

# Specific Human Leukocyte Antigen DQ Influence on Expression of Antiislet Autoantibodies and Progression to Type 1 Diabetes

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**Context:** Human leukocyte antigen (HLA) DQ haplotypes have the strongest genetic association with type 1 diabetes (T1DM) risk.

**Objective:** The objective of the study was to analyze whether HLA DQ alleles influence the development of antiislet autoantibodies, the progression to T1DM among autoantibody-positive relatives, or both.

**Design:** The Diabetes Prevention Trial-1 screened more than 90,000 nondiabetic relatives of patients for cytoplasmic islet-cell autoantibody (ICA) expression between 1994 and 2002.

**Setting:** The study was conducted in the general community.

**Participants:** The Diabetes Prevention Trial-1 found 2817 ICA-positive relatives who were tested for biochemical autoantibodies (GAD65, ICA512, and insulin) and HLA-DQ haplotypes, and 2796 of them were followed up for progression to diabetes for up to 8 yr (median, 3.6 yr).

**Main Outcome Measure:** Progression to T1DM was measured.

**Results:** High-risk DQ haplotypes and genotypes were associated with a higher percentage of relatives expressing multiple biochemical autoantibodies and higher T1DM risk (e.g., respectively, 59 and 36% at 5 yr for carriers of the DQA1\*0301-DQB1\*0302/DQA1\*0501-DQB1\*0201 genotype). The number of autoantibodies expressed significantly increased T1DM risk and across different DQ genotypes, autoantibody positivity directly correlated with diabetes risk. However, multivariate analyses indicated that the influence of most genotypes on T1DM risk was not independent from autoantibody expression, with the possible exception of DQA1\*0102-DQB1\*0602. Specific genotypic combinations conferred 5-yr diabetes risks significantly lower (e.g., 7%-DQA1\*0201-DQB1\*0201/DQA1\*0501-DQB1\*0201 and 14%-DQA1\*0301-DQB1\*0301/DQA1\*0501-DQB1\*0201) than when those haplotypes were found in other combinations.

**Conclusion:** HLA DQ alleles determine autoantibody expression, which is correlated with diabetes progression. Among autoantibody-positive relatives, most HLA DQ genotypes did not further influence T1DM risk. (*J Clin Endocrinol Metab* 91: 1705–1713, 2006)

THE MAJOR HISTOCOMPATIBILITY complex accounts for approximately 50% of the familial aggregation of type 1 diabetes (T1DM) (1). High-risk alleles, haplotypes, and genotypes as well as protective alleles, haplotypes, and genotypes have been identified. DQ haplotypes have the strongest association with diabetes, but DR alleles can modify risk.

Most studies assessing the influence of human leukocyte antigen (HLA) haplotypes on T1DM have analyzed populations of individuals with known T1DM. The Diabetes Prevention Trial Type 1 (DPT-1) (2) afforded the opportunity of

prospectively evaluating progression to diabetes. We aimed to analyze the influence of HLA class II haplotypes and genotypes on antiislet autoantibody expression and, whether in relatives with positive biochemical autoantibodies, T1DM risk is influenced by HLA class II. Given the size of the DPT-1 cohort expressing cytoplasmic islet-cell autoantibodies (ICA) who were all HLA typed ( $n = 2817$ ), we were able to analyze the influence of haplotypes when found in specific genotypic combinations (DQ of both sixth chromosomes) on autoantibody expression and progression to diabetes.

## Subjects and Methods

### Subjects

**Identification.** The DPT-1 screened 97,272 first- and second-degree relatives of individuals with T1DM who had been diagnosed before age 45 yr. To have been eligible for screening, an individual must have been a first-degree relative of a patient with T1DM and between ages 3 and 45 yr or second-degree relative between ages 3 and 20 yr. A flow chart depicting the ascertainment and follow-up of the cohort is illustrated in

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Abbreviations: GAD65, Glutamic acid decarboxylase; GAD65A, GAD65 autoantibody; HLA, human leukocyte antigen; HR, hazard ratio; ICA, cytoplasmic islet-cell autoantibodies; ICA512A, ICA512 autoantibody; LD, linkage disequilibrium; mIAA, microinsulin autoantibody assay; OGTT, oral glucose tolerance test; T1DM, type 1 diabetes.

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Fig. 1. Relatives who were found to be ICA+ were staged to further assess risk. Staging consisted of repeat ICA testing, determination of insulin antibodies, HLA-DQ typing, an iv glucose tolerance test, and oral glucose tolerance testing (OGTT). Relatives with DQA1\*0102-DQB1\*0602 [in linkage disequilibrium (LD) with DR2] were excluded (noneligible) from the DPT-1. Here we report the results of the 2817 relatives who were HLA typed, were ICA positive, and were also tested, on the screening sample, for glutamic acid decarboxylase (GAD65) autoantibodies (GAD65A), insulin autoantibodies, and ICA512 autoantibodies (ICA512A). Of the 2817, 51% were males, 88% were non-Hispanic white, and 89.8% were first-degree relatives.

**Follow-up.** Of the 2817 ICA+ subjects, 2796 subjects were followed up for up to 8 yr (median 3.6 yr). Of these 2796 subjects, those who were enrolled in DPT-1 intervention studies included those either with confirmed positivity for ICA and insulin autoantibodies or those with confirmed ICA+ and low first phase of insulin release or abnormal, but not diabetic, OGTT at baseline visits. The development of diabetes in these DPT-1 subjects enrolled in intervention studies was assessed by formal OGTT every 6 months or confirmed diabetes by American Diabetes Association criteria. For the remaining subjects, the development of diabetes was assessed via a follow-up questionnaire, which was sent to the nondiabetic screened subjects. For the DQA1\*0102-DQB1\*0602 subjects, the source of follow-up came from either available staging data or the follow-up questionnaire (3). The analyses presented here include the development of T1DM in 441 subjects.

Written consent was obtained from all subjects or their parents.

#### Antiislet autoantibodies

Autoantibodies against GAD65, ICA512 (IA-2), and insulin (termed biochemical autoantibodies for this manuscript, as opposed to cytoplasmic islet-cell autoantibodies, or ICA) were measured in an ancillary study of DPT-1 (4, 5). Cytoplasmic ICAs were determined on frozen sections of human pancreas (6). Samples were considered positive at 10 or more Juvenile Diabetes Foundation units. In the Immunology of Diabetes Society Combinatorial Autoantibody Workshop (Orvieto, Italy, November 1995), this ICA assay had 100% specificity and 74.4% sensitivity for new-onset patients aged younger than 30 yr.

