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Research Article

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Two Subsets of HLA-DQA1 Alleles Mark Phenotypic Variation in Levels of Insulin Autoantibodies in First Degree Relatives at Risk for Insulin-dependent Diabetes

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Abstract

Levels of insulin autoantibodies (IAA) vary among different first degree relatives of insulin-dependent diabetes mellitus patients, suggesting genetic regulation. We previously reported elevated IAA among DR4-positive at risk relatives. In this study, 72/82 at risk relatives were IAA positive, of whom 75% (54/72) carried DR4 versus 20% (2/10) of IAA-negative relatives ($P = 0.0004$). However, 69% (18/26) of DR4-negative relatives were IAA positive. Since DR4 did not account for all IAA positivity, we analyzed DQA1 and DQB1 alleles. Homozygosity for DQA1 alleles deriving from the evolutionary lineage 4 (*0401, *0501, *0601) was associated with low IAA levels, while lineage 1–3 alleles (*0101, *0102, *0103, *0201, *0301) correlated with higher levels. Most (93%, 65/70) relatives with lineage 1–3 alleles were IAA positive (mean = 360±63 SEM nU/ml). Only 7/12 relatives homozygous for lineage 4 alleles were IAA-positive, with lower levels than relatives with lineage 1–3 alleles (mean = 55±15 SEM nU/ml, $P < 0.0001$; 7/12 vs 65/70, $P = 0.004$). The amino acid sequences of lineage 1–3 alleles uniquely share glutamic acid (E) and phenylalanine (F) at positions 40 and 51 (EF alleles). Lineage 4 alleles have glycine (G) and leucine (L) at those positions (GL alleles). 90% (65/72) of IAA-positive relatives had an EF allele, while only 75% (54/72) had DR4 ($P = 0.01$). Homozygosity for GL alleles (often DQA1*0501 on DR3 haplotypes) correlated with little or no humoral response to insulin. Thus, HLA-DQB1 GL alleles, or other genes on haplotypes (e.g., DR3) that carry these DQA1 alleles, may confer recessive low responsiveness to insulin. (*J. Clin. Invest.* 1994. 93:2447–2452.) Key words: autoimmunity • diabetes mellitus, insulin-dependent • insulin autoantibodies • HLA class II antigens • HLA-DQA1 antigens

Introduction

Type I diabetes (IDDM)[1] is considered a chronic autoimmune disease resulting in the destruction of insulin-producing cells in the pancreas (1–3). Several islet molecules are targeted during the autoimmune response, and autoantibodies against these molecules are detectable in the sera of most prediabetics or newly diagnosed IDDM patients (1, 2). It is still not known if there is a precise pattern of humoral autoimmunity leading to IDDM, and indeed we are learning that different individuals may have a different combination of autoantibodies with a different order of appearance during the prediabetic period. The heterogeneity of the autoimmune process may be in part determined by genetic factors.

Among the target autoantigens, insulin is the only β cell–specific autoantigen identified to date, and thus immune responsiveness to this molecule may have an important role in the autoimmune process (4, 5). We have reported an inverse log–linear correlation between insulin autoantibody (IAA) levels and the age of diabetes onset, with the highest levels found in children who became diabetic before age 5 (6). Autoantibody-positive relatives of IDDM patients have different levels of IAA. Individual levels of IAA are often stable over long periods of time, changing very little during the prediabetic period (7). These observations suggest that the extent of the immune response to insulin may be at least in part genetically determined. Indeed, we have previously reported a significant association of high levels of IAA with HLA-DR4 haplotypes (8). However, DR4 did not account for all the individuals with high IAA levels.

The aim of our study was to further characterize HLA alleles associated with the humoral response to insulin during the prediabetic period in 82 first degree relatives at risk for IDDM, who were defined at risk if they were either IAA or islet cell antibody (ICA) positive or because they developed IDDM on follow-up regardless of their autoantibody status.

