**Sequence Analysis of the Diabetes-Protective Human Leukocyte Antigen-DQB1*0602 Allele in Unaffected, Islet Cell Antibody-Positive First Degree Relatives and in Rare Patients with Type 1 Diabetes**

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**ABSTRACT**

The human leukocyte antigen (HLA)-DQA1*0102/DQB1*0602/DRB1*1501 (DR2) haplotype confers strong protection from type 1 diabetes. Growing evidence suggests that such protection may be mostly encoded by the DQB1*0602 allele, and we reported that even first degree relatives with islet cell antibodies (ICA) have an extremely low diabetes risk if they carry DQB1*0602. Recently, novel variants of the DQB1*0602 and *0603 alleles were reported in four patients with type 1 diabetes originally typed as DQB1*0602 with conventional techniques. One inference from this observation is that DQB1*0602 may confer absolute protection and may never occur in type 1 diabetes. By this hypothesis, all patients typed as DQB1*0602 positive with conventional techniques should carry one of the above diabetes-permissive variants instead of the protective DQB1*0602. Such variants could also occur in ICA/DQB1*0602-positive relatives, with the implication that their diabetes risk could be significantly higher than previously estimated. We therefore sequenced the DQB1*0602 and DQA1*0102 alleles in all ICA/DQB1*0602-positive relatives (n = 8) previously described and in six rare patients with type 1 diabetes and DQB1*0602. We found that all relatives and patients carry the known DQB1*0602 and DQA1*0102 sequences, and none of them has the mtDNA A3243G mutation associated with late-onset diabetes in ICA-positive individuals. These findings suggest that diabetes-permissive DQB1*0602/3 variants may be very rare. Thus, although the protective effect associated with DQB1*0602 is extremely powerful, it is not absolute. Nonetheless, the development of diabetes in individuals with DQB1*0602 remains extremely unlikely, even in the presence of ICA, as confirmed by our further evaluation of ICA/DQB1*0602-positive relatives, none of whom has yet developed diabetes. (J Clin Endocrinol Metab 84: 1722–1728, 1999)

**T**ype 1 diabetes is an autoimmune disease resulting in pancreatic β-cell destruction and absolute insulin deficiency (1, 2). The disease mostly develops in genetically susceptible individuals, and at least 50% of the genetic risk of developing diabetes is conferred by the IDDM1 susceptibility locus, mapped to specific alleles of the human leukocyte antigen (HLA)-DQ and DR loci (3, 4). These highly polymorphic loci encode the HLA-DQ and -DR class II antigens, heterodimers known to play a key role in antigen presentation and immune recognition (5). In Caucasians, the HLA-DQ molecules encoded by the DQA1*0301/DQB1*0302 and DQA1*0501/DQB1*0501 alleles on DR4 and DR3 haplotypes have the strongest association with the disease (6–9), although DRB1 alleles significantly modulate DR4-associated susceptibility (9–15).

In contrast, HLA-DR2 haplotypes are rarely observed among patients with type 1 diabetes. Most of the HLA-DR2-associated type 1 diabetes in Caucasians is accounted for by three neutral haplotypes: DQA1*0102/DQB1*0502/DRB1*1601, DQA1*0103/DQB1*0601/DRB1*1501, and DQA1*0103/DQB1*0601/DRB1*1502 (6, 16–20). The DQA1*0102/DQB1*0602/DRB1*1501 haplotype, the most common DR2 haplotype among Caucasians, is the only DR2 haplotype conferring dominant and almost absolute protec-

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tion from type 1 diabetes among Caucasian and other racial groups. Indeed, patients carrying this haplotype are extremely rare (4, 6, 16, 18–30). The characterization of both common and rare recombinant DR2 haplotypes observed in patients with type 1 diabetes also indicates that DQB1*0602 is the only class II allele exclusively found on diabetes-protective DR2 haplotypes. This suggests that protection is mostly, although not exclusively, conferred by DQB1*0602 (which together with DQA1*0102 codes for a protective DQ heterodimer) (18, 31, 32). Moreover, our previous studies indicate that DQB1*0602 confers strong diabetes protection even among islet cell antibodies (ICA)-positive first degree relatives of patients with type 1 diabetes (33). In fact, approximately 7% of ICA-positive relatives identified through autoantibody screening carry DQB1*0602, and in our family study, none of such relatives has developed diabetes on follow-up. Because of the apparent dominance of the protective effect (19, 20) and the extremely low risk ascertained in prospective studies (33, 34), ICA/DQB1*0602-positive first degree relatives are presently excluded from receiving treatment in the Diabetes Prevention Trial–Type 1, a major ongoing trial involving several centers in the United States (35).