GAD65A and ICA512A levels were measured simultaneously in a combined GAD65A/ICA512A radioassay, as previously described (full-length GAD65 and ICA512bdc cDNA clones) (7). The assay was per-

formed in 96-well filtration plates with autoantibody bound [<sup>3</sup>H]GAD65 and [<sup>35</sup>S]ICA512 precipitated with protein A-Sepharose. The cut points were set at indices of 0.032 (GAD65A) and 0.049 (ICA512A), the 99th percentiles of 198 controls. The interassay coefficients of variation were 6.5 and 9.6%, respectively, for GAD65A and ICA512A assays. In the Immunology of Diabetes Society Combinatorial Workshop, for patients younger than 30 yr, the assay specificity was 99 and 100%, and sensitivity was 83.7 and 74.4% for GAD65A and ICA512A, respectively. In the 2003 Diabetes Antibody Standardization Program proficiency testing, the assay specificity was 98 and 100%, and sensitivity was 84 and 58% for GAD65A and ICA512A, respectively.

Insulin autoantibodies were measured with <sup>125</sup>I-insulin in standard 96-well filtration plates that quantitate precipitated <sup>125</sup>I-insulin with a multichannel 96-well  $\beta$ -counter. Scintillation fluid is directly added to the 96-well plate (Top-Counter scintillation counter) (8). An index was determined based on the difference in counts per minute between wells without and with cold insulin. The cut point for positivity was set at the 99th percentile of healthy controls (0.01 and 0.02 index, respectively, for the Denver and Boston laboratories). In the 2003 Diabetes Antibody Standardization Program proficiency testing, the microinsulin autoantibody assay (mIAA) assay sensitivity was 74 and 56% and specificity 90 and 98%, respectively, for the Denver and Boston laboratories, and the correlation coefficient between both laboratories was  $r = 0.90$ ,  $P < 0.0001$ .

Among DPT-1 eligible and screened relatives who were positive for ICA, ICA512A, mIAA, or GAD65A, 21.9, 46, 41, and 34.8% progressed to diabetes, respectively, compared with 10.2, 10.8, 16.1, and 9.5% among relatives who were negative for each single autoantibody (all comparisons  $P < 0.0001$ ). Among relatives who were positive for none, one, two, or three biochemical autoantibodies (*e.g.* ICA512A, mIAA, GAD65A), 7.9, 27.8, 42.5, and 47.6% progressed to diabetes ( $P < 0.0001$ ).

#### HLA-DQ typing

HLA-DQA and DQB alleles were typed as part of the DPT-1 study using sequence-specific oligonucleotide probes in the DPT-1 HLA Core Laboratory (Denver, CO) (9). The DQA1\*0101-DQB1\*0501 haplotype (in LD with DR1) has been assigned an odds ratio of 1 when haplotypes are compared for diabetes protection or risk (neutral haplotype) (10).

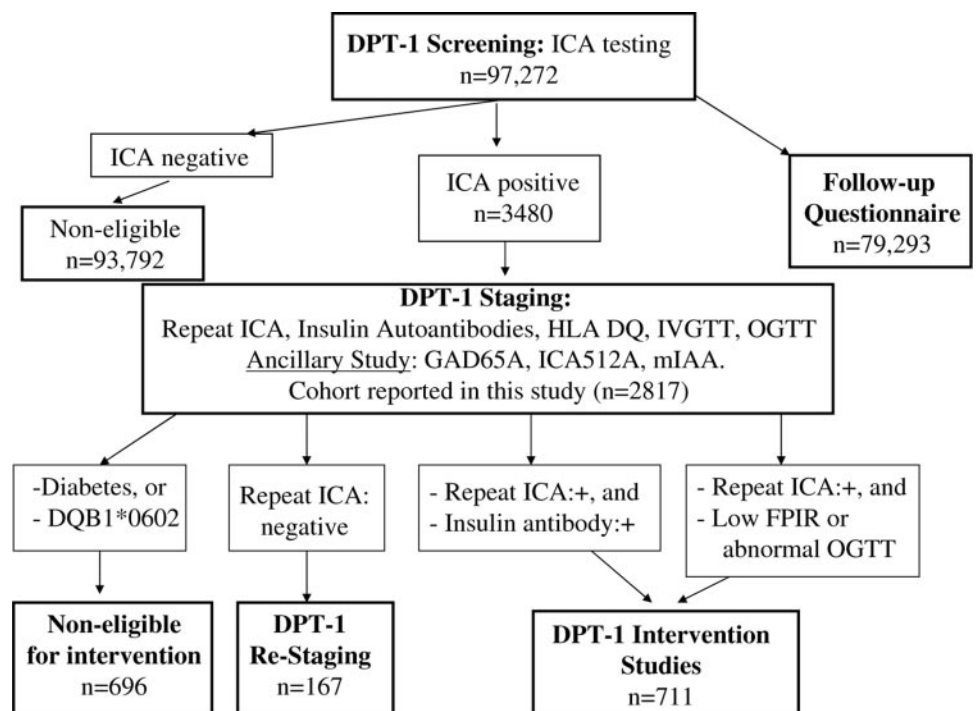


FIG. 1. Flow chart describing the cohort and its follow-up.

### Statistical analysis

Categorical variables were analyzed using  $\chi^2$  tests or Fisher's exact tests, depending on cell size. Time-to-event analytic methods included Kaplan-Meier estimates (with log-log confidence intervals) and log-rank. Life table analysis of progression to diabetes among autoantibody-positive relatives was based only on screening sample results. Additionally, time to progression to T1DM was modeled using both univariate and multivariate Cox-proportional hazard models. Independent variables considered included DR status and number of positive antibodies observed on all available samples (*e.g.* screening and staging). Statistical analyses were performed using SAS (SAS Institute, Cary, NC), True Epistat (Epistat Services, Richardson, TX), and Prism (GraphPad Software, San Diego, CA) software using a two-sided significance level of 0.05.

### Results

In this group of 2817 relatives of patients with T1DM who screened positive for ICA, the most frequent haplotypes expressed were the high-risk DQA1\*0301-DQB1\*0302 (in LD with DR4) in 50% and DQA1\*0501-DQB1\*0201 (in LD with DR3) in 46%. The protective haplotypes DQA1\*0102-DQB1\*0602 (in LD with DR2), DQA1\*0201-DQB1\*0303 (in LD with DR7) and DQA1\*0101-DQB1\*0503 (in LD with DR14) were found, respectively, in 8, 2, and 2%. Almost 20% of the relatives had the highest risk genotype DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302, and all other genotypes were present in less than 7% of relatives.

