Specific Association of HLA-DR4 With Increased Prevalence and Level of Insulin Autoantibodies in First-Degree Relatives of Patients With Type I Diabetes

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First-degree relatives of patients with insulin-dependent (type I) diabetes (n = 264 from 106 families) were evaluated with HLA typing and determination of competitive insulin autoantibodies (CIAAs) and islet cell autoantibodies (ICAs). The levels of CIAAs in 30 relatives exceeded our upper limit of normal (≥39 nU/ml), and 30 had high-titer ICAs (≥40 Juvenile Diabetes Foundation units [JDF U]). Eleven of the HLA-typed relatives developed diabetes during follow-up. Twenty-three percent (28 of 123) of the relatives with at least one HLA-DR4 allele were CIAA$^+$ (CIAA ≥39 nU/ml) versus 4% (6 of 141) among DR4$^-$ relatives ($P < 0.0001$). Twenty-one of 22 of the highest CIAA values were all in the DR4$^+$ group (DR4$^+$ vs. DR4$^-$, $P = 0.003$, Wilcoxon's rank-sum test). HLA-DR3 did not correlate with the level of CIAAs, and neither DR3 nor DR4 correlated with titer of ICAs measured in JDF U. We conclude that, in first-degree relatives of patients with type I diabetes, there is a striking association with HLA-DR4 in both the prevalence of relatives exceeding the normal CIAA range and in the level of CIAAs. These data suggest that a gene on HLA-DR4 haplotypes contributes to the level of anti-insulin autoimmunity, and we hypothesize that DR4-associated diabetes susceptibility, distinct from DR3-associated susceptibility, may be secondary to this influence. *Diabetes* 40:709–14, 1991

Insulin-dependent (type I) diabetes mellitus appears to result from an autoimmune process with immunogenetic factors contributing to pathogenesis in animal models and in humans (1). The appearance of autoantibodies to different antigens often precedes the onset of diabetes by years (2–5). Palmer et al. (2) initially described insulin autoantibodies (IAAs) in non-insulin-treated new-onset type I diabetic subjects. The levels of these competitive insulin autoantibodies (CIAAs) correlate with the rate of progression to overt diabetes in islet cell antibody–positive (ICA$^+$) relatives (6,7). Levels >80 nU/ml in our radioimmunoassay for CIAAs (upper limit of normal 39 nU/ml) are associated with both long-term persistence of antibodies and progression to overt diabetes (8,9). This observation has clinical implications in that CIAA levels between 40 and 80 nU/ml, although exceeding the upper limit of normal (see METHODS), might not have the same predictive value as CIAA values >80 nU/ml.

Type I diabetes is associated with the HLA types DR3 and DR4 in whites, and subtyping of DR4 haplotypes at the DQβ-locus indicates that DQw8 (lacking an Asp at position 57 of the HLA-DQ β-chain) is strongly associated with diabetes susceptibility (10–14). It has been reported that DR3/DR4-heterozygous children may have accelerated β-cell destruction, whereas DR3 is associated with a slower progression of type I diabetes (15,16). The highest risk for diabetes occurs in DR3/DR4-heterozygous individuals, suggesting that each haplotype (DR3-expressing haplotype vs. DR4) has a distinct contribution to diabetes susceptibility. The nature of such distinct influences on susceptibility is unknown.

Conflicting data have been reported as to whether specific HLA-DR types are associated with the presence of IAAs in new-onset diabetic subjects (17–21). In a preliminary analysis at the time 9 IAA$^+$ first-degree relatives of patients with type I diabetes were typed for HLA (both DR4$^+$ and DR4$^-$), we noted that a preponderance of relatives with high levels of IAAs expressed HLA-DR4 (for the initial 9 relatives, mean ± SE CIAA: DR4$^+$ vs. DR4$^-$ 199.8 ± 56.6 vs. 79.7 ± 9.2 nU/ml, P = 0.2, Wilcoxon's rank-sum test). With these preliminary data, we have prospectively analyzed further relatives to test the hypothesis that DR4 is associated with elevated levels of IAAs. In this study, we typed 264 relatives of patients with type I diabetes from 106 families for HLA

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and measured CIAAs and ICAs. Forty-one relatives were either CIAA$^+$ or ICA$^+$ or both.

