HLA-D–related (DRw) Antigens in Juvenile Diabetes Mellitus

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We studied the distribution of HLA-D–related (DRw) antigens in 40 patients with juvenile diabetes mellitus (JDM) and 79 matched controls. We found that DRw2 was significantly decreased in the JDM group, suggesting a protective effect of the antigen and that the decrease observed in B7 was secondary. HLA-DRw3 and HLA-DRw4 were increased in the diabetic group, and, as with B8/B15, these two antigen predisposed to the disease additively. The susceptibility for JDM was found to be more strongly related to HLA-DRw3 than to B8. On the other hand, B15 rather than DRw4 showed the stronger association with JDM. Moreover, we found that this second diabetogenic gene is associated primarily with B15 and only secondarily with Cw3, which is in linkage disequilibrium with B15. This study further emphasizes the immunogenetic heterogeneity of JDM. DIABETES *28*:552–557, June 1979.

It is now accepted that, in most populations studied, strong associations are found between certain of the HLA B-locus antigens and juvenile diabetes mellitus (JDM). Thus, the disease is associated with HLA-B8, -B15, -B18, and -Cw3 in Caucasians[1–7] and -Bw54 (formerly Bw22 J) in the Japanese.[8] The combinations B8/B15[1–4] and B8/B18[1,7] appear to increase the susceptibility of the disease in an additive manner. More recently, the disease was found to be even more strongly associated with the HLA-D antigens Dw3 and Dw4 (as defined by homozygous cell typing)[1–4] in Caucasians and DYT in the Japanese;[9] again, the influence of Dw3 and Dw4 in the former population on the susceptibility to JDM acted in an additive manner.[1–4]

In this study we report the distribution of HLA-DRw antigens, as defined by sera developed in our laboratory, in a group of well characterized patients with JDM.

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MATERIALS AND METHODS

73 unrelated Caucasian Newfoundlanders suffering from JDM were studied with respect to HLA-A and -B antigens and 28 for antigens of the C locus. Additionally 40 of the 73 patients were typed for HLA-DRw antigens. Criteria for inclusion in the study were abrupt onset of symptoms, continued need for insulin therapy, and onset before 35 yr of age.[7] Controls consisted of 214 healthy persons from the same geographic area; 60 of these were typed for the C locus and 79 for the HLA-DRw locus antigens.

Peripheral leukocytes were typed for at least 26 HLA-A and -B antigens using a microcytotoxicity method;[9] however, in 79 individuals, 42 HLA-A and -B antigens could be typed. This was achieved with the benefit of sera that split previously established antigens or identified new ones. It was also possible to identify six HLA-C antigens in 60 healthy controls. Bone marrow–derived (B) lymphocytes were used to type for seven HLA-DRw specificities of the Seventh International Histocompatibility Testing Workshop. The method for cell separation and establishment of the specificity of the sera employed for the cell typing for HLA-DRw antigens were described in detail elsewhere.[10]

Relative risk for the disease (x) and chi-square tests for significance of association were calculated.[11,12]

RESULTS

Table 1 shows the HLA-A, -B, and -C antigens in which significant deviations were observed to occur in the JDM group compared with controls. The C-locus antigen Cw3 resulted in a relative risk of 2.85; however, statistical significance in this series was not achieved when the number of antigens was taken into consideration. Table 2 shows three B-antigen combinations found to be increased in the JDM group. Only in the case of the combination B8/B15 did significance persist after correction of the data for the number of possible combinations. It was possible to

TABLE 1
Distribution of HLA-A, -B, and -C antigens in which important deviations were observed

	JDM (73)*	Control (214)	x	χ^2 (Woolf)
B7	7	44	0.4098	4.265
B8	34	54	2.583	11.284†
B15	24	19	5.027	21.76†
B18	10	12	2.672	4.730
Cw3	13 (28)	14 (60)	2.848	4.625

x = relative risk calculated as follows:

$$\frac{\text{No. of patients positive for antigen (a)}}{\text{No. of patients negative for antigen (b)}} \Big/ \frac{\text{No. of controls positive for antigen (c)}}{\text{No. of controls negative for antigen (d)}}$$

* The numbers in parentheses represent the number of patients and controls tested. The number tested for C-locus antigens are shown in parentheses for Cw3.