We find evidence that two different subsets of DQA1 alleles derived from evolutionary lineages 1–3 and 4 (9, 10) are associated with phenotypic variation in levels of IAA. In particular, homozygosity for lineage 4 alleles (including DQA1*0501 found on DR3 and DR5 haplotypes, as well as the much rarer *0401 and *0601) is associated with low levels of IAA. In contrast, lineage 1–3 DQA1 alleles (including DQA1*0301 found on DR4 haplotypes) are associated with high levels of IAA. Of note, only lineage 1–3 DQA1 alleles carry glutamic acid at position 40 and phenylalanine at position 51 of their second exon. Therefore, the presence of these residues is shared only by those DQA1 alleles associated with high IAA levels both on DR4 and non-DR4 haplotypes. Our data lead us to speculate that these DQA1 sequences shared by lineage 1–3 alleles may facilitate the presentation of insulin to the immune system and determine higher levels of autoantibodies once tolerance to insulin is broken during the prediabetic period.

Methods

IAA and ICA assays. IAA was measured by a competitive radioimmunoassay as described previously (6). Our upper limit of normal is 39 nU/ml (3 SD above normal). Reported IAA values represent the indi-

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1. *Abbreviations used in this paper:* C. I., confidence interval; DQA1 EF, lineage 1–3 DQA1 alleles; DQA1 GL, lineage 4 DQA1 alleles; IAA, insulin autoantibodies; ICA, islet cell antibodies; IDDM, insulin-dependent diabetes mellitus; SSO, sequence-specific oligonucleotide; TMAC, tetramethylammonium chloride.

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vidual mean level of several autoantibody measurements before insulin therapy. ICA was determined as reported previously (11) including the restricted and nonrestricted subtypes (12). According to this classification, relatives with restricted ICA have a lower risk of diabetes compared with relatives with nonrestricted ICA. However, three relatives with restricted ICA were included in this analysis, since one of them became diabetic on follow-up at age 68, and the other two had elevated IAA levels.

Subjects. After cytoplasmic ICA and IAA screening of ~ 6,000 first degree relatives of IDDM patients, we have analyzed the HLA type and IAA level of 82 individuals at increased risk for IDDM. Subjects were defined at risk for IDDM if they had one or a combination of the following: (*a*) they had nonrestricted ICA > 20 JDF units; (*b*) they had been followed to the development of overt IDDM; and (*c*) they had a mean IAA level > 39 nU/ml in the absence of other antiislet autoantibodies.

Table I illustrates the autoantibody status of the above 82 relatives subdivided by progression to overt diabetes. 72 of 82 relatives had IAA levels > 39 nU/ml, of which 40 were ICA positive, and 32 were ICA negative. 49 relatives were ICA positive (46 had nonrestricted ICA, and 3 had restricted ICA). Of the 34 (41.4%) relatives who became diabetic on follow-up, 20 were IAA and ICA positive, 7 had only IAA, 6 had only ICA (one of which was the only relative with restricted ICA who developed IDDM at age 68), and 1 relative was IAA and ICA negative.

57 normal individuals, 30 females and 27 males, were studied as a control group.

HLA typing. HLA-DR and -DQ typing was performed by serological methods. DQA1, DQB1, and DRB1 alleles were identified by DNA typing with a dot-blot technique using oligonucleotide sequence-specific probes (SSO). Two different protocols were used to ensure accuracy.

The first protocol and reagents were from the XI International HLA Workshop and were used for DQB1 typing (13). 1 μg of DNA was amplified by PCR with specific primers. PCR conditions for the amplification of the second exon of the DQB1 gene were: 30 cycles, 94–96°C for 30 s, 56°C for 60 s, and 72°C for 60–90 s. PCR amplification products were spotted onto prewet nylon membranes (Hybond N; Amersham Corp., Arlington Heights, IL) with a dot-blot apparatus (Bio-Rad Laboratories, Hercules, CA). After ultraviolet cross-linking (0.12 J/cm^2 for 30 s), membranes were hybridized with ^{32}P-labeled SSO. Each probe (5–10 pmol) was labeled in a 25-μl reaction with 60 μCi of [γ-^{32}P]ATP (sp act 6,000 Ci/mmol) in the presence of 20 U of T4 polynucleotide kinase. Hybridization was performed in 50 mM Tris-HCl (pH 8.0), 3 M tetramethylammonium chloride (TMAC), 2 mM EDTA (pH 8.0), 5 × Denhardt's solution, 0.1% SDS, and 100 μg/ml heat-denatured salmon sperm DNA for 1–3 h at 54°C (18-mer oligonucleotide probes). After hybridization, membranes were washed twice in 2 × sodium chloride sodium phosphate EDTA buffer, 0.1% SDS at room temperature and twice in TMAC washing buffer (50 mM Tris-HCl [pH 8.0], 3 M TMAC, 2 mM EDTA [pH 8.0], 0.1% SDS) at 56–58°C (for 18-mer oligonucleotide probes). Membranes were subjected to autoradiography by exposure to Kodak X-OMAT-AR films for 1–3 h at − 80°C.