RESEARCH DESIGN AND METHODS

For this study, 264 first-degree relatives of patients with type I diabetes were typed for MHC markers and were screened for CIAAs and cytoplasmic ICAs (168 siblings, 14 twins, and 3 relatives who are both offspring and siblings of a type I diabetic patient; 11 offspring; and 68 parents). For 78 families (n = 195 relatives), HLA typing was performed as part of a study evaluating extended HLA haplotypes associated with type I diabetes; thus, the antibody status of the relatives was unknown at the time of HLA typing. Five of these relatives were found to be autoantibody positive. In addition, 69 members of 28 families in which family screening had identified an ICA$^+$ or CIAA$^+$ relative were typed for HLA. The criteria for inclusion in this study were the availability of both HLA-DR typing and measurement of CIAAs/ICAs and/or development of type I diabetes after autoantibody screening.

Sera stored at −20°C were tested for CIAAs with a radioassay described previously (9,22). The displaced amount of insulin (in nU/ml) by cold ligand is calculated by the difference in percent binding with and without excess unlabeled insulin. Negative CIAA values are computational products where the radioactive counts per minute (cpm) with cold competition are slightly greater than without unlabeled insulin. The upper limit of normal of our assay is 39 nU/ml, 3SD above the normal mean of 74 control subjects without a family history of diabetes. None of 81 additional control subjects measured after the normal range was defined exceeded this upper limit, and our assay took part in the 1990 international Immunology in Diabetes Workshop (IDW) comparison of IAA assays. The interassay coefficient of variation is 10%.

The assays for ICAs were done on frozen sections of human and Wistar-Furth rat pancreas with fluorescein isothiocyanate– or peroxidase-conjugated protein A or, more recently, to increase sensitivity with peroxidase-conjugated anti-IgG, in a blinded manner as previously described (23–25). The assays give positive results in <1 of 400 population control subjects, 2% of first-degree relatives of type I diabetic subjects, and 60% of new-onset diabetic subjects and correlate with consensus Juvenile Diabetes Foundation units (JDF U) in IDW comparisons (26). ICA$^-$ in this study is defined as <40 JDF U.

For MHC typing, blood was collected into a Vacutainer tube containing K$^+$-EDTA and into a heparinized syringe. Plasma, which was centrifuged (2000 rpm for 10 min) from the EDTA-containing blood, was either used immediately or frozen at −80°C. The separation of lymphocytes was done by Ficoll-Hypaque centrifugation, after which the cells were frozen and stored in vapor-phase liquid N_2 until analysis. The microlymphocytotoxicity assay was used for typing for HLA-A, HLA-B, HLA-C, HLA-DR, and HLA-DQ (27). Properdin factor B (BF) typing was carried out by immunofixation after agarose-gel electrophoresis of plasma or serum samples with goat anti-human factor B (28). C2 patterns were generated by isofocusing of serum or plasma samples in a polyacrylamide gel and development of patterns in a C2-sensitive agarose gel containing indicator antibody–sensitized sheep erythrocytes (29). For typing of C4A and C4B, neuraminidase-treated plasma samples were subjected to agarose-gel electrophoresis and immunofixation with goat anti-C4 (30). Complotypes are given in arbitrary order as BF, C2, C4A, and C4B alleles in abbreviated form; e.g., SCO1 denotes *BF*S, C2*C, C4A*QO, C4B*1* (31).

The CIAA values were not normally distributed. Thus, nonparametric statistical analyses were used with Fisher's exact test, χ^2-test, log-likelihood ratio, and Wilcoxon's rank-sum test in the SAS statistical package for personal computers.

