A Particular Subset of HLA-DR4 Accounts for All or Most of the DR4 Association in Type I Diabetes

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SUMMARY

Two human T-lymphocyte clones, derived from a mixed leukocyte culture (MLC) with stimulating cells from a type I diabetic patient, define a subset of HLA-DR4, tentatively called “DR4S.” In testing of 69 random type I diabetic subjects and 69 random controls, 79% (37/47) of DR4-positive patients, but only 44% (8/18) of DR4-positive controls, had DR4S (P < 0.01). The relative risk of type I diabetes for DR4S+ individuals was 8.8, while that for DR4+ DR4S~ individuals was only 1.0. Thus, in the population tested, DR4S accounts for all or most of the increased frequency of HLA-DR4 in type I diabetes.

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Many reports have shown elevated frequencies of HLA-DR3 and -4 in type I diabetes, and some show an altered distribution of DR41-2 subtypes among type I diabetic subjects compared with DR3- or DR4-positive controls. Owerbach et al.1 found a skewed distribution of DR4 subtypes using restriction-fragment length polymorphisms (RFLPs), and Bach et al.2 made a similar observation upon testing for HLA-D antigens in mixed leukocyte culture. Our approach has been to systematically produce reagents, in the form of human T-lymphocyte clones, specifically sensitized in vitro to detect leukocyte antigens of type I diabetic patients. The rationale is as follows: we assume that the altered frequency of certain DR antigens in type I diabetes most likely derives from the role of DR molecules in antigen presentation to T-cells. Thus, human T-cell clones, detecting individual epitopes of DR and other class II HLA molecules of type I diabetic patients, may be ideal for detection of diabetes-relevant variation in these molecules.

Our study design has other unique features relative to many HLA-disease association studies. First, for comparison with the Wisconsin patients, we selected only control subjects who were born in Wisconsin to minimize ethnic variation between patient and control samples. Second, we took pains to select and maintain random patient and control groups, to avoid possible genetic bias in either group. A key element in the maintenance of random groups was the production of permanent lymphoblastoid cell lines (LCLs) from all subjects to insure that the samples could not become selectively depleted of certain individuals' cells.

Here we report both a preliminary study of 19 type I diabetic subjects and 19 controls, in which the DR4S antigen was identified, and a prospective study of 50 patients and 50 controls, indicating that DR4S accounts for all or most of the DR4-diabetes association in the population studied.

MATERIALS AND METHODS

Subjects. Patients had classic insulin-requiring type I diabetes and attended the Pediatric Diabetes Clinic at University Hospital in Madison (N = 54) or were newly diagnosed type I diabetic patients (N = 15) from the Wisconsin Diabetes in Youth Study (WDYS) conducted in a 14-county area of Southern Wisconsin by Dr. Donn D'Alessio. Controls were random Wisconsin-born, Madison area residents (N = 54) or age- and sex-matched “best friend” controls from the WDYS (N = 15).

Cells. Cells tested for the 138 subjects were Epstein-Barr-virus-transformed lymphoblastoid cell lines (LCLs).

HLA typing. HLA-DR typing was done by the standard NIH microcytotoxicity technique.3

Production and maintenance of human T-cell clones. Priming in mixed leukocyte culture (MLC) and cloning of T-cells were done by standard techniques as described earlier.4 In the MLC, responding cells were PBMC from a nondiabetic person HLA-A1,28;B37,44;Cw6;DR5, and stimulating cells were from a type I diabetic child HLA-A1, B8, Cw7, DR3/A2, Bw62, Cw3, DR4 (patient's antigens shown as maternal/paternal HLA haplotypes). Cells were cloned after

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that person's cells by the DR4S-defining clones were either positive, or ambiguous by cluster analysis, as described. An additional (10,000r), including approximately equal numbers of both positive, or one positive and the other ambiguous. Individual was defined as DR4S-positive if PLT responses to PLT results were classified as positive, negative, or ± (ambiguous). Actual results corresponding to these categories are as follows (expressed as percent of the highest single response for each clone):

| Clone  | Expt. 1 | 70%–100% | 3%–18% | ±100% (in cpm) |
|--------|---------|----------|--------|----------------|
| Clone 60 | +       | 73%      | 1–14%  | 6,844          |
| Clone 72 | +       | 70%      | 3–18%  | 7,560          |
| Clone 72 | +       | 66%      | 2–10%  | 32,792         |

**RESULTS**

Preliminary study. Of 91 T-cell clones derived from a control-antidiabetic MLC, 2 were found to define a subset of HLA-DR4, hereafter referred to as "DR4S" (Figure 1). In preliminary testing, of 19 patients and 19 controls, DR4S comprised a greater subset of DR4 among the patients (DR4S/DR4 = 9/13) than among the controls (DR4S/DR4 = 3/7).

