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A specific HLA-DPβ allele is associated with pauciarticular juvenile rheumatoid arthritis but not adult rheumatoid arthritis

(autoimmunity/polymerase chain reaction/sequence-specific oligonucleotide)

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ABSTRACT **Nonradioactive sequence-specific oligonucleotide probes specific for the HLA-DPβ locus have been used in a simple dot-blot format to type samples amplified by the polymerase chain reaction from 44 patients with pauciarticular juvenile rheumatoid arthritis, 32 patients with adult rheumatoid arthritis, and 50 random controls. The sequences of four new DPβ alleles derived from these patients and controls are reported, bringing the total number of alleles identified thus far to 19. The *DPB2.1* allele is significantly increased in juvenile rheumatoid arthritis patients over controls; this allele is not increased in patients with adult rheumatoid arthritis. The association of juvenile rheumatoid arthritis with the *DPB2.1* allele is independent of linkage with previously defined HLA-D region markers of disease. Analysis of the *DPB2.1* sequence shows that it differs from the nonsusceptible *DPB4.2* allele by only 1 amino acid at position 69 in the β1 domain.**

A striking association exists between susceptibility to certain human autoimmune disorders and particular alleles of the HLA class II (HLA-D) region (1). This region encodes three related class II antigens, HLA-DR, -DQ, and -DP, which are cell-surface glycoproteins composed of an α and a β chain (2). With the exception of the DRα and DPα chains, these proteins are highly polymorphic, with the variability localized to the NH_2-terminal extracellular domain encoded by the second exon. The role of the class II molecule in the immune response is twofold. (*i*) Class II molecules present on the stromal cells of the thymus help determine the specificity of the mature T-cell repertoire (3–6). (*ii*) Class II antigens on the cell surface of antigen-presenting cells bind processed antigen and present it to $CD4^+CD8^-$ T cells, which can become activated and initiate an immune response (7–11). Consequently, an individual's ability to respond to an antigen, whether the antigen be foreign or self, is partly determined by the amino acid sequences of its class II molecules. Indeed, susceptibility of certain autoimmune disorders has been associated with particular class II allelic sequences. For instance, the charge of the amino acid at position 57 in the DQβ chain appears to correlate with susceptibility to insulin-dependent diabetes mellitus (12–14), whereas genetic susceptibility to *Pemphigus vulgaris*, an autoimmune disease of the skin, appears associated with sequences in both the DRβI locus (DR4-associated) and the DQβ locus (DRw6-associated) (15–17).

The HLA-DP locus, as defined by the primed lymphocyte typing (PLT) method, a complicated cellular assay, and restriction fragment length polymorphism analyses, has also been implicated in the susceptibility to certain autoimmune diseases (18–22). One such disease is pauciarticular juvenile rheumatoid arthritis (JRA), a type of childhood arthritis of unknown etiology that primarily affects young females, begins with involvement of fewer than five joints, and is associated with antinuclear antibodies in the serum and with inflammatory anterior uveitis. Previous results showed susceptibility to this form of JRA to be associated with an increased frequency of the HLA serotypes DR5, DRw6, and DRw8 (23–28) as well as with the cellularly defined HLA-DP antigen DPw2 (18, 19).

Recently, the polymerase chain reaction method was used to amplify the polymorphic second exons of DPα and DPβ from 34 DPw-typed cell lines (29). Sequence analysis identified 14 DPβ alleles and 2 DPα alleles; this is in contrast to the 6 DPw specificities defined by the PLT method. In addition to suggesting that the cellularly defined specificities, DPw1–DPw6, are determined by polymorphic residues within the β chain, these data also showed that the DPw2 specificity includes 2DPβ alleles, the more frequent *DPB2.1* allele, and the rarer *DPB2.2* allele. These two alleles differ by 3 amino acids in the exon encoding the polymorphic β1 domain (Fig. 1).

Based on the 14 DPβ sequences, a DPβ typing system was developed using nonradioactive DPβ sequence-specific oligonucleotides as probes in a simple dot-blot format to analyze DNA amplified from the second exon of the DPβ locus (29, 30). Here, we report the use of this assay to DPβ-type patients with pauciarticular JRA and classical rheumatoid factor-positive adult rheumatoid arthritis.