RESULTS

Of the total population of first-degree relatives studied, 23% (28 of 123) in the group with at least one DR4 allele were CIAA$^+$ (>39 nU/ml) compared with 4% (6 of 141) in the DR4$^-$ group ($P < 0.0001$, Fisher's exact test) (Fig. 1). Because a CIAA level >80 nU/ml has a better predictive value for progression to diabetes, we also analyzed our data in regard to CIAA levels >80 and >39 nU/ml (8,9). Eighteen percent (22 of 123) of DR4$^+$ relatives had CIAA levels >80 nU/ml versus 2% (3 of 141) in the DR4$^-$ group ($P < 0.00001$, Fisher's exact test). In contrast, grouping relatives by the presence or absence of HLA-DR3 did not result in groups significantly different for CIAA levels: 15% (16 of 109) CIAA$^+$ subjects in the DR3$^+$ group versus 12% (18 of 155) in the DR3$^-$ group.

Figure 2*A* plots individual CIAA values of relatives (mean age 16.3 yr, range 2.5–50 yr for 25 siblings, 8 twins, 3 parents, and 6 offspring) confirmed positive (≥40 JDF U) for cytoplasmic ICAs or CIAAs (>39 nU/ml) or both with repeat measurements, except 3 who developed diabetes after only one autoantibody determination (n = 2) or after testing negative for autoantibodies (n = 1). Twenty-one of the 22 highest CIAA levels were in the group of relatives with at least 1 DR4 allele (P = 0.003, Wilcoxon's rank-sum test). Deleting the initial 9 CIAA$^+$ relatives from this analysis does not change the statistical result (P = 0.003, Wilcoxon's rank-sum test). Among these 42 autoantibody-positive relatives were six pairs of first-degree relatives. Excluding the oldest or young-

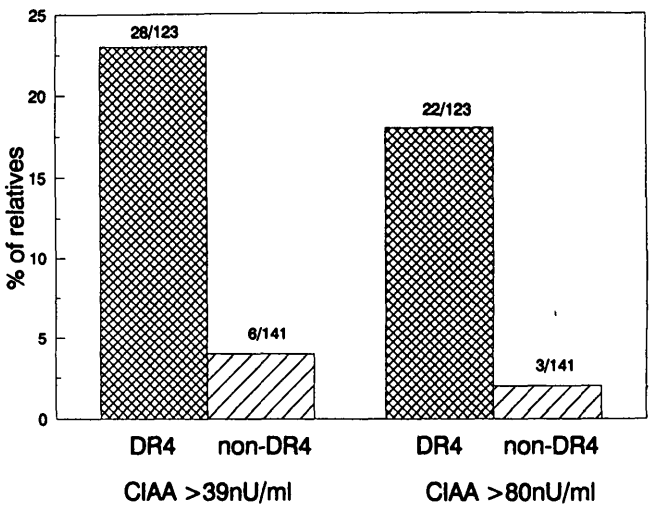

FIG. 1. Percentage of 1st-degree relatives in this study exceeding upper limit of normal for competitive insulin autoantibodies (CIAA; >39 nU/ml) and those having CIAA level >80 nU/ml subdivided into relatives having at least 1 HLA-DR4 allele vs. those having no DR4 allele.