† Significance at 5% level maintained after correction of number of antigens tested.

compare the numbers of B8- and/or B15-positive patients at the time of diagnosis on three winter-spring intervals (October–March 1974/75, 1975/76, and 1977/78). In the 1974/75 season, of six patients typed, four were B8-positive and one was B15-positive; in 1975/76, five patients were available for study: two were positive for both B8 and B15 and one for B8 only. Of the five patients diagnosed in 1977/78, two each were positive for B8 or B15; none were positive for both. However, in view of the small numbers of patients studied, no firm conclusions can be drawn.

The distribution of HLA-DRw antigens is shown in Table 3. HLA-DRw3 and HLA-DRw4 were increased in the JDM group compared with controls resulting in relative risks of 3.39 and 4.93, respectively. Contrariwise, DRw2 was decreased among the diabetic patients, 32 (80%) of whom were positive for DRw3 and/or DRw4 compared with 32 (40%) of the control group. Ten patients (25%) were positive for both antigens, whereas only three controls were (3.8%). From these figures it can be calculated that the DRw3/DRw4 combination results in a relative risk of 7.62 ($\chi^2 = 8.595$, $P < 0.005$), suggesting that the two antigens contribute to the susceptibility for the disease in an additive manner. The fact that the observed number of patients who were DRw3- and DRw4-positive (25%) is close to that expected on the basis of random associations (29%) attests to the fact that their influence is additive rather than merely a reflection of the higher frequency of the two antigens in the JDM compared with controls.

In four control subjects (4.9%) and two patients (5%), DRw3 was identified as the only antigen. Because a significant proportion of antigens at the DRw locus cannot be presently identified (blanks), it was not possible to completely exclude homozygosity without genotyping these individuals. Of the patients typed 3, X, homozygosity was excluded in one by a family study. Likewise, HLA-DRw4 was the only antigen to be detected in each of three controls (3.3%) and two patients (5%). Thus, presumptive homozygotes for DRw3 or DRw4 are not increased in JDM.

It is observed from the above results that, whereas the relative risks conferred by HLA DRw3 were greater than those for B8, B15, and DRw4, they appeared to contribute to susceptibility equally. However, in order to obtain statistical comparisons between risks contributed by antigens of the two HLA series, we tested the presence versus the absence of one of the antigens in patient and controls in individuals with and without the other antigen. Table 4 shows that DRw3 is increased in B8-positive patients and much more so in B8-negative patients compared with the corresponding controls. In contrast, B8 is distributed in the same frequency in DRw3-negative patients and controls. Thus, JDM is more strongly associated with DRw3 than B8, the increase of the latter being secondary to that of DRw3.

In contrast to B8/DRw3, in the case of B15/DRw4, significant deviations were seen only when the influence of DRw4 was neutralized, while examining the contribution of B15 to the susceptibility to the disease (Table 5, upper panel). More detailed examination of the data shows that the association of B15 with JDM is mainly attributable to the excess of DRw4-positive, B15-positive patients as compared to controls.

In view of the reported association of Cw3 with JDM[2,6] and because of the linkage disequilibrium of this antigen with B15, it was important to determine which of these two antigens was primarily related to the disease. We therefore statistically neutralized the effect of each of these two antigens in turn while examining the influence of the other on disease susceptibility (Table 6). It is apparent that

TABLE 2
HLA-B–antigen combinations found to be increased in JDM

Combinations	JDM	Controls	χ^2	x
B8/B15				
+	10	3	19.17*	9.99
–	63	211		
B8/B18				
+	4	1	7.707	9.215
–	69	213		
B15/B40				
+	4	1	7.707	9.125
–	69	213		

* Significance maintained after correction for possible antigen combinations.

