles, haplotypes or genotypes, the *p*-values were not corrected for the number of comparisons since only two different groups of patients were compared. Patient characteristics were compared by using Student's *t*-test (two-tailed) except for the urinary albumin concentration, where the Mann-Whitney U-test was used.

Results

Alleles. HLA-DR and -DQ alleles were compared between patients with and without severe retinopathy (Table 2). DRB1*0401 and *0301 occurred frequently in both groups. DQA1*03 and *0501 dominated comparing alleles of the DQA1 locus but no allele showed a significant difference. There was no significant difference when comparing DQB1*0201 (39 vs 27%; NS), though *0302 occurred more frequently in both groups.

Genotypes. HLA genotypes were compared between the two groups (Table 3). DRB1*0301/0401 was frequent in patients with than without severe retinopathy (43 vs 21%; NS). More patients without severe retinopathy were positive for *0401/0401 (18 vs 3.6%; $p < 0.05$). Of the DQA1

Table 2. HLA alleles in IDDM patients with (Ret. +) and without (Ret. -) severe retinopathy

Allele	Ret. + <i>n</i> = 56	Ret. - <i>n</i> = 56	Chi square	<i>p</i> -value
DRB1				
*0301	21 (38 %)	14 (25 %)	9.6	NS
*0401	20 (36 %)	23 (41 %)	3.4	NS
*0404	4 (7.1 %)	6 (11 %)	0.75	NS
*0101	3 (5.4 %)	3 (5.4 %)	0	NS
*0402	1 (1.8 %)	1 (1.8 %)	0	NS
*0701	1 (1.8 %)	1 (1.8 %)	0	NS
*0801	0	3 (5.4 %)	3.2	NS
*09012	0	1 (1.8 %)	1.0	NS
*1101	2 (3.6 %)	0	NA	NA
*1102	1 (1.8 %)	0	NA	NA
*1301	0	1 (1.8 %)	1.0	NS
*1302	2 (3.6 %)	3 (5.4 %)	0.35	NS
*14	1 (1.8 %)	0	NA	NA
DQA1				
*0501	24 (43 %)	14 (25 %)	9.5	NS
*03	25 (45 %)	31 (55 %)	2.6	NS
*0101	2 (3.6 %)	3 (5.4 %)	0.35	NS
*0102	4 (7.1 %)	3 (5.4 %)	0.35	NS
*0103	0	1 (1.8 %)	1.0	NS
*0201	1 (1.8 %)	1 (1.8 %)	0	NS
*0401	0	3 (5.4 %)	3.2	NS
DQB1				
*0302	25 (45 %)	29 (52 %)	1.5	NS
*0201	22 (39 %)	15 (27 %)	4.5	NS
*0301	3 (5.4 %)	1 (1.8 %)	4.1	NS
*0303	0	1 (1.8 %)	1.0	NS
*0402	0	3 (5.4 %)	3.17	NS
*0501	3 (5.4 %)	3 (5.4 %)	0	NS
*0603	0	1 (1.8 %)	1.0	NS
*0604	3 (5.4 %)	3 (5.4 %)	0	NS

NS, not significant; NA, not applicable

genotypes, *03/0501 dominated in patients with severe retinopathy (54 vs 29%; $p < 0.05$) and *03/03 occurred frequently in patients without severe retinopathy (29 vs 11%; NS). A large number of patients with severe retinopathy was positive for DQB1*0201/0302 (57 vs 25%; $p < 0.01$). Patients in both groups were also positive for *0302/0302, but the difference was not significant.

Haplotypes. DRB1-DQA1-DQB1 haplotypes of the two groups were compared (Table 4). More patients with than without severe retinopathy were positive for DR3-DQ2 (DRB1*0301-DQA1*0501-DQB1*0201) (38 vs 25%). A number of patients were also positive for DR4-DQ8 (DRB1*0401-DQA1*03-DQB1*0302), but the difference was not significant.