The second typing protocol used primers and horseradish peroxidase–labeled oligonucleotide probes developed by Cetus Corp. (Emeryville, CA), as described previously (14), for DQA1, DQB1, and DRB1 typing. For some relatives, DQA1 alleles were typed using SSO probes developed by Cetus Corp. for forensic typing that are able to discriminate DQA1 alleles differing at positions 40 and 51.

Statistical analysis. Chi square, Fisher's exact test, and rank sum test were used for statistical comparisons among the studied groups. Only two-tailed *P* values are reported.

Table I. Clinical Characteristics of 82 at Risk First Degree Relatives

Relatives	IAA+/ICA+	IAA+/ICA−	IAA−/ICA+	IAA−/ICA−	Total
All	40	32	9	1	82
Prediabetic*	20	7	6	1	34
Nondiabetic	20	25	3	0	48

* Followed to the development of overt diabetes.

Results

The majority (87.8%, 72/82) of first degree relatives at risk for diabetes had IAA levels > 39 nU/ml. Extending our previous report (8), 75% (54/72) of IAA-positive relatives carried a DR4 haplotype vs 20% (2/10) of IAA-negative relatives (*P* = 0.0004, 54/72 vs 2/10, 95% confidence interval [C.I.] = 0.02–0.4, chi square). As shown in Fig. 1 *A*, DR4-positive relatives had significantly higher IAA levels than DR4-negative relatives (mean = 370±70 SEM vs mean = 198±84 SEM nU/ml; *P* = 0.001, rank sum test). DR4 haplotypes carrying different DRB1 alleles (*0401, *0402, *0404, *0405) were equally associated with high IAA levels (data not shown).

18 IAA-positive individuals could be identified among 26 DR4-negative relatives. Table II shows their HLA-DR and -DQA1 alleles and their IAA levels ranging from 40 to 2,230 nU/ml. The observation that 25% (18/72) of IAA-positive relatives lacked DR4 indicated that DRB1 alleles on DR4 haplotypes do not account for all relatives with high IAA levels.

Since alleles at the DQ (DQA1 and DQB1) loci are significantly associated with diabetes susceptibility (15–19), we investigated potential associations between IAA levels and DQ alleles. With only one well-documented exception (19), the alleles found in our relatives did not differ from those predicted

