Brief Definitive Report

SPECIFIC GENOMIC MARKERS FOR THE HLA-DQ SUBREGION DISCRIMINATE BETWEEN DR4$^+$ INSULIN-DEPENDENT DIABETES MELLITUS AND DR4$^+$ SEROPOSITIVE JUVENILE RHEUMATOID ARTHRITIS

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HLA-DR4, like most other HLA-DR specificities, is a serologically defined public specificity present on a number of different haplotypes. Haplotypes that share the DR4 specificity may differ considerably; polypeptide products of HLA class II genes in the DR and DQ regions vary among different DR4$^+$ cells (1–4) and restriction endonuclease fragment length polymorphisms (RFLP) identify nucleotide variation among different DR4$^+$ haplotypes (5). Polymorphic variations within HLA class II molecules are implicated in the regulation of immune response, and are associated with susceptibility to a number of different diseases, including insulin-dependent diabetes mellitus (IDDM) and seropositive juvenile rheumatoid arthritis (JRA), which are both associated with HLA-DR4 (6–9). Using RFLP to analyze specific genotypic markers associated with HLA-DR4 in these two clinically disparate diseases, we have identified a specific DQβ variant highly associated with IDDM. Different DQβ genomic patterns were found in JRA, implicating different genetic elements contributing to HLA-linked disease susceptibility for these two diseases.

Materials and Methods

Patient Selection and HLA Typing. JRA patients were chosen from those attending the Arthritis Clinic at Children's Orthopedic Hospital and Medical Center, Seattle, WA. The diagnosis of seropositive JRA was made using previously described criteria (8). HLA typing of these patients has been previously reported (9); all patients used in this report are homozygous for HLA-DR4. IDDM patients were selected from those attending the Diabetes Center at Pacific Medical Center, Seattle, WA, and fulfill the criteria for juvenile onset insulin-dependent diabetes. We selected 17 DR4$^+$ patients for this study. All of the DR4$^+$ haplotypes were positive for HLA-DQw3. However, no specific HLA-B antigens or class III markers suggesting preferred extended haplotypes were observed.

Analysis of Genomic DNA. DNA was obtained from 4×10^7 peripheral blood leukocytes either freshly drawn or following in vitro culture for 7 d in the presence of PHA and IL-2. For some studies, EBV-transformed B lymphoblastoid lines prepared from patients'

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lymphocytes were used. After endonuclease digestion for 18 h at 37°C (Bam HI) or 65°C (Taq I), DNA digests were precipitated with ethanol and resuspended in 10 mM Tris, 1 mM EDTA, pH 7.6. Digested DNA was loaded at 12 μg per lane on a 0.7% agarose gel, and electrophoresed at 30 V for 18 h. After electrophoresis, gels were denatured and neutralized before transfer to nitrocellulose (Schleicher & Schuell, Inc., Keene, NH) by the method of Southern (10). After transfer, filters were baked for 18 h at 80°C, prehybridized at 42°C, and hybridized with nick-translated, P-32-labeled DQ cDNA probes. The DQβ cDNA probe (11) and the DQα cDNA probe (12) were used as previously described (5). After 48 h of hybridization at 42°C, blots were washed at 60°C in 1.5 mM NaCl, 0.015 mM sodium citrate, and 0.1% SDS three times for 20 min each. Autoradiographs are shown after exposure of the gels to Kodak XAR-5 film for 2–4 d.

Results and Discussion

In previous studies, we have identified specific nucleotide variation within the DQβ genomic region which identifies two allelic DQβ variants on HLA-DR4$^+$ haplotypes, DQ3.1 and DQ3.2 (13). A number of restriction endonucleases, including Bam HI, Hind III, Taq I, Pvu II, Xba I, and Sst I, generate different cleavage fragments on genomic DNA from these DQw3$^+$, DR4$^+$ individuals. DQ-reactive mAb 9w790, which defines a variant of DQw3 designated TA10, reacts with cells carrying the DQ3.1 allelic variant, but not the DQ3.2 (13), and restriction mapping of genomic DNA with a DQβ synthetic oligonucleotide probe (14) confirms the assignment of the DQβ variant RFLP to the DQβ2 (or DCβ) locus.