DRB1*04-DQB1 genotypes. DR4 subtypes in combination with different DQB1 genotypes were compared (Table 5). Since DQA1 is in linkage disequilibrium with DQB1 (Fig. 1a), the extended genotype was determined. DR4-DQ8/DR3-DQ2 (DRB1 *0401-DQA1*03-DQB1*0302/DRB1-*0301-

Table 3. HLA genotypes in IDDM patients with (Ret. +) and without (Ret. -) severe retinopathy

Genotype	Ret. + <i>n</i> = 28	Ret. - <i>n</i> = 28	Chi square	<i>p</i> -value
DRB1a/DRB1b				
*0301/0401	12 (43 %)	6 (21 %)	7.6	NS
*0401/0401	1 (3.6 %)	5 (18 %)	3.9	< 0.05
*0301/0101	2 (7.1 %)	1 (3.6 %)	1.1	NS
*0301/0301	2 (7.1 %)	2 (7.1 %)	0	NS
*0301/0402	1 (3.6 %)	1 (3.6 %)	0	NS
*0301/0404	1 (3.6 %)	0	NA	NA
*0301/09012	0	1 (3.6 %)	1.0	NS
*0301/1101	1 (3.6 %)	0	NA	NA
*0301/1301	0	1 (3.6 %)	1.0	NS
*0401/0101	1 (3.6 %)	2 (7.1 %)	0.54	NS
*0401/0404	1 (3.6 %)	1 (3.6 %)	0	NS
*0401/0701	1 (3.6 %)	0	NA	NA
*0401/0801	0	2 (7.1 %)	2.2	NS
*0401/1101	1 (3.6 %)	0	NA	NA
*0401/1302	2 (7.1 %)	2 (7.1 %)	0	NS
*0404/0404	1 (3.6 %)	2 (7.1 %)	0.54	NS
*0404/1302	0	1 (3.6 %)	1.0	NS
*0701/0801	0	1 (3.6 %)	1.0	NS
DQA1a/DQA1b				
*03/0501	15 (54 %)	8 (29 %)	8.6	$p = 0.05$
*03/03	3 (11 %)	8 (29 %)	4.4	NS
*0102/03	3 (11 %)	3 (11 %)	0	NS
*1102/14	1 (3.6 %)	0	NA	NA
*0101/03	0	2 (7.1 %)	2.2	NS
*0101/0501	2 (7.1 %)	1 (3.6 %)	1.1	NS
*0102/0501	1 (3.6 %)	0	NA	NA
*0103/0501	0	1 (3.6 %)	1.0	NS
*0201/03	1 (3.6 %)	0	NA	NA
*0201/0401	0	1 (3.6 %)	1.0	NS
*03/0401	0	2 (7.1 %)	2.2	NS
*0501/0501	3 (11 %)	2 (7.1 %)	0.54	NS
DQB1a/DQB1b				
*0201/0302	16 (57 %)	7 (25 %)	15	$p = 0.01$
*0302/0302	4 (14 %)	7 (25 %)	1.7	NS
*0302/0604	2 (7.1 %)	3 (11 %)	0.37	NS
*0201/0201	2 (7.1 %)	2 (7.1 %)	0	NS
*0201/0501	2 (7.1 %)	1 (3.6 %)	1.1	NS
*0201/0303	0	1 (3.6 %)	1.0	NS
*0201/0402	0	1 (3.6 %)	1.0	NS
*0201/0603	0	1 (3.6 %)	1.0	NS
*0301/0302	0	1 (3.6 %)	1.0	NS
*0301/0604	1 (3.6 %)	0	NA	NA
*0302/0402	0	2 (7.1 %)	2.2	NS
*0302/0501	1 (3.6 %)	2 (7.1 %)	0.54	NS

NS, not significant; NA, not applicable

DQA1 *0501-DQB1*0201) (Fig. 1b) occurred more frequently in patients with than without severe retinopathy (43 vs 21 %). The DR4-DQ8 homozygote (DRB1*0401-DQA1*03-DQB1*0302/DRB1 *0401-DQA1*03-DQB1*0302) was only seen in patients without severe retinopathy (14 vs 0 %).