Of interest, a few novel variants of the DQB1*0602 and DQB1*0603 alleles were recently reported by Hoover et al. (36) in four rare patients originally typed as DQB1*0602-positive with conventional sequence-specific oligonucleotide (SSO) typing techniques (20). Such variants, not distinguished by the initial panel of SSO probes, appear to be permissive for the development of diabetes. This observation suggests the hypothesis that DQB1*0602 may confer absolute protection and may never occur in patients with type 1 diabetes. If this hypothesis is, in fact, correct, all or most of those rare patients typed as DQB1*0602 with conventional techniques could carry one of the above diabetes-permissive variants instead of the protective DQB1*0602. It is also conceivable that such variants may occur in the previously described ICA/DQB1*0602-positive relatives (33); if so, this could have significant prognostic implications, as their risk of developing diabetes may be higher than previously estimated.

In addition, a mitochondrial DNA mutation (mtDNA A3243G) has been recently associated with the development of diabetes in ICA/DQB1*0602-positive individuals.

We therefore investigated the occurrence of diabetes-permissive DQB1*0602 variants and the mtDNA A3243G mutation in the eight ICA-positive relatives previously identified (33) and in six rare patients with type 1 diabetes, all typed as DQB1*0602-positive with conventional techniques.

### Subjects and Methods

#### Subjects

Unaffected ICA/DQB1*0602-positive first degree relatives (n = 8) were identified through autoantibody screening of family members of patients with type 1 diabetes at the Joslin Diabetes Center and the Barbara Davis Center for Childhood Diabetes, as previously reported (33). All relatives were Caucasians. Their clinical characteristics and updated follow-up information are shown in Table 1. ICA positivity was defined as measurements of 20 Juvenile Diabetes Foundation units or more on at least two occasions, and the ICA titer shown in Table 1 is the highest level observed for each individual.

We also studied six DQB1*0602-positive patients, four Caucasians and two African-Americans, diagnosed as having type 1 diabetes (Table 2) according to the criteria of the National Diabetes Data Group (42). The diagnosis was confirmed in four patients by the presence of autoantibodies against islet cell antigens (serum was unavailable for testing the other two patients). Table 2 shows the autoantibody profiles for the four patients tested (two Caucasians and two African-Americans). One of the African-American patients (no. 17865) also had stiff-man syndrome (SMS) and developed diabetes at age 35 yr (43, 44). SMS is a rare neurological disorder reportedly associated with type 1 diabetes and autoimmune responses to the GAD65 autoantigen (glutamic acid decarboxylase, 65-kDa isof orm), as in this case (45, 46). This patient was particularly interesting and unusual because he developed diabetes despite having DQB1*0602, an allele that we reported as diabetes protective among patients with SMS (43, 44). The remaining two Caucasian patients with DQB1*0602 were identified through the Human Biological Data Interchange repository, a collection of families with type 1 diabetes (47), as already mentioned, no serum samples from these patients were available for autoantibody testing.

#### HLA typing

Genomic DNA samples were extracted from heparinized blood samples with phenol/chloroform. HLA typing for DQA1, DQB1, and DRB1 alleles was performed on all subjects by the PCR and hybridization with SSO probes as previously described (4, 33). For one patient, DRB1 alleles were determined by direct sequencing. The second exon of the DRB1 gene was amplified using forward primers for the DRB1*15/16 or DRB1*04 subtypes paired with a generic DRB1 reverse primer in separate PCR reactions. PCR products were cloned and sequenced with the same strategy described below for DQA1*0102 and DQB1*0602 alleles.