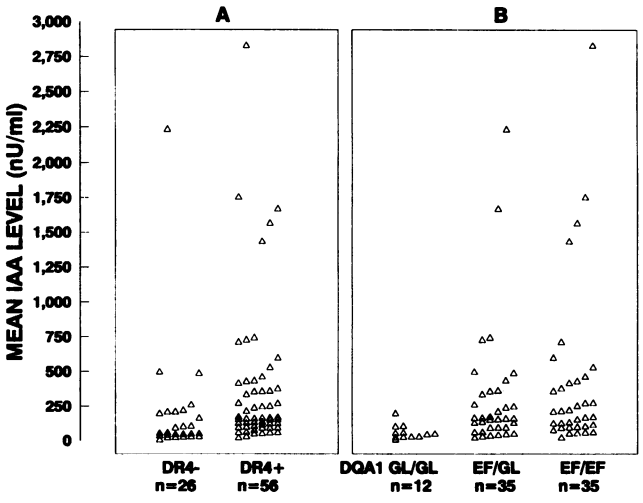

Figure 1. IAA levels in 82 first degree relatives at risk for diabetes subdivided by the presence/absence of DR4 and of DQA1 subtypes. (*A*) Most (75%, 54/72) of IAA-positive relatives carried a DR4 haplotype. 98% (54/55) of DR4-positive relatives had IAA levels above normal with significantly higher IAA levels than DR4-negative relatives (mean = 370±70 SEM vs mean = 198±84 SEM nU/ml; *P* = 0.001, rank sum test). (*B*) Most relatives (70/82, 85%) carried DQA1 EF alleles (with no difference in the IAA level distribution between 35 EF/EF homozygotes and 35 EF/GL heterozygotes), and 93% (65/70) of them had IAA levels > 39 nU/ml (mean = 360±63 SEM nU/ml). In contrast, among 12 relatives homozygous for GL alleles, only 7 had IAA levels above normal (*P* = 0.004, 7/12 vs 65/70, 95% C.I. = 0.07–0.49, Fisher's exact test; mean = 55±15 SEM nU/ml, *P* < 0.0001, rank sum test). Of note, 90% (65/72) of IAA-positive relatives had a DQA1 EF allele (including DR4-positive relatives), and only 75% (54/72) had a DR4 haplotype (*P* = 0.01).

Table II. HLA-DR and -DQA1 Alleles and IAA Levels for 18 DR4-negative Relatives with IAA Levels above Normal

Relative	Mean IAA	DR	DQA1	DQA1 allele type	DQB1
	nU/ml				
1	40	3,3	0501,0501	GL, GL	0201,0201
2	41	5,7	0501,0201	GL, EF	0301,0201
3	43	3,8	0501,0401	GL, GL	0201,0402
4	45	7,6	0201,0102	EF, EF	0201,0604
5	49	3,3	0501,0501	GL, GL	0201,0201
6	52	3,3	0501,0501	GL, GL	0201,0201
7	88	3,1	0501,0101	GL, EF	0201,0501
8	97	3,3	0501,0501	GL, GL	0201,0201
9	101	3,5	0501,0501	GL, GL	0201,0301
10	160	3,6	0501,0102	GL, EF	0201,0604
11	193	3,3	0501,0501	GL, GL	0201,0201
12	205	7,6	0201,0102	EF, EF	0201,0604
13	206	3,9	0501,0301	GL, EF	0201,0303
14	216	7,6	0201,0102	EF, EF	0201,0605
15	256	3,2	0501,0102	GL, EF	0201,0502
16	482	3,7	0501,0102	GL, EF	0201,0604
17	492	3,2	0501,0102	GL, EF	0201,0602
18	2230	3,1	0501,0101	GL, EF	0201,0501

IAA levels are expressed as the mean of multiple measurements for each individual. Analysis of DQA1 alleles indicates that 11 of the above relatives (7 of whom had the highest IAA levels) carried at least one DQA1 allele sharing sequences at positions 40 and 51 with DQA1*0301 (see Fig. 2 for sequences).

by the linkage disequilibrium patterns among class II alleles (DR-DQ) in Caucasians (17).

Analysis of DQB1 subtypes among 56 DR4-positive at risk relatives revealed that 46 had the IDDM susceptibility allele DQB1*0302 (15, 16) and 11 carried the neutral allele DQB1*0301 (8 relatives were DR4 homozygous and 2 were also DQB1*0302/DQB1*0301 heterozygous). However, the mean IAA level was not statistically different for these two groups of relatives (DQB1*0302 mean = 367±81 SEM nU/ml, n = 46; DQB1*0301 mean = 505±166 SEM nU/ml, n = 11, P = 0.30, NS).

Since all Caucasian DR4 haplotypes carry the same allele at the DQA1 locus (DQA1*0301), we compared the sequence of DQA1*0301 with DQA1 alleles in the 18 IAA-positive relatives lacking a DR4 haplotype (Table II). Among the seven relatives with highest IAA levels in this group, one relative carried the same DQA1*0301 allele usually linked to DR4 on a DR9 haplotype, and six relatives had a DQA1*01 allele (DQA1*0102, n = 5; DQA1*0101, n = 1). Considering the whole group of 18 relatives, DQA1*01 alleles were found in 9 of 18 relatives (DQA1*0102, n = 8; DQA1*0101, n = 2), and DQA1*0201 (associated with DR7) was present in 4 relatives.

