Primary Association of HLA-DQw8 With Type I Diabetes in DR4 Patients

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DNA from 164 Caucasian type I (insulin-dependent) diabetic patients and 200 Caucasian nondiabetic control blood donors were analyzed by the polymerase chain reaction technique for HLA-DR4 and the associated Dw and DQB subtypes of DR4. The DQw8 subtype of HLA-DR4 was associated with type I diabetes in all DR4 subgroups (DR4/3 and DR4/non-3). Dw subtypes of DR4 other than Dw10 did not confer additional association with type I diabetes. Thus, the DQ region appears to provide the primary major histocompatibility association with type I diabetes in most DR4 patients. *Diabetes* 38:942–45, 1989

The serological type HLA-DR4 is associated with five alleles of the DRB1 locus (Dw4, Dw10, Dw13, Dw14, and Dw15) and two alleles of the DQB1 locus (DQw7 and DQw8) (1,2). The DQw8 serological specificity, previously called DQw3.2, is associated with type I (insulin-dependent) diabetes, whereas the DQw7 specificity, previously called DQw3.1, is not associated with type I diabetes (3–9). Tait et al. (10) recently reported that the DQw8 association exists only in type I diabetic individuals who type as HLA-DR4/3. DR4 subtypes (Dw4 and Dw10) are also associated with type I diabetes (11–13); however, the role of other Dw specificities remains controversial (11–14).

To further clarify the specific major histocompatibility associations with type I diabetes, we typed 164 type I diabetic patients, of which 120 typed as HLA-DR4, and 200 blood donor controls, of which 55 typed as HLA-DR4, for DQB1 and DRB1 subtypes. The association of DQw8 and type I diabetes was seen in all DR4 subgroups analyzed (DR4/3 and DR4/non-3). The distribution of Dw subtypes, other than Dw10, was similar in our HLA-DR4 type I diabetic patients and nondiabetic control subjects. With these data, we calculated that the absolute risks for type I diabetes in unrelated individuals homozygous for DR4 (DQw8) or heterozygous for DR4 (DQw8) /DR3 (DQw2) are 6.7 and 8.5%, respectively, frequencies approaching those seen in family studies (14). Thus, the presence of DQw8 appears to be the primary association with type I diabetes in DR4 patients.

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RESEARCH DESIGN AND METHODS

Subjects. Eighty type I diabetic subjects were patients enrolled and followed in the Diabetes Control and Vascular Disease Study (Boston, MA; 15–18). An additional 84 type I diabetic subjects were patients followed at the Diabetes Center at Texas Children's Hospital (Houston, TX). All 164 were unrelated Caucasian subjects with onset of diabetes at <18 yr of age. Because the frequencies of the DRB1 and DQB1 loci and ages at diagnosis for type I diabetes did not differ between the Boston and Houston patients, the two patient populations were considered together in comparisons with the control population. Control subjects were 200 blood donors provided by the Gulf Coast Regional Blood Center in Houston, Texas. All blood donors were Caucasian and >18 yr of age.

Polymerase chain reaction (PCR) amplification. DNA was isolated from peripheral blood leukocytes or transformed lymphoblastoid cell lines by standard procedures (19). DNA (1 μg) was amplified with the PCR (20) with *Taq* I polymerase, an automated DNA thermal cycler, and reactions were described by the manufacturer (Perkin-Elmer-Cetus, Norwalk, CT). The following primers were used in separate amplification reactions: DQA (5′-GGA TCC ATT GGT GGC AGC GGT AGA, 5′-GAA TTC TAT GTG GAC CTG GAGA), DQB (5′-TGC TAC TTC ACC AAC GGG AC, 5′-GGT AGT TGT GTC TGC ACA CC), and DRB (5′-GGA GCA GGT TAA ACA TGAG, 5′-TCG CCG CTG CAC TGT GAA GC). The DQA and DQB primers are common to all the subtypes of DQA