### TABLE 1. Clinical characteristics of unaffected, ICA/DQB1*0602-positive first degree relatives of patients with type 1 diabetes

<table>
<thead>
<tr>
<th>Subject ID</th>
<th>Sex</th>
<th>DQA1</th>
<th>DQB1</th>
<th>DRB1</th>
<th>DR</th>
<th>ICA &lt;20</th>
<th>IAA &lt;42</th>
<th>GAD65 &lt;0.032</th>
<th>ICA512 &lt;0.071</th>
<th>Follow-up (yr)</th>
<th>Age (yr)</th>
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<tbody>
<tr>
<td>9280</td>
<td>M</td>
<td>0102/0201</td>
<td>0602/0201</td>
<td>1501/0701</td>
<td>2/7</td>
<td>80</td>
<td>29</td>
<td>0.001</td>
<td>0.001</td>
<td>8.1</td>
<td>41.2</td>
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<td>726</td>
<td>M</td>
<td>0102/0501</td>
<td>0602/0301</td>
<td>1501/0301</td>
<td>2/3</td>
<td>640</td>
<td>273</td>
<td>1.260</td>
<td>0.002</td>
<td>12.8</td>
<td>19.7</td>
</tr>
<tr>
<td>1371b</td>
<td>F</td>
<td>0102/0301</td>
<td>0602/0302</td>
<td>0301/0401</td>
<td>2/4</td>
<td>320</td>
<td>490</td>
<td>1.130</td>
<td>1.148</td>
<td>12.2</td>
<td>55.1</td>
</tr>
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<td>0602/0302</td>
<td>1501/0401</td>
<td>2/4</td>
<td>320</td>
<td>490</td>
<td>1.130</td>
<td>1.148</td>
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<td>0602/0501</td>
<td>1501/0101</td>
<td>2/1</td>
<td>640</td>
<td>29</td>
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<td>0.003</td>
<td>14.2</td>
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<td>0.007</td>
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<td>0.002</td>
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<td>0602/0302</td>
<td>1501/0404</td>
<td>2/4</td>
<td>640</td>
<td>4</td>
<td>0.110</td>
<td>0.001</td>
<td>8.5</td>
<td>49.4</td>
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</tbody>
</table>

*a Subject 1271 has an unusual haplotype, DR3, DQB1*0602.

*b Subject 8974 was followed for 4.3 yr and then entered a pilot insulin preventive trial before his HLA type was available. He remains nondiabetic, but his last follow-up date remains fixed to the day he entered the trial.
TABLE 2. Clinical characteristics of rare DQB1*0602-positive patients with type 1 diabetes

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Race</th>
<th>Age of onset (yr)</th>
<th>Autoantibodies</th>
<th>HLA genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>IAA &lt;42 GAD65 &lt;0.032 ICAl52 &lt;0.071</td>
<td>DQA1 DQB1 DRB1 DR</td>
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<tr>
<td>209365</td>
<td>C</td>
<td>9</td>
<td>1492 0.083 0.001</td>
<td>0102/0501 0602/0201 1501/0301 2/3</td>
</tr>
<tr>
<td>211688</td>
<td>AA</td>
<td>21</td>
<td>873 1.377 0.840</td>
<td>0102/0501 0602/0201 1501/0301 2/3</td>
</tr>
<tr>
<td>17865</td>
<td>AA</td>
<td>35</td>
<td>2 1.333 0.001</td>
<td>0102/0301 0602/0201 1503/0901 2/9</td>
</tr>
<tr>
<td>19810104</td>
<td>C</td>
<td>11.7</td>
<td>ND 1.106 0.976</td>
<td>0102/0301 0602/0302 1501/0401 2/4</td>
</tr>
<tr>
<td>HBDI 01128</td>
<td>C</td>
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<td>ND ND ND ND</td>
<td>0102/03 0602/0302 1504/0404 2/4</td>
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<td>C</td>
<td>25</td>
<td>ND ND ND ND</td>
<td>0102/03 0602/0302 1504/0404 2/4</td>
</tr>
</tbody>
</table>

ND, Not determined.