TABLE 3
The distribution of HLA-DRw antigens among diabetic patients and controls

Antigens	JDM (n = 40)	Controls (n = 79)	x	χ^2 (Woolf)	P
DRw1	5	15	0.6095	0.7883	NS
DRw2	1	22	0.097	9.29	<0.005
DRw3	20	18	3.388	8.663	<0.005
DRw4	23	17	4.934	14.374	<0.0005
DRw5	4	20	0.3277	3.608	NS
DRw6	5	16	0.5625	1.078	NS
DRw7	13	24	1.103	0.0557	NS
Blank	9	26	0.5918	1.3710	NS

The three antigens in which deviations from controls were observed remained significant after correction for number of antigens.

NS, not significant.

TABLE 4
Association of HLA-B8 and DRw3 with juvenile diabetes mellitus

		JDM	Controls	χ_1^2	χ_2^2 for significance	χ_2^2 for heterogeneity
DRw3+	B8+	13	14	0.7765		
	B8−	7	4			
DRw3−	B8+	1	6	0.9458		
	B8−	19	55			
Totals				1.7223	0.89738 ($P > 0.60$)	0.02148 ($P > 0.95$)
B8+	DRw3+	13	14	2.677		
	DRw3−	1	6			
B8−	DRw3+	7	4	6.5853		
	DRw3−	19	55			
Totals				9.2622	10.693 ($P < 0.005$)	3.564 ($P > 0.10$)

To determine primacy of association, we examined the relation of HLA-B8 with JDM after dividing the patients and controls into DRw3+ and DRw3− subgroups (upper panel) and that of HLA-DRw3 with JDM calculated after dividing the patients and controls into B8+ and B8− subgroups (lower panel). χ^2 for significance = $\Sigma y^2 w$, where $y = \ln x$ and w is the sum of 1/variance of y. The variance of y is derived as follows: $[1/(a + 1)] + [1/(b + 1)] + [1/(c + 1)] + [1/(d + 1)]$. (Symbols a–d are as outlined in Table 1.) χ^2 for heterogeneity = $\Sigma y^2 w - [(\Sigma wy)^2/\Sigma w]$.

the disease is primarily associated with B15 and only in a secondary manner to Cw3.

No relation was found between the age at diagnosis and the distribution of DRw3 and DRw4.

DISCUSSION

In order to relate the present results to previous reports of HLA–JDM associations, the relationship of HLA-D–related (DRw) antigens to HLA-D specificities as defined by functional assays[10,13] needs to be discussed. The situation may be summarized as follows: whereas there is good correlation between corresponding D and DRw antigens, the agreement is far more complete, suggesting that the two, typing methods do not identify exactly the same gene products.[13] Specificity of reagents notwithstanding, HLA-DRw typing is apparently more sensitive in identifying a given antigen than homozygous cell typing for the corresponding D specificity.[14] Additionally, convincing evidence has been provided that more than one locus control the antigens identified serologically on bone marrow–derived (B) cells.[15,16] Specifically related to the present study, the Seventh International Histocompatibility Workshop recorded significant linkage disequilibria between B7 and Dw2, both B8 and B18 and Dw3, and B15 and Dw4—antigens in which important deviations were found in JDM. On the other hand, the linkages B18/DRw3, B15/DRw4, and Cw3/DRw4 were found in only one or the other of two Caucasian population pools.[17] Data on haplotypic HLA-B/DRw association in 658 haplotypes ascertained from unrelated Caucasians showed no significant B15/DRw4 or B18/DRw3 linkage disequilibria.[18]

In this study, important associations were observed between JDM and HLA-B8, -B15, -B18, and -Cw3; however, only in the first two antigens was significance maintained when the number of antigens tested was considered. Likewise, the combinations B8/B15, B8/B18, and B15/B40 appeared to act in an additive manner in increasing the risk of JDM; only the first combination withstood the test of significance when the possible combinations were taken into account. These results are in general agreement with previous reports from other laboratories as well as with a previous report from this center based on a