MATERIALS AND METHODS

Patients. The 44 pauciarticular JRA patients all meet the standard criteria for this diagnosis, including onset before the age of 16 years of a chronic inflammatory arthritis of at least 6 weeks duration, with fewer than five joints involved in the first 6 months of disease. All 32 adult patients meet the criteria for classic rheumatoid arthritis and, in addition, were positive for serum rheumatoid factor.

DPβ Typing. DPβ typing was done by using sequence-specific probes in a dot-blot format to analyze DNA (prepared according to standard procedures; ref. 31) polymerase chain reaction-amplified (32–34) from the second exon of the DPβ locus. Briefly, the primers UG19 (5′-GCTGCAGGAGAGTGGCGCCTCCGCTCAT-3′) and UG21 (5′-CGGATCCGGCCCAAAGCCCTCACTC-3′) were used in a 200-μl reaction to amplify 0.5 μg of DNA for 35 cycles (denaturation: 96°C for 30 sec; annealing and extension: 65°C for 45 sec). [Note that these primers, in conjunction with this amplification profile, give a much better yield of the polymerase chain reaction product than those described (29).]

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Abbreviations: JRA, juvenile rheumatoid arthritis; PLT, primed lymphocyte typing.

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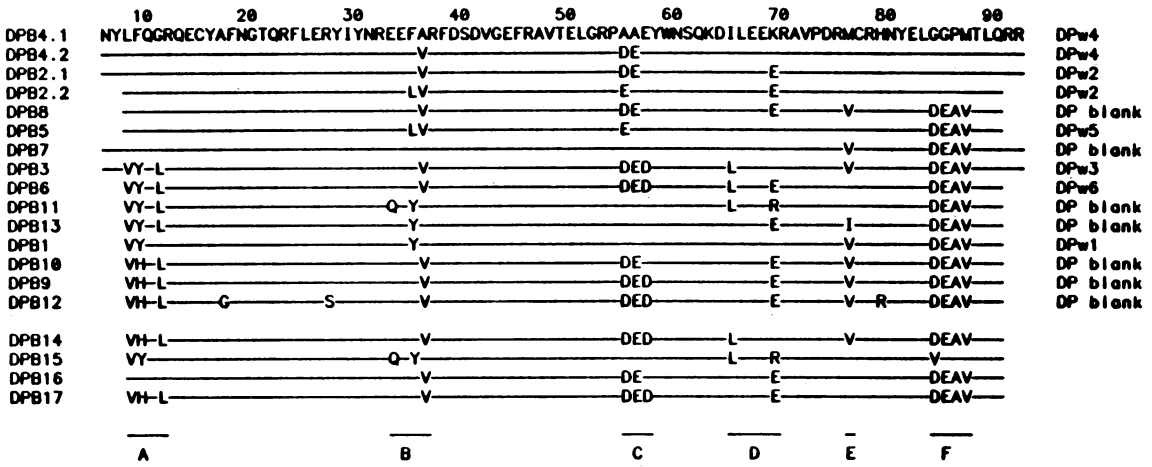

FIG. 1. Alignment of protein sequences of the first domains of the HLA-DPβ genes. The sequences are reported in the one-letter code and aligned with the *DPB4.1* allele. A dash indicates identity with the *DPB4.1* sequence, and a space indicates the sequence was not determined. Numbers above indicate the amino acid position in the mature protein, whereas the six regions of variability (A–F) are indicated below. Designations of alleles are shown at left; their PLT-defined specificities are shown at right. The nucleotide sequences of *DPB1–DPB13* have been reported (29, 30).

Approximately 0.2 μg of the amplified product was dot blotted (35) onto each of 13 separate membranes, and each membrane hybridized to a specific horseradish peroxidase-labeled oligonucleotide probe. The sequence of each probe and conditions for hybridization are reported elsewhere (refs. 29 and 30; T.L.B., A.B.B. and H.A.E., unpublished work).