#### *Influence of HLA haplotypes and genotypes on number of autoantibodies expressed*

There was a wide variation in the percentage of ICA+ relatives expressing biochemical autoantibodies relative to DQ haplotypes, ranging from 57% of 44 relatives with DQA1\*0301-DQB1\*0201 (in LD with DR9) expressing multiple (two or more) biochemical autoantibodies to 8% of 238 relatives with DQA1\*0102-DQB1\*0602 expressing multiple biochemical autoantibodies. Almost 50% of DQA1\*0301-DQB1\*0302 and 38% of DQA1\*0501-DQB1\*0201 relatives were positive for multiple biochemical autoantibodies. Of note, 39% of relatives with the DQA1\*0301-DQB1\*0301 haplotype, which is in LD with DR4 and has aspartic acid at amino acid 57 of DQB1, expressed multiple biochemical antibodies. Almost 80% of relatives with the protective haplotype DQA1\*0102-DQB1\*0602 expressed no biochemical autoantibody, compared with 45% among 2579 relatives without this haplotype ( $P < 0.0001$ ). Similarly, 79 and 71%, respectively, of relatives with DQA1\*0201-DQB1\*0303 or DQA1\*0101-DQB1\*0503 expressed no biochemical antibody, compared with 47% without DQA1\*0201-DQB1\*0303 ( $n = 2749$ ,  $P < 0.0001$ ) and 47% of relatives without DQA1\*0101-DQB1\*0503 ( $n = 2766$ ,  $P < 0.001$ ).

Table 1 indicates the percentage of subjects expressing multiple ( $\geq 2$ ) biochemical autoantibodies by genotypes and odds ratios relative to the highest risk genotype DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302. Of note, the frequency of positivity for multiple biochemical autoantibodies was not different in relatives with DQA1\*0301-DQB1\*0302/DQA1\*0501-DQB1\*0201 (59%) and relatives who were homozygous for DQA1\*0301-DQB1\*0302 (57%,  $P = 0.79$ ), whereas it was higher than relatives who were homozygous for DQA1\*0501-DQB1\*0201 (30%,  $P < 0.0001$ ). The frequency

of positivity for multiple autoantibodies was not significantly different between relatives with DQA1\*0501-DQB1\*0201/DQA1\*0501-DQB1\*0201 (30%), relatives with DQA1\*0501-DQB1\*0201/DQA1\*0101-DQB1\*0501 (26%), and relatives with DQA1\*0101-DQB1\*0501/DQA1\*0101-DQB1\*0501 (26%) ( $P = 0.60$ ). Relatives with DQA1\*0301-DQB1\*0302/DQA1\*0101-DQB1\*0501 had an intermediate prevalence (47%).

Genotypes containing the protective DQA1\*0102-DQB1\*0602 haplotype were associated with low frequencies of multiple autoantibody expressivity, even when combined with high-risk haplotypes DQA1\*0301-DQB1\*0302 or DQA1\*0501-DQB1\*0201 (4 and 10%, respectively, expressed multiple biochemical autoantibodies).

Positivity for each of the biochemical autoantibodies was significantly more prevalent in relatives with the high-risk haplotypes DQA1\*0301-DQB1\*0302 and DQA1\*0501-DQB1\*0201 than in relatives with protective or neutral haplotypes. However, mIAA positivity occurred in 22% of DQA1\*0101-DQB1\*0501 relatives, which is not different from 22% in DQA1\*0301-DQB1\*0302 relatives ( $P = 0.83$ ), and higher than 17% in DQA1\*0501-DQB1\*0201 relatives ( $P < 0.0005$ ). The frequency of positivity for specific biochemical autoantibodies was not significantly different between relatives with the DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302 genotype and relatives who were homozygous for DQA1\*0301-DQB1\*0302. Relatives who were homozygous for DQA1\*0501-DQB1\*0201 were significantly less likely to be positive for each of the biochemical autoantibodies than those who were DQA1\*0301-DQB1\*0302 homozygous (data not shown).

#### *Influence of HLA haplotypes and genotypes on progression to T1DM*

Of the 2817 relatives who were HLA typed and tested for autoantibodies, 2796 subjects were prospectively followed up until progression to T1DM.

*Overall progression to T1DM by HLA haplotypes and genotypes, regardless of autoantibody status.* Life table-estimated risk of progression to diabetes at 5 yr was 32% in relatives with DQA1\*0301-DQB1\*0302, significantly higher than 15% in relatives without this haplotype ( $P < 0.001$ ). After DQA1\*0301-DQB1\*0302, the haplotype with the highest diabetes risk, among those haplotypes that were present in more than 15 subjects and therefore suitable for life table analysis, was DQA1\*0301-DQB1\*0201, with 26% risk at 5 yr. Five-year diabetes risk was 25% in DQA1\*0501-DQB1\*0201 relatives, 23% in DQA1\*0101-DQB1\*0501 relatives, and 20% in DQA1\*0301-DQB1\*0301 relatives. Progression to T1DM at 5 yr in relatives with the protective DQA1\*0102-DQB1\*0602 ( $n = 238$ ) or DQA1\*0201-DQB1\*0303 ( $n = 67$ ) was 1 and 11%, respectively, significantly lower than relatives without those haplotypes (respectively, 25%,  $P < 0.001$  and 24%,  $P < 0.04$ ). However, progression to T1DM in 51 relatives with DQA1\*0101-DQB1\*0503 was 19%, not significantly different from relatives without this haplotype (24%,  $P = 0.288$ ). Life-table analyses of progression to T1DM by different haplotypes are available in supplemental Table 1 published on The