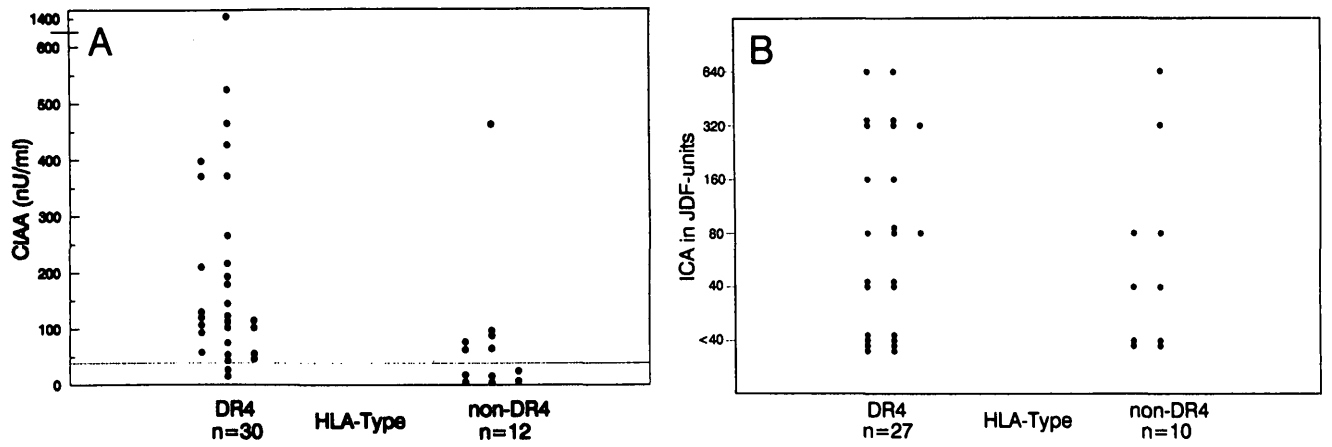

FIG. 2. *A*: distribution of mean competitive insulin autoantibody (CIAA) values in relatives who were islet cell autoantibody positive (ICA$^+$; ≥40 Juvenile Diabetes Foundation units [JDF U]) or CIAA$^+$ (upper limit of normal >39 nU/ml) and/or followed to diabetes. Group is subdivided into relatives having at least 1 HLA-DR4 allele vs. those having no DR4 allele. *Horizontal line*, upper limit of normal of CIAA. *B*: distribution of ICA titer in JDF U for relatives shown in *A* (in 5 positive relatives, sera were not available for JDF titer determination) with relatives subdivided by HLA-DR4 or non-DR4.

est of each pair and reanalyzing the remaining 36 relatives does not appreciably alter the statistical significance of the DR4 association (total group χ^2-test as per Table 1, P = 0.0026; excluding oldest of pair, P = 0.016; excluding youngest of pair, P = 0.0029). There was no difference in age between the two groups. As shown in Fig. 2*B*, subdividing relatives by expression of HLA-DR4 was not associated with different titers of cytoplasmic autoantibodies as expressed in JDF U (Fig. 2*B*). In particular, both DR4$^+$ and DR4$^-$ relatives had a similar distribution of anti-cytoplasmic ICA titers. Note that relatives in Fig. 2 include the CIAA$^+$ relatives of Fig. 1 and those CIAA$^-$ but ICA$^+$. Subdividing relatives into DR3$^+$ and DR3$^-$ groups did not correlate with CIAA levels (P > 0.05, Wilcoxon's rank-sum test; P = 0.3, Fisher's exact test; for CIAA >40 nU/ml) or ICA titer. Of the CIAA$^+$ relatives, 17 were DR4$^+$, 5 DR3$^+$, and 11 DR3$^+$/4$^+$ (6 DR4$^-$ and DR3$^-$). In the group of ICA$^+$ relatives, 12 were DR4$^+$ versus 5 DR3$^+$, and 10 were DR3$^+$/4$^+$ (3 DR4$^-$ and DR3$^-$). Nine relatives were DR4$^+$ and both CIAA$^+$ and ICA$^+$; 3 in this group were DR3$^+$, and 9 were DR3$^+$/4$^+$.