We used these specific genotypic markers to investigate the genomic basis of the association of DR4 with IDDM and JRA. IDDM is highly associated with DR4$^+$, Dw4$^+$ phenotypes, and the risk of disease is greatest in DR3/4 and Dw3/Dw4 heterozygous individuals (6–7). DNA from 17 patients was analyzed for the presence of specific DQ variants using RFLP analysis with DQβ cDNA probes. As shown in Fig. 1, panel *IA*, using the enzyme Bam HI, polymorphic bands representing the two DQβ alleles associated with DQw3 are apparent among ten representative patients. A 12.0-kb band identifies the DQ3.2 allele, which is present in all but one of the diabetic patients (D12). In contrast, the alternate allele DQ3.1 is represented by a pair of bands at 6.9 kb and 3.7 kb (13). A 10.2-kb band is invariant in both DR3- and DR4-related haplotypes, and a 6.9-kb band is invariant among the DR3-related haplotypes. Because of the DR3-related invariant 6.9-kb band, we must focus on the 3.7-kb band to determine which patients express this allele. All but 1 of the 17 patients studied so far show the DQ3.2 allele, thus showing the predominant expression of the DQ3.2 allele and the very rare expression of the DQ3.1 allele among these DR4$^+$ diabetics.

We contrasted these results with a similar analysis of patients with another DR4-related disease, seropositive juvenile rheumatoid arthritis (JRA). We have previously reported that the strong DR4 association with this form of JRA represents two haplotypes, DR4,Dw4 and DR4,Dw14; many of the JRA patients are heterozygous for these two DR4$^+$ haplotypes (9). Panel *IIA* of Fig. 1 shows DQβ cDNA hybridization of Bam HI digested genomic DNA from 10 of these patients. In cases in which a DR4$^+$,Dw14$^+$ haplotype is expressed, a concomitant DQ3.2 allele is also present, consistent with our previous observation that the 3.2 DQβ variant is in linkage disequilibrium with the Dw14 haplotype (13). The expression of DQβ variants on the DR4$^+$,Dw4$^+$ haplotype permits a direct