Sequencing exon 2 of the DQB1*0602 and DQA1*0102 alleles

The second exon of both the DQA1 and DQB1 genes was sequenced, because this exon encodes the extracytoplasmic polymorphic regions of the HLA-DQ molecule. The DQA1 gene was amplified from genomic DNA by PCR with primers 0102ex2-F 5′-CT GAC CAC GTC TCC TGT TGT-3′ and 0102ex2-R 5′-ATT GGT AGC AGC GGT AGA GTT-3′. Primers were selected at the 5′- and 3′-termini of the second exon (intron sequences are not currently available) and amplify a 150-bp product (annealing, 58°C). The second exon of the DQB1 gene was amplified with primers 0102ex2-F 5′-TC CCC GCA GAG GAT TTC GTG T-3′ and 0102ex2-R 5′-TCC TGC AGG GCC GCG CTC ACC TCT CC-3′ (annealing, 60°C). These primers amplify a 375-bp product including 8 and 18 bp of intronic sequences at the 5′- and 3′-ends, respectively, and allow distinction of the polymorphism reported at codon 9 by Hoover et al. and Williams et al. (36, 48), Primers MADC4 5′-CCT GAC TGA CCG GCC GGT GTG-3′ and UG71 5′-ACA TGT AAA AGG AGC GGG CAG TCT CCT TGT CAG CAG CCG-3′ were also used and allowed discrimination of the codon 9 polymorphism as well (298-bp product; annealing, 55°C). The PCR products were cloned into the TA Cloning Vector (Invitrogen, San Diego, CA). DNA samples extracted from transformed colonies were screened by PCR using sequence-specific primers for the DQB1*0602 and DQA1*0102 alleles (PCR-SSP) as described by Olerup et al. (49). DNA samples from DQB1*0602- and DQA1*0102-positive colonies were then sequenced (both strands, using the SP6 and T7 primers). PCR products generated with the MADC4/UG71 primer pair were sequenced directly using MADC4 as primer and dye terminators FS (ABI). The other strand of each product PCR was also sequenced as a control using the dye primer FS kit (ABI, Foster City, CA) containing the −21M13 primer. Sequence analysis was performed on an ABI 373 automated sequencer.

Typing for the mtDNA A3243G gene mutation

Typing for the A3243G transfer ribonucleic acid Leu (UUR) mutation was performed by PCR-restriction fragment length polymorphism as previously described (50). In brief, approximately 0.5 µg genomic DNA was subjected to PCR amplification (94°C for 60 s, 55°C or 60 s, 72°C for 45 s; 25 cycles) using primers for the mtDNA light strand positions 3116–3134 and heavy strand positions 3353–3333 (51). The DNA fragment produced by the PCR reaction was digested with the restriction enzyme HaelII for 2 h at 37°C. The A→G transition at position 3243 creates a new HaelII site that is diagnostic for the mutation (50). The digestion products were electrophoresed on a 12% nondenaturing polyacrylamide gel and stained with ethidium bromide. This technique can detect mitochondrial mutations with low degree of heteroplasmy in peripheral blood (as low as 5%, below which any significant influence on the mitochondrial function in more relevant tissues is unlikely).

Autoantibody testing

The eight unaffected ICA/DQB1*0602-positive relatives were tested for the presence of ICA with a standardized assay as previously described (33, 52). ICA positivity was defined as measurements of 20 CIE & M • 1999 Vol 84 • No 5

Additional follow-up and autoantibody testing in ICA/DQB1*0602-positive first degree relatives

All of the previously identified ICA/DQB1*0602-positive relatives were tested to determine whether they express other autoantibodies besides ICA. The upper limits of normal are 42 nU/mL for IAA and indexes of 0.032 and 0.071 for phatase-like ICA512 (or IA-2). Insulin autoantibodies (IAA) were determined with a fluid phase RIA using 600 µL serum, with duplicate determinations with and without unlabeled insulin for competition (53). The interassay coefficient of variation for the IAA assay is 10.3% at low positive values. The assay had a specificity of 91% and a sensitivity of 49% for new-onset patients less than age 30 yr in the 1995 Immunology of Diabetes Society (IDS) Combinatorial Workshop. Autoantibodies to GAD65 were measured in triplicate by RIA, using in vitro transcribed and translated GAD65 (clone provided by A. Lemmark) (54, 55). Autoantibody-bound GAD65 was precipitated with protein A-Sepharose. The interassay coefficient of variation of this assay is 6.5%. Assay specificity was 99%, and sensitivity was 83.7% for new-onset patients less than age 30 yr at the IDS Combinatorial Workshop. Autoantibodies to ICA512 were measured using the in vitro transcribed and translated labeled product of a clone termed ICA512bdc (amino acids 256–979 of ICA512/IA-2) (55–58). Autoantibodies were measured in triplicate with protein A-Sepharose precipitation. This assay gave a specificity of 100% and a sensitivity of 74.4% for new-onset patients less than age 30 yr at the IDS Combinatorial Workshop. GAD65 and ICA512 autoantibody levels are expressed as an index calculated from the counts per min for the test sample and the positive and negative control samples. GAD65 and ICA512 autoantibodies were determined simultaneously with differential labeling (125I for ICA512 and 124H for GAD65) in an automated 96-well β-counter.