To further analyze the influence of DR4 on IAAs, a subset of high-risk relatives (n = 30) with high titer (≥40 JDF U) of ICAs or subsequent development of diabetes (and therefore true prediabetic subjects) were identified (Table 1); note that relatives in Table 1 include those of Fig. 2 who are ICA$^+$ but not those who are CIAA$^+$ only. Eight relatives developed diabetes and also had ICA titers ≥40 JDF U; 1 developed diabetes and was ICA$^-$. For 4 relatives who developed diabetes, no sera were available to determine ICA titer, and 17 relatives were high-titer ICA$^+$ and have not yet developed overt diabetes. No significant difference was found between these subgroups regarding the HLA-DR4 distribution or the frequency of CIAA positivity (NS, χ^2-test). Nineteen of 21 (90%) of this group with at least one DR4 allele are CIAA$^+$, and 15 of 21 (71%) have CIAA levels >80 nU/ml compared to 3 of 9 (33%) and 1 of 9 (11%), respectively, of the DR4$^-$ group (P = 0.002, χ^2-test; P < 0.001, Wilcoxon's rank-sum test).

With complete families typed for HLA markers to determine haplotypes, it was possible to compare HLA haplotype sharing with CIAA levels. In two families in which HLA-identical siblings were present, levels of CIAAs were not solely determined by HLA haplotypes (Fig. 3). For instance in family 1, an identical twin (neither twin with diabetes but both HLA identical to a sibling with diabetes) has IAAs (and cytoplasmic ICAs) over 5 yr of follow-up, whereas his twin is autoantibody negative. Family 2 illustrates two HLA-identical siblings, one with a high and one with a moderate level of CIAAs.

Because most of the IAA$^+$ relatives (n = 26) had extensive MHC typing, we also assessed, after evaluating the DR relationship, whether IAA correlated with BF, C2, C4A, C4B, or alleles of HLA-B or specific extended HLA haplotypes known to be associated with type I diabetes (HLA-B8, SCO1, DR3; HLA-B18, F1C30, DR3; HLA-Bw62, SC33, DR4; HLA-B38, SC21, DR4) (32). No specific associations were found with these polymorphic markers of the HLA region and CIAA levels. Examining each HLA marker separately, BF, C2, C4A,

TABLE 1
Subset of high-risk relatives with high-titer islet cell antibodies (ICAs) and/or development of diabetes subdivided by their HLA-DR type and competitive insulin autoantibody (CIAA) level

	DR4	Non-DR4	DR4/DR4	DR4/DR3	DR4/X	DR3/DR3	DR3/X	X/X
CIAA$^-$ (<40 nU/ml)	2	6	1	0	1	1	1	4
CIAA$^+$ (40–80 nU/ml)	4	2	0	2	2	2	0	0
CIAA$^+$ (>80 nU/ml)	15	1	2	8	5	0	1	0

High-titer ICA level is defined as ≥40 Juvenile Diabetes Foundation units. The upper limit of normal for CIAA level is 39 nU/ml. X, any DR allele other than DR3 or DR4. P < 0.01 DR4 vs. non-DR4 by χ^2-test.

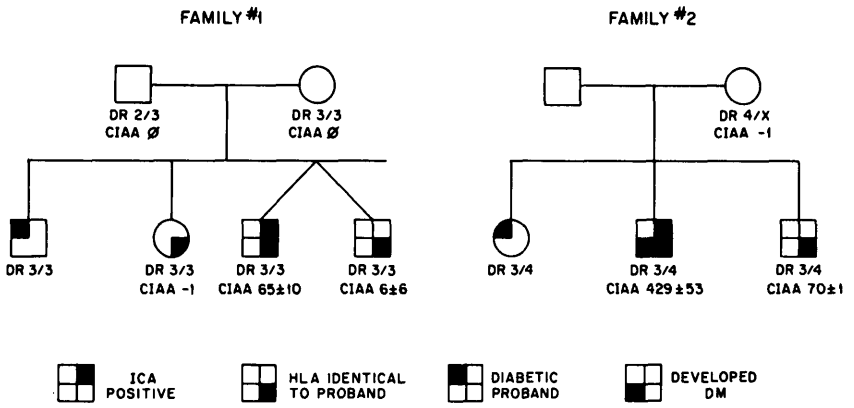

FIG. 3. Family trees of 2 families with HLA-identical siblings. In family 1, there are identical twins: 1 is positive for anti-insulin competitive insulin autoantibodies (CIAA) and islet cell antibodies (ICA); other is negative for both antibodies. Family 2 illustrates HLA-identical siblings: 1 has high CIAA level (429 ± 53); other has moderate level (70 ± 1). DM, diabetes mellitus; X, any DR allele other than DR3 or DR4.

or C4B also failed to show any differences in CIAA levels among carriers of the different alleles.