**TABLE 1.** Expression of multiple ( $\geq 2$ ) biochemical autoantibodies by genotypes

Genotype (DQ)	Proportion (%)	Odds ratio (95% CI)	P value
0501-0201/0301-0302	305/521 (58.5)	N/A (reference)	N/A
0101-0501/0101-0501	5/19 (26.3)	0.249 (0.088–0.702)	0.008
0101-0501/0102-0604	5/16 (12.5)	0.317 (0.109–0.925)	0.038
0101-0501/0102-0602	0/12 (0)	0.028 (0.002–0.474) <sup>a</sup>	2.84E-05
0101-0501/0501-0201	22/85 (25.9)	0.243 (0.145–0.408)	1.61E-08
0101-0501/0301-0302	58/124 (46.8)	0.638 (0.427–0.938)	<0.026
0101-0501/0301-0301	23/39 (59.0)	1.002 (0.517–1.942)	1.0
0101-0501/0501-0301	2/24 (8.3)	0.063 (0.015–0.272)	5.76E-07
0101-0501/0103-0603	2/12 (16.7)	0.139 (0.030–0.643)	0.005
0101-0501/0201-0201	4/18 (22.2)	0.199 (0.065–0.613)	0.003
0102-0602/0102-0604	1/14 (7.1)	0.054 (0.007–0.413)	1.02E-04
0102-0602/0501-0201	7/72 (9.7)	0.075 (0.034–0.167)	2.6E-16
0102-0602/0301-0302	2/56 (3.6)	0.026 (0.006–0.107)	2.4E-17
0102-0602/0301-0301	1/11 (9.1)	0.070 (0.009–0.548)	0.001
0102-0602/0501-0301	1/23 (4.4)	0.032 (0.004–0.237)	8.21E-08
0102-0602/0201-0201	1/11 (9.1)	0.070 (0.009–0.549)	0.001
0102-0502/0501-0201	5/19 (26.3)	0.249 (0.088–0.702)	0.004
0102-0502/0301-0302	8/21 (38.1)	0.429 (0.175–1.053)	0.071
0501-0201/0102-0604	10/45 (22.2)	0.199 (0.097–0.411)	1.4E-06
0501-0201/0101-0503	0/17 (0)	0.020 (0.001–0.333) <sup>a</sup>	4.05E-07
0501-0201/0501-0201	42/138 (30.3)	0.305 (0.204–0.456)	3.02E-09
0501-0201/0301-0301	23/84 (27.4)	0.269 (0.158–0.438)	6.8E-08
0501-0201/0501-0301	14/80 (17.5)	0.148 (0.081–0.270)	2.14E-12
0501-0201/0103-0603	8/41 (19.5)	0.169 (0.077–0.373)	9.1E-07
0501-0201/0201-0201	22/88 (25.0)	0.232 (0.139–0.388)	3.3E-09
0501-0201/0201-0303	7/24 (29.2)	0.140 (0.047–0.414)	6.1E-05
0501-0201/0401-0402	4/27 (14.8)	0.121 (0.041–0.356)	8.6E-06
0301-0302/0102-0604	27/63 (42.9)	0.523 (0.308–0.887)	0.021
0301-0302/0101-0503	4/11 (36.4)	0.398 (0.115–1.378)	0.214
0301-0302/0301-0302	100/175 (57.1)	0.951 (0.673–1.346)	0.79
0301-0302/0301-0301	30/57 (52.6)	0.775 (0.448–1.340)	0.397
0301-0302/0501-0301	44/119 (37.0)	0.409 (0.271–0.617)	1.6E-05
0301-0302/0103-0603	28/60 (46.7)	0.610 (0.357–1.043)	0.069
0301-0302/0201-0201	34/83 (41.0)	0.484 (0.302–0.775)	0.003
0301-0302/0401-0402	18/38 (47.4)	0.627 (0.324–1.214)	0.176
0301-0302/0301-0201	10/12 (83.3)	3.485 (0.756–16.067)	0.135
0301-0301/0102-0604	11/22 (50.0)	0.697 (0.297–1.637)	0.508
0301-0301/0301-0301	9/13 (69.2)	1.568 (0.477–5.159)	0.574
0301-0301/0501-0301	2/20 (10)	0.078 (0.018–0.337)	2.2E-05
0301-0301/0103-0603	3/11 (27.3)	0.261 (0.069–0.997)	0.059
0301-0301/0201-0201	7/20 (35.0)	0.375 (0.147–0.956)	0.039
0501-0301/0102-0604	2/13 (15.4)	0.127 (0.028–0.578)	0.003
0501-0301/0501-0301	0/11 (0)	0.030 (0.002–0.517) <sup>a</sup>	6.7E-05
0501-0301/0103-0603	2/12 (16.7)	0.139 (0.030–0.643)	0.005
0501-0301/0201-0201	4/24 (16.7)	0.139 (0.047–0.414)	6.1E-05
0501-0301/0401-0402	0/13 (0)	0.026 (0.002–0.437) <sup>a</sup>	1.2E-05
0201-0201/0102-0604	5/20 (25.0)	0.232 (0.083–0.649)	0.003
0201-0201/0401-0402	9/20 (45)	0.570 (0.232–1.400)	0.251

Odds ratios are relative to DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302 (in LD with DR3/4). Only genotypes present in more than 10 subjects are included. CI, Confidence interval; N/A, not applicable.

<sup>a</sup> Corrected logit estimators.

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Relatives bearing the DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302 genotype ( $n = 521$ ) had a significantly higher risk of T1DM (36% at 5 yr) than relatives without this genotype (20%,  $P < 0.001$ ) (Fig. 2A). Diabetes risk at 5 yr was 36% in 175 relatives homozygous for DQA1\*0301-DQB1\*0302, and 34% in 138 relatives homozygous for DQA1\*0501-DQB1\*0201 but only 14% in 19 relatives with DQA1\*0101-DQB1\*0501/DQA1\*0101-DQB1\*0501, 3% for 72 relatives with DQA1\*0102-DQB1\*0602/DQA1\*0501-DQB1\*0201, and 2% for 56 relatives with DQA1\*0102-DQB1\*0602/DQA1\*0301-DQB1\*0302. There were only seven subjects with DQA1\*0102-DQB1\*0602/DQA1\*0102-DQB1\*0602, and, at 5 yr of follow-up, none had developed diabetes. Lifetable

analyses of progression to T1DM by different genotypes are available in supplemental Table 2 published on The Endocrine Society's Journals Online web site at <http://jcem.endojournals.org>.

*Progression to T1DM by HLA haplotype and genotype in autoantibody-positive relatives.* Univariate analysis indicated that, regardless of genotype, an increasing number of positive autoantibodies significantly increased the hazard ratio (HR) of progression to T1DM relative to positivity for ICA only: HR of 0.161 for no positive autoantibodies ( $SE = 0.60$ ,  $P < 0.002$ ), 2.106 for one autoantibody ( $SE = 0.331$ ,  $P < 0.024$ ), 4.924 for two autoantibodies ( $SE = 0.187$ ,  $P < 0.0001$ ), 9.901 for three autoantibodies ( $SE = 0.171$ ,  $P < 0.0001$ ), and 9.822 for four autoantibodies ( $SE = 0.184$ ,  $P < 0.0001$ ).