Results

Sequencing exon 2 of the DQB1*0602 and DQA1*0102 alleles

We studied eight unaffected, ICA-positive first degree relatives (Table 1) and six rare patients with type 1 diabetes (Table 2), all previously typed as DQB1*0602 positive with standard SSO typing techniques. We performed direct sequence analysis of the second exon of their DQB1 and DQA1 alleles to investigate whether any of the diabetes-protective DQB1*0602 variants recently described (36) occurred among these subjects. All subjects carried conventional DQB1*0602 and DQA1*0102 exon 2 sequences. Thus, all subjects carry DQA1 and DQB1 alleles coding for a diabetes-protective HLA-DQ heterodimer.

Typing for the mtDNA A3243G gene mutation

None of the subjects studied carried the mtDNA A3243G mutation reportedly associated with the development of late-onset diabetes in ICA-positive individuals.
GAD65 and ICA512 autoantibodies, respectively. Autoantibody levels shown in Tables 1 and 2 are the mean levels for each individual. As illustrated in Table 1, seven of eight relatives have GAD65 autoantibodies but only two of eight and one of eight have autoantibodies against insulin and ICA512, respectively. None of these relatives have developed type 1 diabetes to date with further extended follow-up (Table 1; mean follow-up ± sd, 8.55 ± 4.93 yr; a mean increase of 2.6 yr in follow-up length since our first report) (33).

Discussion

The HLA-DQB1*0602 allele confers strong diabetes protection, even among ICA-positive first degree relatives of patients with type 1 diabetes (33). In our family study, approximately 7% of ICA-positive relatives were found to carry DQB1*0602 using standard SSO typing techniques. However, such techniques cannot distinguish DQB1*0602 from the putative diabetes-permissive DQB1*0602/3 variants recently described by Hoover et al. (36). Moreover, a form of slowly progressive diabetes has been associated with late age of onset in Japanese ICA-positive patients carrying the mtDNA A3243G mutation (37–39). The presence of such DQB1*0602 variants or of the above mtDNA mutation may have significant diagnostic implications, as the risk of diabetes for ICA/DQB1*0602-positive relatives would certainly be higher than previously estimated. We therefore investigated the occurrence of diabetes-permissive DQB1*0602 variants and of the mtDNA A3243G mutation among all the previously identified, unaffected, ICA-positive first degree relatives, all identified as DQB1*0602 positive (33). We found that all ICA-positive relatives studied have conventional sequences for both DQB1*0602 and DQA1*0102, thus coding for a protective HLA-DQ heterodimer. Moreover, none of them carries the mtDNA A3243G mutation reportedly associated with late-onset diabetes in ICA-positive subjects (37).

Among ICA/DQB1*0602-positive relatives, 7 of 8 have GAD65 autoantibodies but only 2 of 8 and 1 of 8 have autoantibodies against insulin and ICA512, respectively (Table 1). Thus, ICA reactivity appears to be mainly directed against GAD65 in most ICA/DQB1*0602-positive relatives (59, 60). In contrast, GAD65 autoantibodies were detected only in 2 of 13 ICA-positive patients from Japan with the mtDNA A3243G mutation, none of whom had DQB1*0602 (37). As diabetes risk also correlates with the number of autoantigens targeted by antilcell immune responses (61), it is apparent that ICA/DQB1*0602-positive relatives express a low risk phenotype with only limited loss of tolerance to islet cell autoantigens (60). Indeed, despite ICA positivity, none of these relatives has developed type 1 diabetes to date during further extended follow-up. Moreover, most relatives (5 of 8) are now over 40 yr old. Together, these observations confirm that ICA/DQB1*0602-positive relatives are a distinct group of individuals with extremely low risk of diabetes, even at an older age. These findings validate our previous report that DQB1*0602 significantly protects from type 1 diabetes even in the presence of ICA/GAD65 autoantibodies (33), an observation that has significant implications for the design of prevention trials.