DISCUSSION

In previous studies, we have reported that the presence of CIAAs measured in fluid-phase radiobinding assays is associated with more rapid progression to diabetes and that high levels of CIAAs are associated with persistence of the antibody in ICA$^+$ first-degree relatives. By life-table analysis, we found an increased risk for developing diabetes in ICA$^+$ first-degree relatives and in ICA$^-$ or low-titer ICA$^+$ relatives with a level of CIAAs >80 nU/ml (7–9).

In a study by Atkinson et al. (18), IAAs in first-degree relatives occurred in relatives with high-risk HLA alleles DR3 or DR4. Similar to this study, a review of their published data subdivided by DR4$^+$ versus DR4$^-$ shows a prevalence of IAAs of 17 of 131 (13%) in the DR4$^+$ group versus 5 of 114 (4%) in the DR4$^-$ group (P = 0.03). Held et al. (19) reported a higher prevalence of IAAs in DR4$^+$ in contrast to DR4$^-$ new-onset type I diabetic subjects. Five of 6 of their CIAA$^+$ patients typed for at least one DR4 allele (19). In contrast, Karjalainen et al. (17), who determined HLA types, IAAs (with a radiobinding assay different from the one we use), ICAs, and β-cell function in 115 new-onset diabetic subjects, did not find an association between HLA type and the prevalence of IAAs or ICAs (17). A study by Spinas et al. (20) with an enzyme-linked immunosorbent assay (ELISA) for IAAs did not find an association between IAAs and HLA-DR types. IDW comparisons indicate that such ELISA assays measure different antibodies than current radiobinding assays (20,21).

In type I diabetic subjects receiving insulin, studies have shown that the development of secondary insulin antibodies also seems to have an HLA-DR association. McEvoy et al. (33) found that HLA-DR3–homozygous diabetic subjects had the lowest levels of antibodies, and Asplin et al. (34) described that DR4$^+$ diabetic subjects were more frequent in the group of their upper third of insulin antibodies, but otherwise no association was found. Similar results had been published by Reeves et al. (35) in that DR3$^+$ diabetic subjects had lower insulin antibody levels, whereas DR7$^+$ individuals showed the highest levels of secondary insulin antibodies. A study by Kahn et al. (36) identified a high percentage of DR7$^+$ diabetic subjects who had an abnormal immune response to insulin, characterized by cutaneous and/or systemic reactions and insulin resistance. Interestingly, the two relatives in our study with the highest levels of IAAs were both HLA-DR4$^+$/DR7$^+$.

In studies of ICAs in the prediabetic period of first-degree relatives of type I diabetic subjects, Vexiau et al. (37) reported that presence of ICAs in nondiabetic relatives was associated with DR3 and/or DR4 compared with DR3$^-$ or DR4$^-$. Pagano et al. (38) reported that DR3/DR4-heterozygous patients had an increased frequency of ICAs in new-onset diabetic subjects, whereas Spinas et al. (20) found no correlation between HLA types and the prevalence of ICAs in their first-degree relatives. Our results are similar to those of Spinas et al. in that we found no association between ICAs and HLA-DR type.