In relatives carrying the DQA1\*0301-DQB1\*0302,

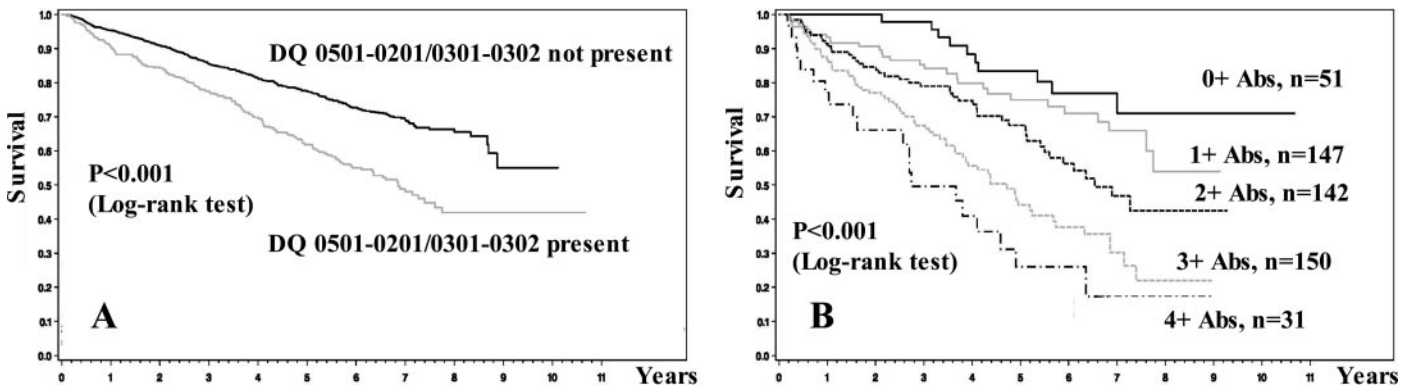


FIG. 2. A, Survival analysis of progression to T1DM among 2796 DPT-1 eligible and tested subjects with and without the high-risk genotype DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302. B, Survival analysis of progression to T1DM among 521 relatives with the high-risk genotype DQA1\*0301-DQB1\*0302-DQA1\*0501-DQB1\*0201, by number of positive autoantibodies (ICA, GAD65A, ICA512A, mIAA) at screening.

DQA1\*0501-DQB1\*0201 or DQA1\*0101-DQB1\*0501 haplotypes, development of T1DM was significantly different by number of positive autoantibodies (higher number of positive autoantibodies increased T1DM risk) (data not shown). Among relatives with DQA1\*0102-DQB1\*0602, progression to diabetes was also different by number of positive autoantibodies ( $P < 0.05$ ), with higher number of positive autoantibodies affording greater diabetes risk, but the overall risk was very small (3% with multiple autoantibodies).

A Cox-model prediction of T1DM indicated an independent influence of both the presence of DQA1\*0102-DQB1\*0602 (relative to its absence) (HR = 0.258,  $P = 0.0071$ ) and the number of positive autoantibodies (relative to ICA positivity) (HR and  $P$  values, for no, one, two, three, and four autoantibodies expressed, respectively, were 0.165 and 0.0026, 2.027 and 0.0325, 4.689 and  $< 0.0001$ , 9.219 and less than 0.0001, and 9.063 and  $< 0.0001$ ).

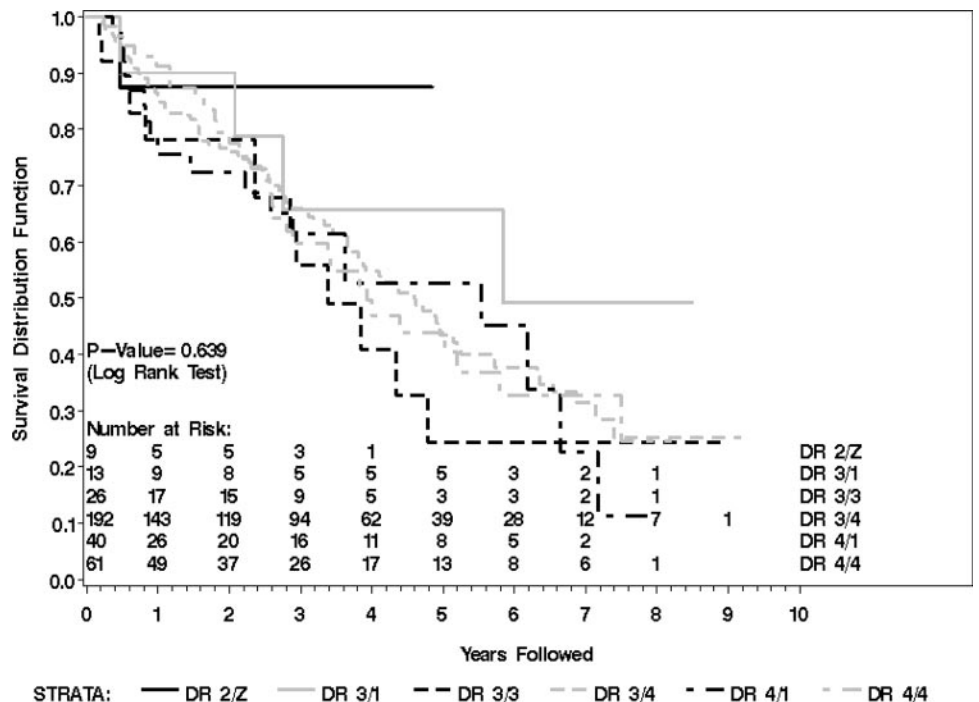
The number of positive autoantibodies significantly in-

creased diabetes risk in relatives with the highest risk genotype DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302 ( $P < 0.001$ ) (Fig. 2B), DQA1\*0301-DQB1\*0302/DQA1\*0301-DQB1\*0302, or DQA1\*0501-DQB1\*0201/DQA1\*0501-DQB1\*0201 (data not shown).

For relatives who were positive for multiple biochemical autoantibodies at screening, survival analysis of progression to T1DM was not different among those who had the highest risk genotype DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302, those who were homozygous for DQA1\*0301-DQB1\*0302, those who were homozygous for DQA1\*0501-DQB1\*0201, those with DQA1\*0101-DQB1\*0501/DQA1\*0301-DQB1\*0302, those with DQA1\*0101-DQB1\*0501/DQA1\*0501-DQB1\*0201, or those with the DQA1\*0102-DQB1\*0602 haplotype (combined with any other haplotype, *i.e.* DR2/Z). For the latter group of relatives, it should be noted that the number was small (Fig. 3).

A series of multivariate analysis for prediction of T1DM, each Cox model including a specific genotype alone and

FIG. 3. Survival analysis of progression to T1DM among relatives with multiple positive biochemical autoantibodies (two or more positives of GAD65A, ICA512A or mIAA) at screening only, by HLA genotype. Numbers at the bottom indicate numbers of relatives still followed up at each time point.



positivity for no, one, two, three, or four autoantibodies, indicated that the effect of most genotypes on progression to T1DM was not independent from autoantibody positivity. Exceptions were genotypic combinations of DQA1\*0501-DQB1\*0201 with DQA1\*0301-DQB1\*0301 (HR = 0.375, SE = 0.366,  $P < 0.007$ ), DQA1\*0501-DQB1\*0301 (HR = 0.373, SE = 0.427,  $P < 0.021$ ), or DQA1\*0201-DQB1\*0201 (HR = 0.326, SE = 0.458,  $P < 0.016$ ). Table 2 illustrates a summary of these analyses, for each genotype and positivity for all four autoantibodies.