**Prospective study.** To determine if the excess of DR4S among DR4-positive type I diabetic patients versus controls was a general phenomenon, we tested an additional 50 patients and 50 controls. In this prospective series (Table 1, line 2), DR4S was again overrepresented in the DR4-positive patient group (P = 0.041).

**Analysis of combined studies.** Since the DR4S/DR4 ratios were so similar in the preliminary and prospective series (Table 1, lines 1 and 2), we also analyzed the pooled data (Table 1, line 3). The relative risk of 8.3, for DR4S among DR4-positive individuals, implies that DR4S+ individuals are roughly 8 times as likely to develop type I diabetes as are DR4+DR4S− individuals.

Table 2 compares the frequencies of DR4, DR4S, and the complementary DR4 subset (DR4+DR4S−) among all tested individuals. As expected from Table 1, the relative risk of diabetes is somewhat higher for DR4S than for DR4, although the etiologic fraction (an index of the degree to which a disease can be attributed to the factor in question) is slightly lower for the DR4S subset than for DR4 as a whole. More striking is the finding that the DR4+DR4S− subset was equally frequent (14%) in diabetic and control groups (relative risk = 1, etiologic fraction = 0), suggesting that the DR4S-negative subset lacks a critical diabetes-susceptibility determinant. A similar conclusion is suggested by the fact that the relative risk associated with DR4S among DR4-positive subjects (RR = 8.3, Table 1) is nearly the same as that for DR4S among all subjects (RR = 8.8, Table 2).

**DISCUSSION**

We used two human T-lymphocyte clones in a proliferative assay to define an HLA-DR4 subtype that is significantly more common in DR4-positive type I diabetic patients than in DR4-
TABLE 2
Frequencies of DR4, DR4S, and the complementary subset of DR4 in type I diabetic patients and controls

|                | Patients (N = 69) | Controls (N = 69) | Relative risk | Etiologic fraction |
|----------------|------------------|------------------|---------------|-------------------|
| DR4+DR4S+      | 37 (54%)         | 8 (12%)          | 8.8           | 0.48              |
| DR4+DR4S-      | 10 (14%)         | 10 (14%)         | 1.0           | 0.0               |
| Total DR4      | 47 (68%)         | 18 (26%)         | 6.1           | 0.57              |

positive controls. DR4S was found in 54% of random diabetic patients versus 12% of random controls, while the remaining DR4-positive subjects (DR4+DR4S-) comprised 14% of both patient and control groups (Table 2). This suggests that, in the population studied, DR4-associated antigens other than DR4S may occur in type I diabetes only by chance, having little or no etiologic significance.

Other considerations, however, suggest that another DR subset, relatively uncommon in southern Wisconsin, also is diabetogenic. First, since DR3 (not shown) and DR4S are increased in frequency among the diabetic patients, irrelevant antigens should show a compensatory decrease. That this did not happen for the remaining DR4 antigens (they were equally frequent in patients and controls) suggests that some of these may be relevant to diabetes. This is reflected in part by the slightly lower etiologic fraction for DR4S than for DR4 (Table 2). Second, in a Boston study (M. Sheehy et al., unpublished), we found that most Ashkenazi Jewish type I diabetic patients had HLA-Dw10, a DR4 subset that does not overlap with DR4S (M. Sheehy and J. Rowe, unpublished observation). Although we have just begun to test for Dw10 in Wisconsin diabetic patients and controls, Dw10 clearly constitutes a minority of the DR4+DR4S- subset in the sample reported here.

Together, the results described here, together with our unpublished results for HLA-Dw10 in Ashkenazi Jews (described above), indicate that two DR4 subtypes, but not the remaining DR4 subtypes, are important in the etiology of type I diabetes. Thus, the primary association is neither with DR4 nor with any single DR4 subgroup as currently defined. At this point, it seems likely that a functional epitope, common to DR4S and Dw10 but not to other DR4 subtypes, favors the development of anti-beta-cell autoimmunity. We are currently attempting to produce T-cell clones to detect this hypothetical epitope, and have initiated studies to determine the relationship of DR4S to the MLC-defined HLA-D antigens and to DR4-associated RFLPs.

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