We also investigated the occurrence of diabetes-permissive DQB1*0602 variants in six rare patients with type 1 diabetes and DQB1*0602 (as defined with conventional typing techniques). Although a number of patients with DQB1*0602 may suffer from rare genetic forms of diabetes rather than type 1 diabetes [e.g. Wolfram’s syndrome, type 1 autoimmune polyendocrine syndrome, type 1B diabetes, and maturity onset diabetes of the young (MODY)], the clinical diagnosis of type 1 diabetes was confirmed in four of six patients by the presence of autoantibodies against ICA. The diagnosis was exclusively based on clinical criteria for the two patients identified through the Human Biological Data Interchange repository of families with type 1 diabetes (42). We did not observe the diabetes-permissive variants reported by Hoover et al. (36), and all six patients carry normal DQB1*0602 and DQA1*0102 sequences. Moreover, none of the patients has the A3243G mtDNA mutation. Thus, our findings do not support the hypothesis that the above variants may commonly occur and be specifically associated with diabetes development in conventionally typed DQB1*0602-positive subjects.

Our results also imply that the protective effect associated with DQB1*0602 is not absolute, as type 1 diabetes may develop in extremely rare cases in individuals carrying the DQB1*0602 allele. Perhaps other polymorphisms regulating the phenotypic expression of DQB1*0602, including polymorphisms in the DQA1/DQB1 promoter regions, or other genetic loci/environmental factors may modulate the protective effect associated with DQB1*0602. For instance, patient 17865, an African-American with diabetes and SMS, has the DRB1*1503 allele instead of the DRB1*1501 allele. The DRB1*1501 allele, usually found in linkage disequilibrium with DQB1*0602 on protective DR2 haplotypes, may theoretically contribute to the protective effect and, conversely, the DQB1*0602/DRB1*1503 combination may be less protective. However, the analysis of recombinant haplotypes suggests that most of the protection derives from the DQB1*0602 allele (18, 31, 32); moreover, one of the ICA-positive relatives (no. 1271) carries the unusual combination of DQB1*0602 in cis with the diabetes-predisposing DRB1*0301 allele (DR3) and has not developed diabetes despite having ICA/GAD65 autoantibodies for more than 12 yr. We also identified a patient with type 1 diabetes carrying DQB1*0603 in cis with DQA1*0102 and DRB1*1501, once again suggesting that DQB1*0602 is central to diabetes protection (data not shown).

In contrast to our findings, none of the five DQB1*0602-positive patients with type 1 diabetes originally described by Baisch et al. (20) were found to carry DQB1*0602 by Hoover et al. (36); one patient was initially mistyped and had DQB1*0603, and four patients carry alleles with a sequence closely related to that of DQB1*0602 (n = 1) and DQB1*0603 (n = 3). Of note, DQB1*0603 is usually found on haplotypes bearing the DQA1*0103 and DRB1*1301 (DR6) alleles, and such haplotypes are significantly less protective than the DQA1*0102/DQB1*0602/DRB1*1501 (DR2) haplotype. It remains unclear whether the DQB1*0603 variants described by Hoover (termed 0603a, 0603b, and 0603c) are linked to DRB1*1501 (DR2) or DRB1*1301 (DR6) alleles; however, in all instances those variants were found in cis with DQA1*0103,
and based on linkage disequilibrium, one would predict that none of those patients carries a DRB1*1501 (DR2) haplotype. The allele variant directly related to DQB1*0602, also reported by Williams et al. and now termed DQB1*0611 (48), differs from DQB1*0602 at codon 9, where a T→A substitution determines a change in the amino acid sequence (Phe→Tyr). Molecular modelling studies suggest that such sequence variation may alter the peptide-binding site of the HLA-DQ molecule (36), and it is also of interest that the same codon 9 polymorphism is shared with the most common susceptibility alleles (DQB1*0302 and DQB1*0201) and other diabetes-permissive alleles (DQB1*0502, DQB1*0604, and DQB1*0603c) (36, 62). However, the same polymorphism is also found in alleles that are not associated with increased susceptibility (i.e., DQB1*0603 and DQB1*0301), and susceptible alleles such as DQB1*0401 share codon 9 sequence with DQB1*0602 instead. It should also be noted that none of the diabetes-permissive variants differs from DQB1*0602 at positions 57 and 70, the combined variation of which reportedly modulates peptide binding and diabetes susceptibility (36, 62).