Cloning of the PCR Product into M13 and Sequence Analysis. One hundred microliters of the original polymerase chain reaction was phenol/chloroform-extracted twice with one-half volume, chloroform-extracted once with one-half volume, and dialyzed through a Centricon 30 microconcentrator (Amicon) with 2 ml of Tris/EDTA buffer by spinning in a Beckman J17 centrifuge for 25 min at 3440 × *g*. The sample was recovered, and the DNA was digested for 3 hr with the restriction enzymes *Bam*HI and *Pst* I (New England Biolabs) in a total volume of 200 μl by adding 5 units of each enzyme every hour. After digestion, the sample was phenol/chloroform-extracted once, passed through the same Centricon 30 microconcentrator as described above, and brought to a final volume of 20 μl. Three microliters of the sample was put into a 10-μl ligation reaction with 200 ng of *Bam*HI/*Pst* I-cut m13mp18 and ligated overnight at 16°C with T4 DNA ligase (BRL). Transformations into *Escherichia coli* JM101 were done according to standard procedure (31), and positive plaques were selected by hybridization to a DPβ cDNA. [The cDNA used, pDB117, was isolated from the cell line LG2, which is homozygous for *DPB4.1* (T.L.B. and H.A.E., unpublished results).] A minimum of six independent clones were sequenced from each patient by the dideoxy chain-termination method (36) with ^{35}S-labeled dATP and Sequenase (United States Biochemical).

Contingency Table Test for Heterogeneity. A commonly employed method to examine the association of a genetic marker with disease is to evaluate the relative frequency of the marker in disease and control populations. When this method is used on a marker locus having more than two alleles, the evaluation of the significance of individual tests becomes difficult. We overcame this problem by using the contingency table test for heterogeneity (37), a row-by-column test of independence that simultaneously tests for heterogeneity among all the alleles in the diseased and control groups. When this test is statistically significant, a simultaneous test procedure is used to localize the source of heterogeneity by identifying nonsignificant subgroups of alleles. The *G*-test statistic or logarithm likelihood ratio test (37) is then used to estimate *P* values; this is a more rigorous method for calculating *P* values than the χ^2 test.

RESULTS AND DISCUSSION

To determine whether susceptibility to pauciarticular JRA can be associated with a particular DPβ allele, 44 patients were DPβ-typed by using the dot-blot assay (Table 1), and the results were compared to a control panel of 50 unaffected individuals by using the contingency table test for heterogeneity (Table 2). The analysis shows the frequency of *DPB2.1* in the JRA population to be significantly greater than that found in the control panel ($G = 15.2$, $P < 0.1$). Twenty-four (55%) of the patients were positive for *DPB2.1* compared with 8 (16%) of the controls (data not shown), which gives the overall relative risk associated with the presence of the *DPB2.1* allele in patients with pauciarticular JRA a value of 6.3. None of the patients or controls were positive for the *DPB2.2* allele. The *DPB2.2* allele is relatively rare; it has been

Table 1. Allele frequencies in patients and controls

DPβ allele	Control (100*), %	JRA (88*), %	ARA (64*), %
1	6	3	5
2.1	10	30	12
2.2	0	0	0
3	6	11	11
4.1	56	40	41
4.2	10	6	22
5	2	0	2
6	2	2	2
7	0	0	0
8	0	0	0
9	0	1	0
10	1	1	2
11	3	1	0
12	0	0	0
13	1	1	0
14	2	0	2
15	0	2	2
16	0	1	0
17	1	0	2

ARA, adult rheumatoid arthritis.
*Total number of chromosomes tested.

Table 2. Contingency table test for heterogeneity comparing the distribution of DPβ alleles in JRA and adult rheumatoid arthritis populations to a control population

DPβ allele	JRA (88*) *n* (%)	Control (100†) *n* (%)	ARA (64*) *n* (%)
−1	3 (3)	6 (6)	3 (5)
−2.1	26 (30)	10 (10)	8 (12)
−3	10 (11)	6 (6)	7 (11)
−4.1	35 (40)	56 (56)	26 (41)
−4.2	5 (6)	10 (10)	14 (22)
Other†	9 (10)	12 (12)	6 (9)
		G‡ = 15.2, df = 5, $P < 0.01$	G‡ = 7.0, df = 5, $P > 0.1$

ARA, adult rheumatoid arthritis.
*Total number of chromosomes examined.
†Alleles are classified into five distinct alleles plus one combined "other" group that consists of the less common alleles (*DPB2.2* and *DPB5-17*) to achieve a minimum *expected* frequency of four in each individual category.
‡G, like the common χ^2 test, approximates the χ^2 distribution (37). The vertical line between the JRA and control frequencies defines those alleles included in a nonsignificant subgroup in comparisons of JRA and control individuals; only the *DPB2.1* allele is excluded.

detected in only 9 of the 322 samples that we have DPβ-typed (T.L.B. and A.B.B., unpublished results).