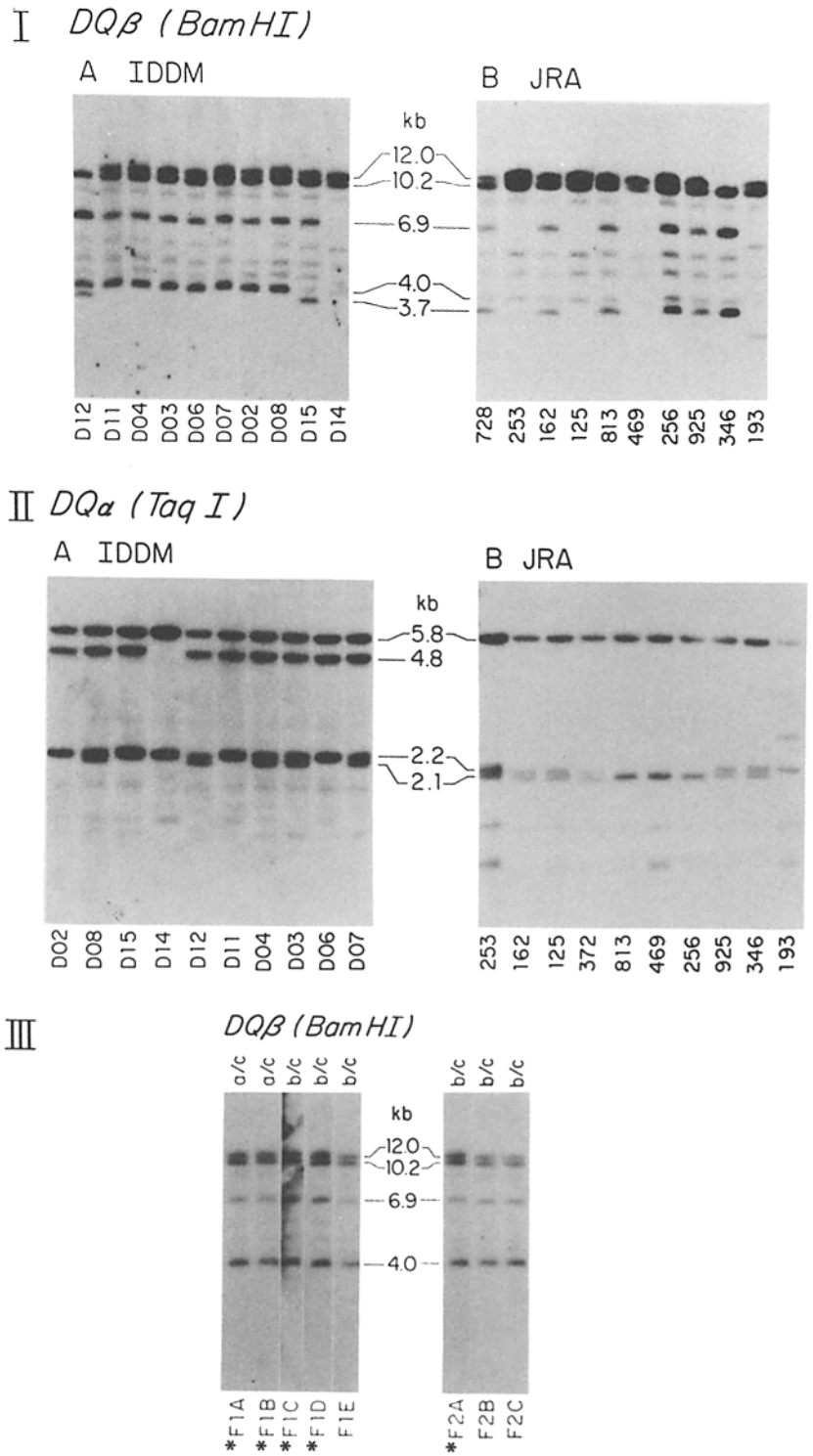

FIGURE 1. (*I*) Genomic DNA from patients with either IDDM or JRA (*B*) digested with Bam HI endonuclease and probed with a DQβ cDNA probe. DR types of the patients are: lanes *1–8*, DR3,4; lane *9*, DR4,X; lane *10*, DR4; lanes *11–20*, DR4,4. (*II*) Genomic DNA from patients with either IDDM (*A*) or JRA (*B*) digested with Taq I and probed with a DQα probe. Again, all IDDM patients are DR3,4, except in lane *3* (DR4,X) and lane *4* (DR4); all JRA patients are DR4,4. (*III*) Genomic DNA from IDDM patients and siblings from two families, digested with Bam HI and probed with DQβ. The *left* side represents five siblings from a single family with the haplotypes as shown. *a*: A1, B8, DR3, *b*: A26, B15, DR3; *c*: Aw24, B40, DR4; and *d*: Aw30, Bw35, DR4. The *right* side shows three siblings from a second family. Haplotype *a*: A2, B15, DR4; *b*: A10, B27, DR1, *d*: A3, B7, DR5; and *c*: A3, B8, DR3. (*) individuals with clinical IDDM.

comparison with the analogous IDDM haplotype. 8 of 12 $Dw4^+$ haplotypes among the JRA patients are associated with the DQ3.1 variant characterized by 3.7-kb and 6.9-kb Bam HI hybridizing fragments. This is in marked contrast to the distribution in the $DR4^+$,$Dw4^+$ IDDM population, in which an extremely high (>90%) incidence of HLA DQ3.2 is observed. The HLA DQ3.2 marker occurs in association with a wide variety of HLA-B and class III alleles, as noted in Materials and Methods. Therefore this DQ polymorphism is not a marker for a single extended haplotype associated with DR4. This conclusion is consistent with reports of associated haplotypes in IDDM in which $DR3^+$ extended haplotypes frequently occur, but most $DR4^+$ haplotypes are "nonextended" (15).