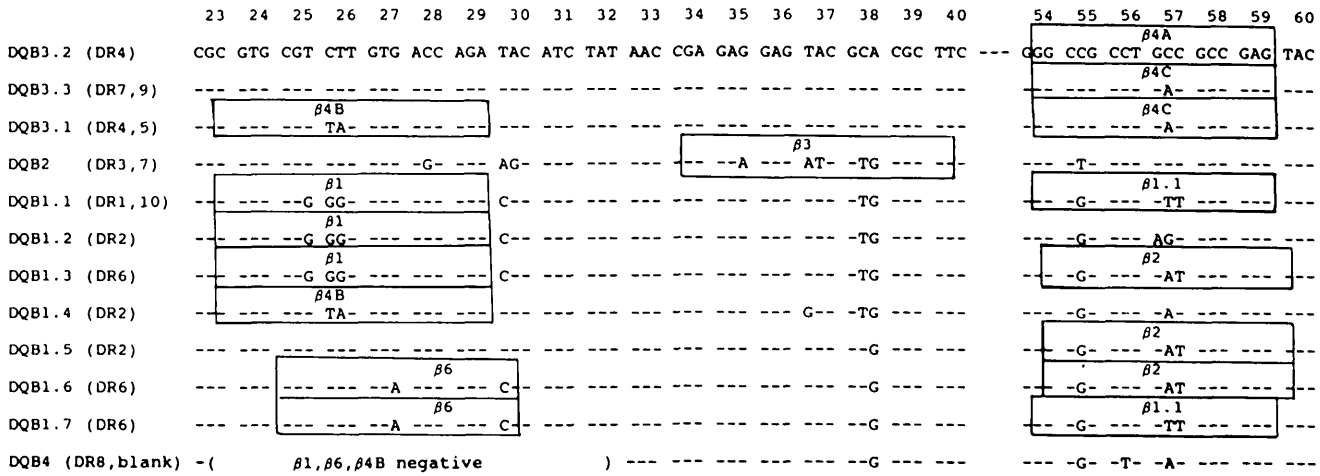

FIG. 1. DQB oligonucleotide probe sequences. Nucleotide sequences are shown for exon 2 of DQB3.2 gene encoding amino acids 23–40 and 54–60. Nucleotide differences are shown for 11 other DQB genes. *Boxed regions* were used to make oligonucleotide probes. Associated DR types are shown for each DQB gene.

and DQB, whereas the DRB primers are specific for the DR4 subtype. Thirty rounds of cycling (denaturation, 1 min at 93°C; annealing, 2 min at 60°C; and extension, 3 min at 72°C) were carried out. Five percent of the amplified material was applied to 1% low-melt agarose gels (Bio-Rad, Richmond, CA) and analyzed by electrophoresis. UV fluorescence of ethidium bromide–stained gels showed DQA-, DQB-, or DR4-amplified sequences at 172 basepairs (bps), 207 bps, or 259 bps, respectively. Plasmid pBr322-*Hae* III–cut DNA (Boehringer Mannheim, Indianapolis, IN) was used as a molecular size standard.

Dot-blot analysis. Approximately equal amounts of the amplified products (5–20% of the total reaction, judged by visualization of UV fluorescence of the ethidium bromide–stained gels) was bound to Zetabind membranes (AMF, Meriden, CT) with an apparatus and procedure provided by Schleicher and Schuell (Keene, NH). Filters were baked for 2 h at 80°C, pretreated in 0.1× sodium chloride–sodium citrate (SSC) and 0.5% sodium dodecyl sulfate (SDS) for 1 h at 65°C, and prehybridized for ≥3 h at 42°C. Prehybridization and hybridization buffers consisted of 900 mM NaCl, 6 mM EDTA, 90 mM Tris (pH 7.4), and 5× Denhardt's buffer (19). Oligonucleotide primers were labeled with standard procedures (19) with [γ-^{32}P]ATP to specific activities of 1 × 10^9 cpm/μg. Approximately 1 ng of probe was added per ml hybridization solution, and filters were incubated at 42°C for 16 h. Filters were washed three times in 6× SSC at room temperature for 15 min/wash, followed by a final wash for 40 min at a temperature determined for each probe by the formula (no. of G and C bps × 4) + (no. of A and T bps × 2). Filters were air dried and exposed to Kodak XAR-5 film for autoradiography. Filters were stripped and reused after incubation in 0.4 M NaOH at 42°C for 1 h followed by treatment in 0.1× SSC, 0.5% SDS, and 0.2 M Tris (pH 7.5) at 42°C for 30 min.

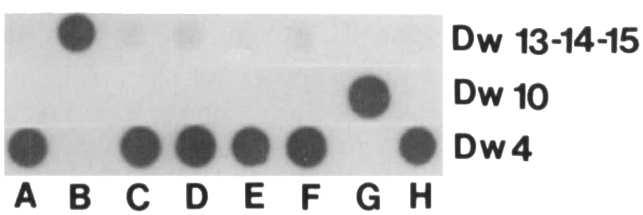

FIG. 2. Dot-blot analysis for Dw subtypes with specific oligonucleotide probes. Individuals A, C–F, and H are positive for Dw4. Individuals B and G are positive for Dw13–15 or Dw10, respectively.

Oligonucleotide probes. We used eight different DQβ probes to detect 12 different DQB genes (Fig. 1). For example, β4B and β4C probes detect sequences present in DQB3.1, whereas DQB3.3 contains only β4C sequences. The nomenclature for the 12 DQB genes and their HLA-DR associations were previously described (21). The DQB3.1 and DQB3.2 genes are associated with the DQw7 and DQw8 specificities, respectively. In addition, the DQB1.1 gene is associated with DR1 and DRw10; however, the DRw10 specificity is very rare.