Diabetes risk at 5 yr for given genotypes correlated with the percentage of relatives expressing no ( $r^2 = -0.71$ ,  $P < 0.0001$ ), two ( $r^2 = 0.67$ ,  $P < 0.0001$ ), three ( $r^2 = 0.35$ ,  $P < 0.0003$ ), at least one ( $\geq 1$ ) ( $r^2 = 0.69$ ,  $P < 0.0001$ , Figure 4), or multiple ( $\geq 2$ ) ( $r^2 = 0.69$ ,  $P < 0.0001$ ) biochemical autoanti-

bodies on the screening sample. In Fig. 4, only relatives with DQA1\*0102-DQB1\*0502 (in LD with DR2) combined with DQA1\*0301-DQB1\*0302 had an apparent higher risk of progression to diabetes (45%) than would be predicted by having 57% of relatives with a biochemical autoantibody, but there were only 21 DQA1\*0102-DQB1\*0502/DQA1\*0301-DQB1\*0302 relatives with one or more positive autoantibodies who were followed up for progression to diabetes.

#### *Influence of specific haplotypic combinations (genotypes) on autoantibody expression and progression to T1DM*

The prevalence of positivity for multiple biochemical autoantibodies was significantly different among relatives who had DQA1\*0201-DQB1\*0201 (in LD with DR7) combined

**TABLE 2.** Prediction of T1DM based on positivity for four autoantibodies (ICA, GAD65A, mIAA, and ICA512A), relative to ICA positivity, and DQ genotype, relative to DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302

Genotype	By genotype		By positivity for 4 autoantibodies		Global $P$ value (df = 6)
	HR (SE)	$P$ value	HR (SE)	$P$ value	
0101-0501/0101-0501	0.447 (0.713)	0.258	3.709 (0.362)	<0.0003	<0.0001
0101-0501/0102-0604	0.979 (0.717)	0.981	3.922 (0.362)	<0.0002	<0.0001
0101-0501/0102-0602	0.0 (390.1)	0.985	3.609 (0.361)	<0.0004	<0.0001
0101-0501/0501-0201	1.211 (0.266)	0.473	5.062 (0.361)	<0.0001	<0.0001
0101-0501/0301-0302	1.109 (0.187)	0.578	4.992 (0.350)	<0.0001	<0.0001
0101-0501/0301-0301	0.891 (0.313)	0.712	3.590 (0.348)	<0.0002	<0.0001
0101-0501/0501-0301	0 (616.0)	0.982	3.609 (0.361)	<0.0004	<0.0001
0101-0501/0103-0603	0 (930.0)	0.988	3.609 (0.361)	<0.0004	<0.0001
0101-0501/0201-0201	1.560 (0.603)	0.461	4.464 (0.368)	<0.0001	<0.0001
0102-0602/0102-0604	0 (888.6)	0.987	3.609 (0.361)	<0.0004	<0.0001
0102-0602/0501-0201	0.317 (0.730)	0.115	4.049 (0.369)	<0.0002	<0.0001
0102-0602/0301-0302	0.101 (1.012)	0.103	3.427 (0.351)	<0.0004	<0.0001
0102-0602/0301-0301	0 (392.4)	0.982	3.609 (0.361)	<0.0004	<0.0001
0102-0602/0501-0301	0 (827.5)	0.986	3.609 (0.361)	<0.0004	<0.0001
0102-0602/0201-0201	0 (915.5)	0.987	3.609 (0.361)	<0.0004	<0.0001
0102-0502/0501-0201	0.553 (0.712)	0.405	3.678 (0.362)	<0.0003	<0.0001
0102-0502/0301-0302	1.562 (0.387)	0.250	4.217 (0.358)	<0.0001	<0.0001
0501-0201/0102-0604	0.667 (0.391)	0.301	3.822 (0.352)	<0.0001	<0.0001
0501-0201/0101-0503	0.479 (1.026)	0.473	3.508 (0.355)	<0.0001	<0.0001
0501-0201/0501-0201	1.099 (0.192)	0.623	4.612 (0.349)	<0.0001	<0.0001
0501-0201/0301-0301	0.375 (0.366)	0.007	3.738 (0.349)	<0.0002	<0.0001
0501-0201/0501-0301	0.373 (0.427)	0.021	3.188 (0.325)	<0.0004	<0.0001
0501-0201/0103-0603	0.787 (0.425)	0.317	3.442 (0.338)	<0.0003	<0.0001
0501-0201/0201-0201	0.326 (0.458)	0.016	4.245 (0.361)	<0.0001	<0.0001
0501-0201/0201-0303	0.388 (0.715)	0.185	3.836 (0.361)	<0.0002	<0.0001
0501-0201/0401-0402	0.521 (0.370)	0.374	4.063 (0.370)	<0.0002	<0.0001
0301-0302/0102-0604	1.013 (0.271)	0.963	3.793 (0.345)	<0.0001	<0.0001
0301-0302/0101-0503	0.482 (1.000)	0.467	3.649 (0.361)	<0.0003	<0.0001
0301-0302/0301-0302	1.101 (0.152)	0.530	5.342 (0.332)	<0.0001	<0.0001
0301-0302/0301-0301	0.730 (0.357)	0.276	3.830 (0.357)	<0.0002	<0.0001
0301-0302/0501-0301	1.061 (0.202)	0.770	4.452 (0.296)	<0.0001	<0.0001
0301-0302/0103-0603	0.871 (0.280)	0.622	4.413 (0.359)	<0.0001	<0.0001
0301-0302/0201-0201	0.826 (0.246)	0.436	3.466 (0.333)	<0.0002	<0.0001
0301-0302/0401-0402	1.473 (0.280)	0.281	3.965 (0.359)	<0.0001	<0.0001
0301-0302/0301-0201	0.876 (0.390)	0.734	3.669 (0.360)	<0.0003	<0.0001
0301-0301/0102-0604	0.653 (0.455)	0.350	3.886 (0.361)	<0.0002	<0.0001
0301-0301/0301-0301	0.560 (0.584)	0.320	3.770 (0.359)	<0.0002	<0.0001
0301-0301/0501-0301	0.726 (0.590)	0.587	3.597 (0.352)	<0.0003	<0.0001
0301-0301/0103-0603	0.310 (1.006)	0.244	3.658 (0.298)	<0.0001	<0.0001
0301-0301/0201-0201	0.342 (0.712)	0.132	3.311 (0.349)	<0.0006	<0.0001
0501-0301/0102-0604	0.818 (1.027)	0.844	3.884 (0.368)	<0.0002	<0.0001
0501-0301/0501-0301	0 (833.1)	0.987	3.609 (0.361)	<0.0004	<0.0001
0501-0301/0103-0603	0.483 (1.013)	0.473	3.869 (0.362)	<0.0002	<0.0001
0501-0301/0201-0201	0.238 (1.006)	0.154	3.777 (0.360)	<0.0002	<0.0001
0501-0301/0401-0402	0 (649.2)	0.984	3.609 (0.361)	<0.0004	<0.0001
0201-0201/0102-0604	0.642 (0.584)	0.449	3.638 (0.346)	<0.0002	<0.0001
0201-0201/0401-0402	0.624 (0.512)	0.356	3.694 (0.349)	<0.0002	<0.0001