In this study, we found a strong correlation between anti-IAA prevalence and level with HLA-DR4 in nondiabetic first-degree relatives of type I diabetic subjects. The correlation between the level of CIAAs and HLA-DR4 was striking, with 21 of 22 relatives with the highest CIAA levels having a DR4$^+$ HLA haplotype. Homozygous and heterozygous DR4 combinations were equally distributed among the CIAA$^+$ relatives compared with the whole study population: 2 of 11 (18%) DR4/DR4 and 26 of 112 (23%) DR3/DR4 or DR4/DRX (X indicates any DR allele other than DR3 or DR4 [NS, Fisher's exact test]). One DR4 allele appears sufficient in exerting this influence on CIAAs, because only 2 of 30 CIAA$^+$ DR4$^+$ individuals were DR4/DR4.

Up to 98% of new-onset type I diabetic subjects in white populations express a DR4 and/or DR3 allele (10). Note that HLA-DR3/DR4 heterozygotes even among identical twins have the highest concordance for type I diabetes, suggesting, at least in part, that different genes with distinct influences of DR3 and DR4 haplotypes may determine diabetes susceptibility. Haplotypes expressing DR4 and DR3 have been subdivided with various techniques, including DNA analysis, primed lymphocyte typing, and evaluation of extended HLA haplotypes with typing for class I and complement alleles. Among DR4$^+$ type I diabetic subjects, 95% express DQw8 (with Ala instead of Asp at position 57 of DQβ1) versus 60% of nondiabetic subjects with DR4 (13). We did not find that all DR4$^+$ high-risk first-degree relatives were CIAA$^+$ (93% [28 of 30]). Of the two DR4$^+$ high-risk relatives who were negative for CIAAs (and thus exceptions),

one became diabetic on follow-up and, of interest, one was DQw7/DQw9 (DQβ1 Asp/Asp homozygous at position 57, kindly typed by H. Erlich, Cetus, Emoryville, CA), and the other was repeatedly ICA$^+$ and DQw8 homozygous. Another interesting individual is the one relative with constantly high CIAA levels (462 ± 89 over 5.5 yr) who is DR4$^-$. This individual, who is DR2/DR3, is DQw1.5/DQw2 and thus not DQw8. Studies of the nature of the IAAs of this individual by competition with different insulin analogues in a project to define the antigen epitope did not show any difference compared with the DR4$^+$ individuals (39). Further DQβ subtyping is underway to determine whether IAAs will more closely correlate with such subtypes versus DR4.

Within families, where we have directly evaluated sharing of HLA haplotypes (and thus, in the absence of rare recombination events, sharing of all HLA genes), it is clear that HLA genes are not the sole determinant of IAA levels (Fig. 3). For instance, many HLA-identical relatives have no evidence of autoimmunity and are CIAA$^-$ (including identical twins), and we have observed different levels of IAAs in HLA-identical siblings. Thus, other factors (genetic or environmental) must contribute to the presence and levels of anti-IAAs.

The finding that an MHC class II allele is associated with elevated CIAA levels in humans is consistent with many data obtained in experimental animals concerning the influence of class II or immune-response genes (40). Although there were identifiable extended haplotypes (HLA-Bw62, SC33, DR4, DQw8; and HLA-B38, SC21, DR4, DQw8) known to be increased among type I diabetic subjects present in our high-risk relatives, most of the DR4 in CIAA$^+$ relatives is not on recognizable extended haplotypes, as is also true of diabetic patients (32).

Our results may contribute to the resolution of the question of immunogenetic contribution of HLA-DR4 versus DR3 haplotypes to the development of type I diabetes. Larsson et al. (41) have reported that insulin is expressed on the surface of intact β-cells and thus may be a primary target of antibodies or T lymphocytes. A gene closely associated with HLA-DR4 (e.g., DQw8) may be directly responsible for the level of IAAs. Because the rate of progression to overt diabetes correlates with levels of anti-IAAs (7), such an allele by its influence on immune response to insulin (humoral or cell mediated) may accelerate β-cell destruction. This influence of DR4 haplotypes among the relatives we studied appears to be unique, and thus the diabetogenic influence of DR3 and DR4 haplotypes may be distinguishable.

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