TABLE 5
Association of HLA-DRw4 and B15 with juvenile diabetes mellitus

		JDM	Controls	χ_1^2	χ_2^2 for significance	χ_2^2 for heterogeneity
DRw4+	B15+	14	3	5.892		
	B15−	9	14			
DRw4−	B15+	3	3	2.8629		
	B15−	14	59			
Totals				8.7548	9.4300 ($P < 0.005$)	0.22 ($P > 0.80$)
B15+	DRw4+	14	3	2.2714		
	DRw4−	3	3			
B15−	DRw4+	9	14	3.848		
	DRw4−	14	59			
Totals				6.119	3.6128 ($P > 0.10$)	0.17 ($P > 0.90$)

To determine primacy of association, we examined the relation of HLA-B15 with JDM after dividing the patients and controls into DRw4+ and DRw4− subgroups (upper panel) and that of HLA-DRw4 with JDM calculated after dividing the patients and controls into B15+ and B15− subgroups (lower panel).

TABLE 6
Association of B15 and Cw3 with juvenile diabetes mellitus

		JDM	Control	χ_1^2	χ_2^2 for significance	χ_2^2 for heterogeneity
B15+	Cw3+	10	4	0.7618		
	Cw3−	2	0			
B15−	Cw3+	3	11	0.0083		
	Cw3−	13	45			
Totals				0.7702	0.3579 ($P > 0.90$)	0.095 ($P > 0.95$)
Cw3+	B15+	10	4	7.0957		
	B15−	3	11			
Cw3−	B15+	2	0	6.040		
	B15−	13	45			
Totals				13.135	17.803 ($P < 0.0005$)	0.368 ($P > 0.90$)

The procedures for analysis for the separate associations of JDM with B15 and Cw3 are as outlined in Tables 5 and 6. Number of patients analyzed in this table = 28 and the controls = 60.

smaller group of patients.[1-7] Our finding that a seasonal aggregation of JDM is accounted for by a disproportionately greater number of B8-positive patients is in agreement with one retrospective report[19] and contrasts with another prospective study.[20]

Concerning the distribution of HLA-DRw antigens among the JDM group, we found that DRw2, DRw3, and DRw4 showed significant deviations from controls. Whereas the decrease in B7 in the JDM group did not achieve significance,[1] that in DRw2 did, suggesting that the apparent protective influence of B7 against JDM is due to its linkage disequilibrium to DRw2. Ilonen et al. also recently reported a significant decrease of Dw2 in patients with JDM and their unaffected relatives.[21] The decrease in DRw2 in our series is unlikely to be secondary to an increase in other DRw antigens associated with JDM, stressing the protective influence against the disease of immune (non-) response gene in linkage with DRw2.

On the other hand, HLA-DRw3 and -DRw4 were observed to be significantly increased in diabetics compared with controls. Moreover, as has been previously observed for B8 and B15, the two HLA-DRw series antigens acted in an additive manner in predisposing to JDM. Statistical treatment of the data clearly showed that HLA-DRw3 was more strongly associated with JDM than B8 and the apparent association of the latter antigen was due to its strong linkage to DRw3. The primary nature of the association of JDM with DRw3 is particularly reflected in the excess of B8-negative patients who were DRw3-positive compared with controls. The relative risks calculated for B15 and DRw4 for this series of patients were comparable (5.02 and 4.93). This apparent equal degree of association of JDM with B15 and DRw4 was confirmed when the influence of each antigen in turn was neutralized, and the effect of the other on disease association was examined. We interpret this finding to mean that the putative B15-associated diabetogenic gene is, unlike the B8-associated gene, not more strongly associated with the DRw antigen than with the B antigen.[2] Rather, it appears to be more strongly associated with B15 than DRw4. It is debated whether the association of JDM with HLA B15 is secondary to its linkage disequilibrium with Cw3[6] or vice versa;[1] the data from this study show clearly that the B15-related diabetogenic gene is nearer the B than either the C or DRw loci.