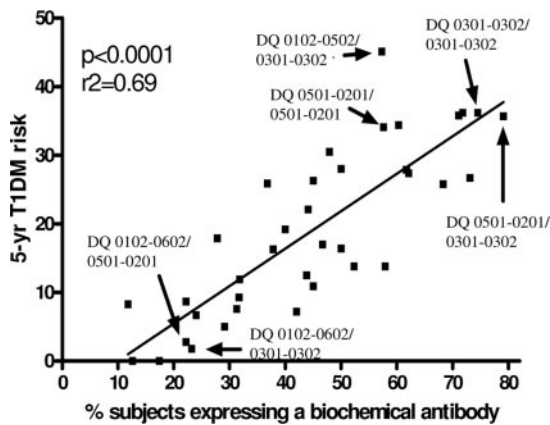


FIG. 4. Correlation between life table-estimated progression to diabetes at 5 yr of follow-up (y-axis) and prevalence of subjects expressing a biochemical autoantibody (of GAD65A, ICA512A, and mIAA) (x-axis), by genotypes. Only genotypes present in more than 15 subjects were included.

with the high-risk DQA1\*0501-DQB1\*0201 (23 of 88, 26%), relatives with DQA1\*0201-DQB1\*0201/DQA1\*0301-DQB1\*0302 (34 of 82, 42%), relatives with DQA1\*0201-DQB1\*0201/DQA1\*0101-DQB1\*0501 (four of 18, 22%), and relatives with DQA1\*0201-DQB1\*0201/DQA1\*0201-DQB1\*0201 (none of five) ( $P < 0.05$ ). Similarly, survival analysis of progression to diabetes indicated that DQA1\*0201-DQB1\*0201/DQA1\*0501-DQB1\*0201 was significantly more protective, with 7% diabetes risk ( $n = 88$ ), than DQA1\*0201-DQB1\*0201/DQA1\*0301-DQB1\*0302, with 28% diabetes risk ( $n = 83$ ,  $P < 0.007$ ), or than DQA1\*0101-DQB1\*0501/DQA1\*0501-DQB1\*0201, with 22% diabetes risk ( $n = 85$ ,  $P < 0.02$ ). Relatives with DQA1\*0201-DQB1\*0201/DQA1\*0501-DQB1\*0201 had lower 5-yr diabetes risk (7%) than those with DQA1\*0201-DQB1\*0201/DQA1\*0101-DQB1\*0501 (18%,  $n = 18$ ), but the difference was not significant ( $P = 0.23$ ). As shown in Table 2, multivariate analysis indicated that the effect of DQA1\*0201-DQB1\*0201/DQA1\*0501-DQB1\*0201 on T1DM risk is independent from multiple autoantibody positivity (HR = 0.326, SE = 0.458,  $P < 0.016$ ), *i.e.* relatives who carried this genotype were protected from diabetes, even when positive for multiple autoantibodies.

Among relatives carrying DQA1\*0301-DQB1\*0301 (in LD with DR4, and with aspartic acid at position 57 of the DQB chain) combined with DQA1\*0501-DQB1\*0201, only 27% expressed multiple biochemical autoantibodies. In comparison, relatives carrying the same DQA1\*0301-DQB1\*0301 haplotype in a different genotypic combination (for instance, with DQA1\*0101-DQB1\*0501, DQA1\*0301-DQB1\*0302 or DQA1\*0301-DQB1\*0301), expressed multiple biochemical autoantibodies in 59, 53, and 69% of the cases, respectively. Similarly, life table-estimated risk of diabetes was significantly different among relatives with DQA1\*0301-DQB1\*0301 in those four genotypic combinations [*e.g.* DQA1\*0301-DQB1\*0301/DQA1\*0501-DQB1\*0201 ( $n = 84$ , 5-yr diabetes risk 14%), DQA1\*0301-DQB1\*0301/DQA1\*0101-DQB1\*0501 ( $n = 39$ , 36%), DQA1\*0301-DQB1\*0302/DQA1\*0301-DQB1\*0301 ( $n = 57$ , 27%), and DQA1\*0301-DQB1\*0301/DQA1\*0301-DQB1\*0301 ( $n = 13$ , 31%) ( $P < 0.015$ )]. As shown in Table 2, multivariate analysis of DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0301 and positivity for four autoantibodies indicated that the effect of this genotype on progression to T1DM is

independent from multiple autoantibody positivity (HR = 0.375, SE = 0.366,  $P < 0.007$ ), *i.e.* relatives who carried this genotype were protected from diabetes even when positive for multiple autoantibodies.

## Discussion

HLA influences progression to T1DM and disease risk can be assigned to a given haplotype or genotype (11). However, it is controversial whether HLA influences the development of positive autoantibodies, the subsequent progression to T1DM, or both stages (12–15). With the screening by DPT-1 of more than 90,000 relatives of T1DM patients for cytoplasmic ICAs (2), we have had the opportunity to analyze HLA DQA1 and DQB1 haplotypes and genotypes of 2,817 ICA+ relatives. This unique data set allowed us to analyze HLA haplotypes and genotypes (combinations of both HLA haplotypes) and their influence on autoantibody expression and progression to diabetes.

As expected, high-risk haplotypes, such as DQA1\*0301-DQB1\*0302 and DQA1\*0501-DQB1\*0201, were the most prevalent among these ICA+ relatives. However, 8% of the relatives had the protective DQA1\*0102-DQB1\*0602 haplotype. The presence of this protective haplotype among ICA+ relatives, at risk for T1DM, is consistent with prior reports (9, 14–16) and confirms that DQA1\*0102-DQB1\*0602-associated protection against autoantibody expression and progression to diabetes is not absolute.