As regards the frequency of the diabetes-permissive variants, DQB1*0611 is extremely rare, as it was detected in only 1 of 30 African-Americans and 0 of 62 non-Hispanic/Hispanic whites with DQB1*0602 (48). No frequency data are available for the other variants (DQB1*0603a, DQB1*0603b, and DQB1*0603c), but one would speculate that these alleles are probably very rare, as they would have been reported much earlier had they occurred at a significant frequency in the population. This is consistent with our findings confirming the presence of DQB1*0602 in all of the relatives and patients studied. Nonetheless, given the significant prognostic implications associated with DQB1*0602, the current typing protocols should be modified to allow the unequivocal identification of DQB1*0602 when typing relatives of patients with type 1 diabetes. This can be easily achieved with sequence-specific primers that selectively amplify DQB1*0602 (PCR-SSP) with a strategy based on the protocols of Olerup et al. (49) and Williams et al. (48), as illustrated in Table 3.

It remains unclear whether DQB1*0602 protects from type 1 diabetes by affecting the shaping of the T cell repertoire in the thymus or by modulating immune responses in the extrathymic periphery. However, the two mechanisms are not mutually exclusive and may both be active in subjects with DQB1*0602. Our finding that the humoral antiislet immune response is mostly limited to the production of GAD65 autoantibodies in relatives with DQB1*0602 suggests that diabetes protection may be at least partially mediated by immunoregulatory mechanisms. Similarly, patients with the type 1 autoimmune polyendocrine syndrome expressing GAD65 autoantibodies do not invariably progress to overt diabetes (63). Type 1 diabetes is reportedly induced by T cells with a Th1 phenotype, and immune modulation strategies resulting in diabetes prevention in animal models are associated with the predominance of immune responses medi-

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The table shows the PCR SSPs designed by Olerup et al. (49) and the nucleotide differences that prevent PCR amplification of any DQB1*06 allele other than DQB1*0602 and DQB1*0611 using an annealing temperature of 65°C (identities with DQB1*0602 sequences are indicated by dashes). Similar to DQB1*0602, the DQB1*0603a, *0603b, and *0603c alleles will not be amplified with these primers. The DQB1*0611 allele can be distinguished with primers discriminating the codon 9 polymorphism as previously reported by Williams et al. (48). The presence of DQB1*0602 can be unequivocally confirmed by running two PCR reactions using both primer sets shown; in the presence of DQB1*0602, both PCR reactions should yield a PCR product as previously described (48, 49).
ated by type 2 helper T cells (64). Thus, anti-GAD65 auto-
toantibodies may be a marker of the predominance of the T
helper cell type 2 immune responses in relatives with
DQB1*0602 (65), and indeed some of our DQB1*0602-positi-
ve relatives were recently found to have higher levels of
circulating interleukin-4, a Th2 cytokine, than DQB1*0602-
negative relatives who progressed to overt diabetes on fol-
low-up (66). Genetic protection could also be mediated by
thymic deletion of autoreactive T lymphocytes. Although
several studies involving transgenic expression of MHC mol-
ecules in mice did not find evidence to support this hypoth-
esis (67–69), this explanation has been revived by a recent
study providing novel evidence for thymic deletion as a
mechanism of protection associated with MHC genes in
transgenic mice (70). Moreover, the recent demonstration
that several islet antigens (including insulin, GAD65,
ICA512, and ICA569) are expressed in human thymus during
development suggests that the DQA1*0102/DQB1*0602
heterodimer may influence the presentation of such antigens
to developing thymocytes and, in turn, affect the shaping of
the T cell repertoire (71, 72). Further studies will be necessary
to elucidate the diabetes-protective effect associated with the
DQB1*0602 allele in humans.

In conclusion, we show that all the ICA/DQB1*0602-posi-
tive relatives previously described carry conventional
DQB1*0602 and DQA1*0102 sequences coding for a protec-
tive heterodimer. Such relatives appear to have a low risk of
type 1 diabetes, as also confirmed by their autoantibody
profiles and extended follow-up data presented. Thus, our
findings provide additional confirmation for the dramatic
protective effect associated with DQB1*0602, even among
relatives with autoantibody positivity. However, our finding
that DQB1*0602 is present in rare patients with type 1 dia-
betes suggests that protection is not absolute, perhaps be-
cause of other unknown genetic or environmental factors
that may modulate the protective mechanisms associated
with DQB1*0602. Finally, although putative diabetes-per-
missive DQB1*0602 variants appear to be extremely uncom-
mon, we suggest a simple typing strategy, based on the
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Acknowledgments