Thirty-two patients with classical rheumatoid factor-positive adult rheumatoid arthritis were also DPβ-typed (Table 1); only 7 (22%) were positive for the *DPB2.1* allele (data not shown). The frequency of *DPB4.2* in adult rheumatoid arthritis patients, however, does appear to be increased. Forty-four percent (14/32) of the patients were positive for the *DPB4.2* allele compared to 18% (9/50) of the controls (data not shown). Using Fisher's exact test, the uncorrected *P* value for the association of *DPB4.2* with adult rheumatoid arthritis is 0.012; however, analysis of these data using the contingency table test for heterogeneity shows that this difference is not statistically significant (Table 2). A larger patient population will be needed to further examine this possible association.

We next examined the possibility that the observed association of pauciarticular JRA with the *DPB2.1* allele was due to linkage disequilibrium with one of the previously defined HLA-DR disease markers, HLA-DR5, -DRw6, or -DRw8 (23–28). Table 3 shows that the frequency of *DPB2.1* does not vary significantly among patients positive for each one of these HLA-DR disease markers. This relationship can also be analyzed by calculating the disequilibrium parameters, *D* and *D'* (38). As Table 4 shows, the disequilibrium of *DPB2.1* with the three DR alleles reveals no significant associations, and in two cases (the haplotypes *DPB2.1*-DR5 and *DPB2.1*-DRw8) the disequilibrium value is negative. There is also no evidence for linkage disequilibrium between the PLT-defined DPw2 specificity and these DR markers in other pauciarticular JRA patient populations (18, 19) or between *DPB2.1* and these DR markers in the normal population (ref. 29; T.L.B. and A.B.B., unpublished results). In addition, we have typed four informative families with *DPB2.1*$^+$ probands. In two of the families examined, the *DPB2.1* allele is not transmitted on the same chromosome as the DR-associated disease marker (data not shown). Together these data suggest that the association of *DPB2.1* with pauciarticular JRA is independent of linkage with the previously reported JRA-associated specificities.

Table 3. Association of *DPB2.1* with HLA-DR5, -DRw6, and -DRw8 in patients with pauciarticular JRA

DPB2.1	Frequency
DRw5$^+$ patients	0.50 (8/16)
DRw6$^+$ patients	0.67 (12/18)
DRw8$^+$ patients	0.52 (11/21)
All patients	0.55 (24/44)

Table 4. Association of the *DPB2.1* allele with the DR locus in the JRA patient population (n = 88 haplotypes) expressed by the disequilibrium parameters *D* and *D'*

	DPB2.1– DR5	*DPB2.1*– DRw6	*DPB2.1*– DRw8	*DPB2.1*– other DRs
Haplotype frequency	0.030	0.106	0.053	0.095
D	−0.03	+0.04	−0.02	+0.01
D'	−0.48	+0.23	−0.28	+0.04
P value	NS	NS	NS	NS

Haplotypes were estimated from the patient population using the method described in ref. 38. $D = X_{ij} - p_i q_j$, where X_{ij} is the observed haplotype frequency, p_i is the frequency of allele i at one locus, and q_j is the frequency of allele j at the second locus. *D'*, a relative measure that reduces the effect of the allele frequency on *D*, ranges from −1.0 when two alleles are never on the same haplotype to +1.0 when an allele of one locus is only found in association with an allele from the second locus. NS, nonsignificant.

It should be noted, however, that only 1 of these 44 patients lacked either DR5, DRw6, or DRw8; this patient also lacked *DPB2.1*. [The DR types of each patient will be reported elsewhere (B.S.N., unpublished work).] Consequently, JRA patients who have the *DPB2.1* allele but not a disease-associated DR marker were not found in this patient population. This suggests that the *DPB2.1* allele by itself does not confer susceptibility to pauciarticular JRA but instead may act to increase the risk of disease in individuals with a DR disease-associated marker. Thus, individuals who are DRw8 (the highest relative risk), DR5, or DRw6 and *DPB2.1* may be at the highest risk for pauciarticular JRA. If the susceptibility conferred by all three of these DR haplotypes reflects a common predisposing sequence, the best candidate would be the DRβI locus because these haplotypes differ in their alleles at the DQα, DQβ, and DRβIII loci (13, 39). The DRβI sequences of these haplotypes, however, are quite similar (39).