Our results of the stronger association of DRw3 than B8 with JDM are in agreement with that established for Dw3 by Neruo et al.[1,4] However, our findings are at variance with the Copenhagen group in that they found a clearly stronger association of Dw4 than B15 with JDM. The differences in results can be explained on the basis of methodologic differences in antigen assignments rather than on the basis of populations of patients studied. Serologically detected HLA-DRw4 appears to be a much broader specificity than is Dw4 determined by homozygous cell typing. Thus, many more healthy individuals were found to be DRw4- than Dw4-positive.[17] Moreover, based on analysis of 658 haplotypes, it was concluded that DRw4 is randomly associated with a large number of B-locus antigens including B15. This contrasts with the linkage disequilibrium previously described for Dw4 and B15. This lack of linkage disequilibrium between DRw4 and B15 may have allowed us to dissociate the influence of these two antigens on disease susceptibility. Compared with the Caucasian frequency of B15 and DRw4 in our control population, the diabetic group studied showed a greater increase in B15 than has been encountered in many other studies. However, it cannot be excluded that this finding is due to variation in sampling,[7] nor will this change the tendency of a closer association of one locus rather than another with the disease.

Our results regarding the association of JDM with HLA-DRw antigens are in general agreement with those of several preliminary reports;[22-26] however, these studies have variously reported a stronger association of JDM with DRw3 than DRw4,[22,25] DRw4 than DRw3,[23] or indeed no association whatsoever with DRw antigens.[26] The basis for this apparent heterogeneity in risk is likely to be the same as has been postulated for JDM–HLA-B antigen associations.[27]

Diseases associated primarily with DRw (or Dw)-locus antigens have in common an autoimmune basis.[28] Such a general scheme seems to be in keeping with the heterogeneous immunogenetic basis of JDM.[2,29-32] Thus, the propensity for developing antipancreatic islet cell antibody and cell-mediated immunity[4] appears to be re-

lated to HLA-B8 and -Dw3 positivity,[4,29] and persistance of these antibodies correlates with B8 (and presumably Dw3) positivity.[30] In contrast, B15 is postulated to predispose to an inherited insensitivity to glucose, reduced β-cell mass, or reduced regenerative capacity following subclinical islet damage.[4] This postulate is in keeping with the fact that the B15 gene is not more strongly associated with DRw4. However, since B15 and *not* B8 is found to be strongly correlated with the capacity to form IgG antibodies against exogenous insulin,[31] it would be of interest to determine whether this immune-response gene is more closely related to the D locus than the B15-associated diabetogenic gene. Of course the possibility exists that the diabetogenic gene and that controlling humoral response to injected insulin may complement each other in inducing the production of anti-insulin antibodies.

A heated debate is currently underway as to whether the susceptibility to all juvenile diabetes can be explained on the basis of a single gene locus requiring two diabetogenic alleles.[1,2,33–35] Our data from this study militate against this view. First, the additive risk of B15 and B8 and DRw3 and DRw4 and the lack of an increase in risk for homozygotes (in a region where HLA homozygosity is not uncommon[27]) of either B8, B15, DRw3, or DRw4 are incompatible with a double dose requirement for susceptibility to diabetes. Secondly, our observation that the B8/DRw3-associated diabetogenic gene is more strongly associated with DRw3 (and hence closer to DR locus), whereas the B15/DRw4-associated gene is closer to the B locus, suggests that these genes map in different locations in the major histocompatibility complex. Conversely, sibpair[1,36] data, showing that both parental haplotypes are necessary for the susceptibility of the disease, do not necessarily conflict with our proposal of a two-gene influence. Coupled with the additive influences of B8/B15 and DRw3/DRw4, it may be postulated that these two genes are not "recessive" in the sense that they both are not necessary for the expression of the disease.

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