Prevalence of expression of multiple biochemical autoantibodies was highest among relatives who carried DQA1\*0301-DQB1\*0201 (in LD with DR9) (57% prevalence) or DQA1\*0301-DQB1\*0302 (in LD with DR4) (49%), whereas it was 38% for DQA1\*0501-DQB1\*0201 relatives. However, the genotypic combinations associated with the highest prevalence of multiple biochemical autoantibodies were DQA1\*0301-DQB1\*0302/DQA1\*0501-DQB1\*0201 (59%) and DQA1\*0301-DQB1\*0302/DQA1\*0301-DQB1\*0302 (57%). The DQA1\*0501-DQB1\*0201 (DR3) haplotype, in other DR3/non-DR4 genotypes, was associated with a significantly lower risk of having multiple autoantibodies.

The risk of T1DM significantly and progressively increased with an increasing number of autoantibodies expressed (*e.g.* no, one, two, three, and four) when the whole study population was considered (regardless of HLA genotype) and also when carriers of a given haplotype or genotype were separately analyzed (as shown in Fig. 2B). Overall, the influence of HLA genotypes on the prevalence of autoantibody positivity and on diabetes risk was similar in direction and magnitude, as shown in Fig. 4. Of note, among multiple autoantibody-positive relatives, progression to T1DM was not different by HLA genotype. Furthermore, multivariate analyses indicated that the influence of most genotypes was not independent from the expression of autoantibodies (Table 2). The number of single or multiple autoantibody-positive relatives may have been larger than we measured, given the relatively low sensitivity of the mIAA assay. Taken together, our observations support the hypothesis that most HLA DQ genotypes influenced the development of autoantibodies in relatives of patients with T1DM, but in relatives expressing multiple autoantibodies,

T1DM risk or protection was not further determined by HLA DQ.

DQA1\*0102-DQB1\*0602 might be an exception: it decreased the risk of single and multiple autoantibody expression, with 80% of relatives with this haplotype being autoantibody negative and only 8% of them being positive for multiple biochemical autoantibodies. It also decreased the risk of diabetes, and we found that this effect was independent from the number of positive autoantibodies. However, it should be noted the small numbers of relatives who carried this haplotype and expressed autoantibodies, and thus, these findings warrant additional studies. In addition, the protective effect was not absolute as some relatives expressed autoantibodies and progressed to diabetes.

Less common haplotypes such as DQA1\*0201-DQB1\*0303 (in LD with DR7) or DQA1\*0101-DQB1\*0503 (DR14) have been previously reported as protective against  $\beta$ -cell autoimmunity (17, 18). We confirmed the protective effect of both haplotypes for autoantibody expression. However, we could not find a significant difference in diabetes risk among relatives with DQA1\*0101-DQB1\*0503. This may be due to small numbers of those individuals or to a weaker protective effect of DQA1\*0101-DQB1\*0503, compared with DQA1\*0102-DQB1\*0602. Alternatively, it may be due to a nondominant effect of this protective haplotype when paired with a high-risk haplotype because in our study population of ICA+ relatives, most subjects with a protective haplotype still had DQA1\*0501-DQB1\*0201 or DQA1\*0301-DQB1\*0302 on the other chromosome. None of the genotypes containing DQA1\*0201-DQB1\*0303 or DQA1\*0101-DQB1\*0503 seemed to influence T1DM risk independently from autoantibody expression. As stated above, this is in contrast to patients carrying DQA1\*0102-DQB1\*0602, who even when autoantibody positive, may be protected from progression to T1DM within the time frame of this study.

The possible protection afforded by DQA1\*0102-DQB1\*0602 seems to be dominant in that even when paired with high-risk haplotypes (DQA1\*0301-DQB1\*0302 or DQA1\*0501-DQB1\*0201), this haplotype still decreased the expression of biochemical autoantibodies and the risk of progression to diabetes. Thus, the 5-yr diabetes risk in DQA1\*0102-DQB1\*0602/DQA1\*0301-DQB1\*0302 and DQA1\*0102-DQB1\*0602/DQA1\*0501-DQB1\*0201 relatives was, respectively, 2 and 3%. Due to the previously described high prevalence of abnormal glucose tolerance in this population, (3), it is possible that progression to disease in those antibody-positive relatives will occur later.

The data indicate that some alleles, even those with the reported protective aspartic acid at position 57 (19, 20), can be associated with high risk of autoantibody expression and progression to diabetes in a genotypic-specific manner, *i.e.* depending on the identity of the second haplotype. An example of how a genotype may confer a risk that is different from that predicted by the two haplotypes expressed is DQA1\*0501-DQB1\*0201/DQA1\*0301-DQB1\*0302, the highest risk heterozygous genotype. Given the large data set, we could analyze a number of less common genotypes with the indication that specific combinations of haplotypes were particularly pathogenic or protective. For example, DQA1\*0201-DQB1\*0201 (DR7) combined with DQA1\*0501-DQB1\*0201 was associated with lower prevalence of expression of multiple biochemical autoantibodies (25%) and diabetes risk (7%

at 5 yr) than when the same haplotype DQA1\*0201-DQB1\*0201 is combined with DQA1\*0301-DQB1\*0302 (41% multiple autoantibody positivity, 28% diabetes risk) or even with the neutral DQA1\*0101-DQB1\*0501 (22% multiple autoantibody positivity, 18% diabetes risk). Similarly, DQA1\*0301-DQB1\*0301 combined with DQA1\*0501-DQB1\*0201 was less diabetogenic (5-yr risk:14%) than DQA1\*0301-DQB1\*0301/DQA1\*0301-DQB1\*0302 (27%), DQA1\*0301-DQB1\*0301/DQA1\*0101-DQB1\*0501 (36%), or DQ0301-DQB1\*0301/DQA1\*0301-DQB1\*0301 (31%). Of note, as illustrated in Table 2, DQA1\*0501-DQB1\*0201 combined with DQA1\*0301-DQB1\*0301, DQA1\*0501-DQB1\*0301, or DQA1\*0201-DQB1\*0201 had a low diabetogenic effect, independent from antibody expression.

In conclusion, the findings in the current study indicate that HLA DQ haplotypes and genotypes predict biochemical autoantibody expression. In relatives expressing multiple autoantibodies, diabetes risk or protection was not further determined by HLA DQ, with the possible exception of DQA1\*0102-DQB1\*0602. Specific combinations of haplotypes (genotypes) were particularly protective (*e.g.* DQA1\*0201-DQB1\*0201/DQA1\*0501-DQB1\*0201 and DQA1\*0301-DQB1\*0301/DQA1\*0501-DQB1\*0201) for autoantibody expression and progression to T1DM.

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