Discussion

Retinopathy is a common complication to diabetes which strikes most IDDM patients [1]. Some IDDM

Table 4. HLA haplotypes in IDDM patients with (Ret. +) and without (Ret. -) severe retinopathy

Haplotype	Ret. + <i>n</i> = 56	Ret. - <i>n</i> = 56	Chi square	<i>p</i> -value
DRB1-DQA1-DQB1				
*0301-0501-0201	21 (38 %)	14 (25 %)	9.1	NS
*0401-03-0302	18 (32 %)	22 (39 %)	1.2	NS
*0404-03-0302	4 (7.1 %)	6 (11 %)	0.75	NS
*0101-0101-0501	2 (3.6 %)	3 (5.4 %)	0.35	NS
*0101-0102-0501	1 (1.8 %)	0	NA	NA
*0401-03-0301	2 (3.6 %)	1 (1.8 %)	1.1	NS
*0402-03-0302	1 (1.8 %)	1 (1.8 %)	0	NS
*0701-0201-0201	1 (1.8 %)	0	NA	NA
*0701-0401-0402	0	1 (1.8 %)	1.0	NS
*0801-0201-0402	0	1	1.0	NS
*0801-0401-0402	0	2 (3.6 %)	2.1	NS
*09012-03-0303	0	1 (1.8 %)	1.0	NS
*1101-0501-0302	2 (3.6 %)	0	NA	NA
*1102-0102-0604	1 (1.8 %)	0	NA	NA
*1301-0103-0603	0	1 (1.8 %)	1.0	NS
*1302-0102-0604	2 (3.6 %)	3 (5.4 %)	0.4	NS
*14-0501-0301	1 (1.8 %)	0	NA	NA

NS, not significant; NA, not applicable

Table 5. Analysis of DR4 subtypes in linkage with DQB1 genotypes in IDDM patients with (Ret. +) and without (Ret. -) severe retinopathy

Genotype	Ret. + <i>n</i> = 28	Ret. - <i>n</i> = 28	Chi square	<i>p</i> -value
DRB1a/b-DQB1a/b				
*0401/0301-0302/0201	12 (43 %)	6 (21 %)	7.6	NS
*0401/0401-0302/0302	0	4 (14 %)	4.7	NS
*0402/0301-0302/0201	1 (3.6 %)	1 (3.6 %)	0	NS
*0404/0301-0302/0201	1 (3.6 %)	0	NA	NA
*0404/0404-0302/0302	1 (3.6 %)	2 (7.1 %)	0.54	NS
*0401/0404-0302/0302	1 (3.6 %)	1 (3.6 %)	0	NS
*0401/0101-0302/0302	1 (3.6 %)	2 (7.1 %)	0.54	NS

NS, not significant; NA, not applicable

patients with early onset of diabetes develop severe retinopathy at a young age [2, 3]. No single predisposing risk factor has yet been identified. It is known that patients with IDDM are more prone to develop severe retinopathy than patients with non-insulin-dependent diabetes (NIDDM) [19], which could be due to several reasons one being an additive effect linked to HLA. A genetic marker could thus offer an explanation and the findings of the present study suggest that the most common risk genotype for IDDM may also be a risk factor for severe retinopathy.

To find out whether certain HLA types are associated with the development of severe retinopathy or not in IDDM patients, we have studied a group of patients who had developed severe retinopathy at young age. Another group was chosen from among IDDM patients with no or only mild signs of retinopathy. Patients in the two groups differed regarding age and age at onset of diabetes, which was earlier in the severe retinopathy group. However, matching patients by age and age at onset of diabetes

Fig. 1. **a** The genomic organization of an HLA haplotype where DQA1 and DQB1 are in linkage disequilibrium. **b** The expressed genes of the most common extended HLA genotype in the present study comprising of 28 IDDM patients with and 28 without severe retinopathy (43 vs 21 %; $p < 0.006$)

rather than duration would make it difficult to exclude that severe retinopathy would in fact develop among these patients. Because of this, patients with severe retinopathy were compared to patients who, despite a long diabetes duration, had no or only mild retinopathy.