To determine whether the *DPB2.1* allele detected in these patients is, in fact, the classic allele defined by sequence and not a variant that is undetectable in the dot-blot assay, samples from four randomly chosen *DPB2.1*$^+$ JRA patients were sequenced. The resulting sequences are identical to those previously reported (Fig. 1). Comparison of the complete amino acid sequence of *DPB2.1* to the other DPβ alleles shows that *DPB2.1* differs from the nonsusceptible *DPB4.2* allele by only one amino acid at position 69 in the mature protein. [The complete amino acid sequences of these two alleles are reported in ref. 40, where they are referred to as DPw2 (*DPB2.1*) and DPw4b (*DPB4.2*).] *DPB2.1* has a positively charged lysine at this position, whereas *DPB4.2* has a negatively charged glutamic acid. The functional significance of this single amino acid difference is evidenced by the fact that the products of these two alleles elicit a different T-cell response in the PLT assay (29). The finding that the charge of a particular amino acid may be correlated with susceptibility to an autoimmune disease is not novel; the charge at position 57 in DQβ has been implicated in susceptibility to insulin-dependent diabetes mellitus (12–14) and *Pemphigus vulgaris* (15–17). While these results may implicate the charge of the amino acid at position 69 of DPβ as an important component in susceptibility to pauciarticular JRA, it is not the sole component, as other nonsusceptible alleles have the same amino acid residue (Fig. 1). As discussed previously (16), it is more likely that it is the entire DPβ allele and not

FIG. 2. Nucleotide sequences of the first domains of *DPB14–DPB17*. Numbers above indicate the corresponding amino acid in the mature protein.

simply the amino acid at position 69 that may confer disease susceptibility.

Finally, dot-blot analysis of this set of 76 patients and 50 control individuals identified several previously unreported patterns of probe hybridization, suggesting the existence of new DPβ alleles. Sequence analysis of these samples identified four new alleles, *DPB14–17* (Figs. 1 and 2). In the DPβ locus, unlike the DRβI, DRβIII, DQα, and DQβ loci, there are very few allele-specific sequences; rather, the DPβ alleles appear to result almost exclusively from the shuffling of a limited number of amino acid sequences in six regions of variability (regions A–F in Fig. 1). This "epitope shuffling" is evident in all four of the new alleles, except region F of *DPB15*, which, because of a single base mutation, has a unique sequence. *DPB16* and *DPB17* are particularly illustrative; they are identical to *DPB8* and *DPB9* except for the same amino acid difference at residue 76. (*DPB16* and *DPB17* have a methionine at position 76, whereas *DPB8* and *DPB9* have a valine.) This amino acid difference corresponds to two nucleotide differences. What this lack of DPβ allele-specific sequences reveals about the evolution of polymorphism at the DPβ locus relative to DQ and DR is not clear; however, sequencing of these genes in other primates should provide valuable information.

The identification of four new alleles brings the total number of DPβ alleles identified to date to 19. Clearly, the DPβ locus is as polymorphic as DRβ and DQβ. The HLA-DP molecule has also been shown to serve as a restriction element for T-cell recognition of viral antigens (41). In addition, occurrences of acute graft-vs.-host disease in bone-marrow transplants of supposed HLA-identical donor–recipient pairs have been shown to correlate with differences in the DP molecule (42, 43). Together these findings suggest that the HLA-DPβ molecule, which has until recently been less well-characterized, may be as important functionally as its well-characterized counterparts DR and DQ. Consequently, that particular DPβ alleles might be associated with susceptibility to certain autoimmune disorders is not surprising.

The mechanism by which particular class II alleles might increase susceptibility to certain autoimmune disorders is unknown, but it is probably related to the function of these molecules in the normal immune response. Class II molecules present on stromal cells of the thymus interact with T-lymphocyte precursors to determine the specificity of the mature T-cell repertoire (3–6). These molecules also bind processed antigen (7–11) to form a complex that is required for the recognition and activation of $CD4^+CD8^-$ T cells. A particular class II allele could therefore contribute to susceptibility to an autoimmune disease by affecting either development of the T-cell repertoire, the binding of antigen, or both. Whatever the mechanism, the most striking finding from this study is the implication that polymorphic residues from both the HLA-DR and -DP loci may contribute to susceptibility to the autoimmune disorder pauciarticular JRA.

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