We also used four different DQA gene probes: α1 (5′-TTC AGC AAA TTT GGA GGTT), α3 (5′-TGT TTG CCT GTT CTC AGAC), α4 (5′-TTC CGC AGA TTT AGA AGAT), and α7 (5′-TTC CAC AGA CTT AGA TTTG). Probe α1 detects DQA1 genes, which are associated with DR1, 2, 6, 8, 10, and blank; α3 detects DQA4 genes associated with DR3, 5, and 8; α4 detects DQA3 genes associated with DR4 and 9; and α7 detects DQA2 genes associated with DR7 (for DQA nomenclature and DR associations, see ref. 21).

In addition, we used three Dw probes that detect subtypes of HLA-DR4: probe Dw4 (5′-GGA GCA GAA GCG GGC CG),

TABLE 1
Frequency of Dw subtypes of DR4 in diabetic and control subjects

Subtypes	DR4 diabetic (n = 120)	DR4 control (n = 55)	Relative risk	χ^2	P
Dw4	70 (58.3%)	38 (69.1%)	0.6	1.8	NS
Dw10	22 (18.3%)	0 (0%)	25.4	11.5	<.001
Dw4 or 10	92 (76.7%)	38 (69.1%)	1.5	1.1	NS
Dw13–15	33 (27.5%)	19 (34.5%)	0.7	0.9	NS

Five diabetic subjects and 1 control subject were heterozygous for 2 different Dw subtypes of DR4; therefore, Dw frequencies do not add up to 100%. Pearson's χ^2-test was used with 1 df. NS = P > .05. Relative risk (odds ratio) calculated according to Haldane (26).

TABLE 2
DR specificities predicted from known associations of DQA and DQB genes (21) in type I (insulin-dependent) diabetic and control subjects

	Predicted DR type	DR4, DQB (*n*)			
		Diabetic		Control	
		3.2+	3.1+	3.2+	3.1+
DQA1/DQB1.1	DR1	12	2	8	8
DQA1/DQB1.2	DR2	0	0	0	0
DQA1/DQB1.3	DR6	0	0	1	0
DQA1/DQB1.4	DR2	1	0	0	0
DQA1/DQB1.5	DR2	0	0	4	4
DQA1/DQB1.6	DR6	4	0	0	0
DQA1/DQB1.7	DR6	6	1	2	1
DQA2/DQB2	DR7	5	2	4	4
DQA4/DQB2	DR3	70	0	5	3
DQA3/DQB3.1	DR4	2	0	1	0
DQA4/DQB3.1	DR5	1	0	5	2
DQA3/DQB3.2	DR4	11	0	1	0
DQA4/DQB3.3	DR9	0	0	0	0
DQA4/DQB4	DR8	3	0	0	0
DQA1/DQB4	DR8	0	0	2	0

probe Dw10 (5′-ATC CTG GAA GAC GAG CG), and probe Dw13–15 (5′-GGA GCA GAG GCG GGC CG). The Dw13–15 probe detects a common specificity associated with Dw13–15 (1).

RESULTS

Dw subtypes of HLA-DR4: Of the 164 type I diabetic patients and 200 control blood donors analyzed by PCR amplification, 120 diabetic patients (73.2%) and 55 blood donors (27.5%) tested positive with oligonucleotide primers specific for HLA-DR4. Figure 2 shows a dot-blot analysis of 8 DR4+ individuals (*A–H*) with 17-mer oligonucleotide probes specific for Dw4, Dw10, or Dw13–15. The frequencies of Dw4 and Dw13–15 were not different in our DR4+ type I patients and DR4+ blood donors (Table 1). The frequency of Dw10, often associated with DR4 in individuals of Ashkenazi Jewish background (11,13), was 18.3% of the DR4+ type I patients and 0% of the blood donors (Table 1; $P < .001$). The frequency of Dw10 in the Boston and Houston diabetic subjects was not significantly different. The combined frequency of Dw4 and Dw10, each of which has been associated with type I diabetes (11–13), was not different in our DR4-matched diabetic and control populations (Table 1). Thus, Dw4 and Dw10 are not more specifically associated with type I diabetes than DR4 alone.

DQB subtypes of HLA-DR4. The 120 DR4 diabetic patients and 55 DR4 control blood donors were subtyped for 12 DQB1 and 4 DQA specificities (Fig. 1; Table 2). DQB3.2 is found in 95.8% of the DR4 diabetic patients compared to only 60% of the DR4 control population (Table 3; $P < .0001$). The association of DQB3.2 with type I diabetes is stronger in the DR4/3 patients than the DR4/non-3 patients (Table 3; $P < .01$). However, the association of DQB3.2 was statistically significant in all DR4 patient subgroups compared to matched DR4 control subgroups (Table 3).