When additional restriction fragment analyses are performed, genotypic differences between IDDM and JRA haplotypes can be shown for DQα as well. Panel *II* shows hybridization with DQα cDNA probes of Taq I—digested cellular DNA from both groups of patients. A 5.8-kb and a 4.8-kb hybridizing band are invariant bands associated with DR4 and DR3 haplotypes, respectively. Bands at 2.2 kb and 2.1 kb are polymorphic, and appear to be allelic to each other. The expression of these RFLP differ in the two patient groups: Only five of the 17 diabetics tested (29%) are positive for the 2.1-kb band, although 100% are positive for the 2.2-kb band. In contrast, 11 of 12 JRA patients (92%) are positive for the 2.1-kb band while 8 of 12 (67%) are positive for the 2.2-kb band.

The use of specific genotypic markers, such as the DQβ and DQα allelic variants described here, permits the molecular characterization of phenotypically similar HLA haplotypes associated with distinct diseases. Our results, in the examples cited above, indicate that while $DR4^+$,$Dw4^+$ haplotypes in IDDM patients consistently show a particular DQβ allelic variant, the analogous $DR4^+$,$Dw4^+$ haplotypes in the JRA patients do not. Previous reports analyzing RFLP differences in IDDM have noted the decrease of a Bam HI 3.7-kb RFLP (16, 17); we can now attribute this observation to the prevalence of the DQ3.2 allele in IDDM. In one report, RFLP differed between IDDM patients and controls, including their normal healthy siblings (18). However, these previous studies did not match the test groups for haplotype identity. We therefore evaluated haplotype-matched siblings from two IDDM families, shown in Panel *III*, again using Bam HI digested cellular DNA probed with DQβ cDNA. None of the healthy family members displays a DQ3.1-associated, 3.7-kb band. However, all of the $DR4^+$ siblings display the 12.0-kb band characteristic of the IDDM-associated DQ3.2 allele, whether or not they express clinical diabetes. Thus, both affected and healthy individuals can carry the same haplotypes and specific allelic variants, which can indicate an increased risk of IDDM but are not in fact disease specific.

We chose to compare IDDM and JRA because both are highly associated with HLA-DR4, and both are even more highly correlated with a heterozygous state, HLA-DR3,Dw3/DR4,Dw4 in IDDM (6, 7) and HLA-DR4,Dw4/DR4,Dw14 in seropositive JRA (9). This heterozygous predisposition might indicate the possibility of two gene effects or gene complementation within the class II region contributing to a mechanism for increased susceptibility. There are marked differences between IDDM and JRA, most notably the difference in end organ susceptibility and clinical course. The different genomic markers reported here

within the HLA DQ region potentially may contribute to such clinical differences, since variations in class II molecules are implicated in a number of important immunoregulatory mechanisms, including immune cell activation, intercellular communication, and target specificity.

Summary

HLA-DR4, Dw4-associated haplotypes associated with IDDM and JRA were compared using genomic DNA restriction fragment analysis to distinguish among DQβ and α alleles linked to DR4. DQβ polymorphisms that subdivide the HLA-DQw3 specificity into DQ3.1 and 3.2 alleles were identified. More than 90% of DR4$^+$ IDDM patients express one of these alleles, DQ3.2; restriction enzyme mapping indicates that the presence of this allele also accounts for the genomic fragment patterns previously reported in IDDM. Furthermore, haplo-identical siblings of DQ3.2 IDDM patients also carry the DQ3.2 allele, regardless of clinical presentation. In contrast, DR4$^+$ JRA patients show no allelic preference at DQβ, implicating different HLA genetic contributions in these two DR4-associated diseases.

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