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References

1. The Expert Committee on the Diagnosis and Classification of Diabetes
Mellitus. 1997 Report of the expert committee on the diagnosis and classifi-
susceptibility and resistance. In: Eisenbarth GS, Lafferty KJ, eds. Type I dia-
betes. Molecular, cellular, and clinical immunology. New York, Oxford: Ox-
ford University Press; 134–152.
of HLA class II genes in insulin-dependent diabetes mellitus: molecular
5. Nepom GT, Erlich H. 1991 MHC class-II molecules, and autoimmunity. Annu
6. Todd JA, Bell JL, McDevitt HO. 1987 HLA-DQ β gene contribution to sus-
ceptibility and resistance to insulin dependent diabetes mellitus. Nature.
of the HLA-DQ loci: relationship to serology, and to insulin-dependent dia-
8. Morel PA, Dorman JS, Todd JA, McDevitt HO, Trucco M. 1988 Aspartic acid at
position 57 of the HLA-DQ β chain protects against type 1 diabetes: a family
lotype is best defined by a combination of HLA-DR, and DQ alleles. J Clin
Invest. 83:830–835.
HLA-DQA sequence polymorphism and genetic susceptibility to IDDM. Di-
betes. 39:96–103.
11. Cold mond BP, Varney MD, Harrison LC. 1995 HLA-DRB1*0401 is
associated with susceptibility to insulin-dependent diabetes mellitus indepen-
12. Cucca F, Muntoni F, Lampis R, et al. 1993 Combination of specific DRB1,
DQA1, DQB1 haplotypes are associated with insulin-dependent diabetes mel-
Different contribution of HLA-DR and -DQ genes in susceptibility and resis-
tance to insulin-dependent diabetes mellitus (IDDM). Tissue Antigens.
molecular properties in a population-based study of Swedish childhood di-
predisposition in IDDM: DR4 subtypes may be associated with different de-
neutral effect of DR2 on IDDM susceptibility in central Italy. Diabetes.
41:904–908.
 tended haplotype is involved in insulin-dependent diabetes mellitus sus-
18. Zeliszewski D, Tiercy J, Boitard C, et al. 1992 Extensive study of DRβ, DQα,
and DQβ gene polymorphism in 23 DR2-positive, insulin-dependent diabetes
in insulin-dependent diabetes mellitus among Blacks, Caucasoids, and Jap-
and their contribution to diabetes susceptibility and resistance in a Swedish
negative and HLA-DQA Arg52 confers susceptibility to insulin-dependent
22. Ronningen KS, Spurkland A, Iwe T, Vartdal F, Thorsby E. 1991 Distribution of
HLA-DRB1,-DQA1 and -DQB1 alleles and DQA1-DQB1 genotypes among
Norwegian patients with insulin-dependent diabetes mellitus. Tissue Antigens.
31:105–111.
HLA-DQ primarily confers protection and HLA-DR susceptibility in type I
(insulin-dependent) diabetes studied in population-based affected families and
24. Hagopian WA, Sanjeevi CB, Kockum I, et al. 1995 Glutamate decarboxylase,
insulin, islet cell antibodies, and HLA typing to detect diabetes in a general
25. Awata T, Kuzuya T, Matsuda A. 1990 High frequency of aspartic acid at
position 57 of HLA-DQ β-chain in Japanese IDDM patients and nondiabetic
chain reaction of histocompatibility leucocyte antigen-DR9-linked suscepti-
blity to insulin-dependent diabetes mellitus. J Clin Endocrinol Metab.
75:1381–1385.
27. Penny MA, Jenkins D, Mijovic CH, et al. 1992 Susceptibility to IDDM in a
HLA-DQA1 and -DQB1 alleles associated with genetic susceptibility to IDDM
29. Sanjeevi CB, Zeidler A, Shaw S, et al. 1993 Analysis of HLA-DQA1 and -DQB1
genes in Mexican Americans with insulin-dependent diabetes mellitus. Tissue
30. Erlich HA, Zeidler A, Chang J, et al. 1993 HLA class II alleles, and suscep-
tibilities of European and Asian populations to insulin dependent diabetes
Implication of specific DQB1 alleles in genetic susceptibility and resistance by identification of IDDM siblings with novel HLA-DQB1 allele and unusual DR2 and DR1 haplotypes. Diabetes. 40:478–481.