DISCUSSION

In this study, we found an association between DR4 and the DQw8 subtype (DQB3.2) in all DR4 subgroups associated with type I diabetes. Although there was a stronger correlation of DR4 and the DQw8 subtype in our DR4/3 patients compared with our DR4/non-3 patients, we found the DR4–DQw8 association with type I diabetes to exist in all the DR4/non-3 patients. These findings are in contrast to a recent study by Tait et al. (10) that reported the DQw8 association only in DR4/3 patients.

We used oligonucleotide probes to distinguish DQw7 and DQw8, whereas Tait et al. used the TA10 antibody. Because restriction-fragment–length polymorphism (RFLP) analysis of genomic DNA shows that DQw7 is associated with DR4 (TA10+) and DQw8 with DR4 (TA10−) (3) and DQw7 and DQw8 typing with either RFLP or oligonucleotide probes is informative (18,22,23), it is unlikely that methodological differences account for the discrepancies between our findings and those of Tait et al.

Our results are also different from those of Bach et al. (12) and Rowe et al. (11) who found overrepresentation of Dw4 and underrepresentation of Dw subtypes other than Dw4 and Dw10 among DR4+ type I patients compared with DR4+ control subjects. Dw4 and Dw13–15 were found in approximately equal frequencies among our DR4+ type I diabetic patients and DR4+ control subjects, respectively. These results cannot be explained simply by the higher frequency of Dw10 in our patient population: the frequency of Dw4 plus Dw10 was not statistically different in our DR4+ patient versus

TABLE 3
Frequency of DQB3.2+ individuals in DR4/X type I (insulin-dependent) diabetic and control subjects

	DQB3.2+						
	Diabetic		Control				
	n	%	*n*	%	Relative risk	χ^2	*P*
Total DR4	115	95.8	33	60.0	14.1	37.1	<.0001
DR4/3	70	100	5	62.5	89.7	27.3	<.0001
DR4/non-3	45	90.0	28	59.6	5.7	12.0	<.001
DR4,Dw4/non-3	26	83.9	15	46.9	5.4	9.5	<.005
DR4/2,4–9	33	91.7	20	64.5	5.4	7.4	<.01
DR4/2,6–9	19	86.4	13	59.1	3.9	4.1	<.05
DR4/1	12	85.7	8	50.0	5.0	4.3	<.05

Frequency of DQB3.2+ in DR4/3 vs. DR4/non-3 subjects: $\chi^2 = 7.3$, $P < .01$. Dw10 and Dw13–15 were associated with DQB3.2 in all subjects except for 3 control subjects who were Dw13–15, DQB3.1+. Pearson's χ^2-test was used with 1 df. Relative risk (odds ratio) calculated according to Haldane (26).

DR4$^+$ control populations. Our results are most similar to those of Platz et al. (14), where HLA-DR4 showed a stronger association with type I diabetes than HLA-Dw4.

Our study shows an unexpectedly high frequency of Dw10 in the Houston patient population (13.1%) compared to the control population (0%). In our patient population, Dw10 does not appear to be preferentially associated with patients of Ashkenazi origin. Furthermore, the strong DQw8 association with type I diabetes is seen independently of Dw10 (Table 3). Because all the Dw10 patients are DQw8$^+$, the data do not allow us to conclude whether Dw10 is primarily or secondarily associated with type I diabetes.

The data in Table 2 can be used to estimate the absolute risk for Caucasian individuals to develop type I diabetes. We used the formula $(hp/hc) \times F$, where *hp* and *hc* are the frequencies of the genotype in question in patients and control subjects, respectively, and *F* is 0.5%, the estimated risk for type I diabetes in a Caucasian population (24). The combinations of DQA3/DQA4/DQB2/DQB3.2 (DR4, DQw8/DR3, DQw2) or DQA3/DQB3.2 (DR4, DQw8) alone provide absolute risks of 8.5 and 6.7%, respectively. Interestingly, the absolute risks associated with DQw8$^+$ DR4/3 heterozygotes or DR4/4 homozygotes are similar, which is in contrast to previous findings with DR typing alone (DR4/3 = 3.0%, DR4/4 = 1.4%; 24). The absolute risks associated with other combinations of DR and/or DQ genes are substantially lower (≤1.9%; 24). However, the absolute risks associated with DQw8$^+$ DR4/4 and DR4/3 approach the risk estimated for diabetes seen in HLA-identical siblings of a type I diabetic proband 12–30%; 14,25).

Our absolute risks of 8.5 and 6.7% in unrelated individuals may be at the upper limit for risk of diabetes associated with the HLA region. These findings suggest that non–HLA-linked genes contribute to susceptibility to type I diabetes. Furthermore, our results suggest that the DQ loci are the primary HLA association with susceptibility to type I diabetes, although additional genes or gene sequences closely linked to DQA or DQB may also be involved.

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