However, other putative risk factors must also be considered. Poor metabolic control is associated with severe retinopathy and improved metabolic control retards both its development and progression [20]. However, improved metabolic control has also been demonstrated to aggravate retinopathy in younger patients [2]. In the present study, we could demonstrate that patients with severe retinopathy had higher HbA_{1c} levels compared to patients with no or only mild signs of retinopathy. This discrepancy in metabolic control is probably not the only explanation for the progression of severe retinopathy seen in these patients. Furthermore, the degree of metabolic control at diagnosis of severe retinopathy and 1 year earlier did not differ. Other known risk factors are high blood pressure and diabetes duration [21–23]. In the present study, there were no differences in blood pressure between the two groups and diabetes duration was longer in patients with no or only mild retinopathy.

In a previous study, DR4 has been described as a genetic risk factor for severe retinopathy [10]. In the present study, by using PCR and hybridization with allele specific probes, HLA-DR and DQ were subtyped and the precise HLA type determined. No significant difference in the frequency of DRB1*0401 or any other DR4 could be found between patients with and without severe retinopathy. A DR4 allele alone could therefore not be associated with severe retinopathy. Instead, *0301 was the only DRB1 allele to show a significant difference between the two groups. In addition, DQA1*0501 and DQB1*0201 were associated with severe retinopathy. There is also a positive association when adding these alleles into the DR3-DQ2 haplotype (DRB1*0301-DQA1*0501-DQB1*0201).

The risk is further increased for severe retinopathy when combining certain alleles in genotypes,

with the exception of DRB1*0401/0401 and DQA1*03/03 which were negatively associated with severe retinopathy. DQB1*0201/0302 was clearly associated with severe retinopathy and DQA1*03/0501 also showed a positive association. In addition, DRB1*0301/0401 indicated the same effect. Similar results have been shown in previous studies [10, 11], where there was an over representation of DR3/4 in patients with severe retinopathy, but the only difference seen was between DR3/4 and other DRX types (DR-non-3/non-4). Even though the studied population was larger than in the present study, the type of diabetes was not clearly defined. A number of NIDDM patients cannot be excluded, as the only criteria for IDDM except for insulin treatment was onset before 30 years. Since NIDDM is not associated with HLA, this could explain why a number of patients were positive for DRX.

We finally compared the extended genotypes of DRB1-DQA1-DQB1 between the two groups. There was an over representation of DR3-DQ2/DR4-DQ8 (DRB1*0301-DQA1*0501-DQB1*0201/DRB1*0401-DQA1*03-DQB1*0302) among patients with severe retinopathy. This HLA type is also associated with IDDM, indicating that the same alleles which show high risk for IDDM also confer a high risk for severe retinopathy. On the contrary, the DR4-DQ8 homozygote (DRB1*0401-DQA1*03-DQB1*0302/DRB1*0401-DQA1*03-DQB1*0302) was only seen in patients without retinopathy. This extended genotype indicates that it protects IDDM patients from severe retinopathy. In conclusion, in IDDM patients the risk for severe retinopathy increases when DRB1*0301, DQA1*0501, and DQB1*0201 occur in certain haplotypes and genotypes. DRB1*0401 alone does not potentiate a higher risk, but in the presence of DRB1*0301 and DQB1*0201/0302, the risk for severe retinopathy increases. It seems as if DQB1*0201/0302 is the strongest genetic marker for severe retinopathy and DRB1*0301/0401 only has a secondary influence when combined with this genotype. The findings of the present study therefore suggest that IDDM patients who are positive for DR3-DQ2/DR4-DQ8 are at greater risk for developing severe retinopathy at young age. Further prospective long-term studies will have to be performed in order to clearly identify those who independently from early age of diabetes onset are at risk of developing severe retinopathy.

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