As Fig. 2 illustrates, all the above DQA1 alleles (DQA1*0101, *0102, *0103, *0201, *0301) derive from evolutionary lineages 1, 2, and 3 and uniquely share glutamic acid (E) at position 40 and phenylalanine (F) at position 51 (second exon) with the allele DQA1*0301 found on DR4 haplotypes (9, 10). Therefore, we designate them DQA1 "EF" alleles. In contrast, DQA1 alleles deriving from the evolutionary lineage 4 (DQA1*0401, *0501, and *0601) usually not associated with high IAA levels carry glycine (G) and leucine (L) at positions 40 and 51 of the second exon (thus abbreviated as "GL" alleles).

As shown in Fig. 1 *B*, 70 of 82 at risk relatives (85%) carried at least one DQA1 EF allele, and 65 of these (93%) had IAA levels > 39 nU/ml (mean = 360±63 SEM nU/ml). There was no difference in the mean IAA levels between EF/GL heterozygous (mean = 300±74 SEM nU/ml) and EF/EF homozygous relatives (mean = 422±102 SEM nU/ml). In contrast, only 7 of 12 relatives homozygous for DQA1 GL alleles (Table III)

EVOLUTIONARY LINEAGES 1, 2, and 3 (EF) ALLELES — **ASSOCIATED DR**

```
     Residue                                  40          51
DQA1*0101: DHVASCGNLYQFYGPSGQYTHEFDGDEEFYVDL E RKETAWRWPE F SKFGGFDPQGALRNMAVAKHNLNIMIKRYNSTAATN   DR1/DR6
DQA1*0102: ----------------------------Q----- E ---------- F ----------------------------------   DR2/DR6
DQA1*0103:             -------F--------Q----- E K--------- F ---------------------------          DR2/DR6
DQA1*0201: -----Y-----S------F-------------- E ----V-KL-L F HRLR*----F--T-I--L------L---S-------   DR7
DQA1*0301: -----Y-----S--------------------- E ----V-QL-L F RR-RR----F--T-I--L------V---S-------   DR4/DR9
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EVOLUTIONARY LINEAGE 4 (GL) ALLELES

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     Residue                                  40          51
DQA1*0401: -----Y-----S---------------Q----- G ----V-CL-V L RQ-R*----F--T-I--L------L---S-------   DR8
DQA1*0501: -----Y-----S---------------Q----- G ----V-CL-V L RQ-R*----F--T-I--L-----SL---S-------   DR3,DR5
DQA1*0601:             S------F--------Q----- G ----V-CL-V L RQ-R*----F--T-I--T------L---S-------
```

Figure 2. Amino acid sequences of second exon for DQA1 EF and GL alleles. With only one exception (11), DR, DQA, and DQB patterns found in our relatives did not differ from those predicted for Caucasians (12). Analysis of DQA1 amino acid sequences of the alleles reported in Table I, including DQA1*0301 allele found on DR4 haplotypes, showed that such alleles derive from the evolutionary lineages 1, 2, and 3. These alleles share glutamic acid at position 40 and phenylalanine at position 51. Therefore, they were named EF alleles. Three alleles derive from the evolutionary lineage 4. Since these alleles differ from EF alleles in that they carry glycine and leucine at positions 40 and 51, they were named GL alleles. Position 51 on the DQ α chain is located in a putative peptide-binding site. Besides positions 40 and 51, EF alleles differ from GL alleles also at positions 47, 50, and 53, where GL alleles all carry cysteine, valine, and glutamine, respectively, at these residues. Thus, the region encompassed by amino acid residues 40–53 is significantly different between the two subtypes of DQA1 alleles.

Table III. DR, DQA1 Alleles and IAA Levels for Relatives Homozygous for DQA1 GL Alleles

Relative	Mean IAA	HLA-DR	DQA1	Allele type	Age DM	Age
	nU/ml					
1	1	3,**8**	0501,**0401**	GL, GL	23	38
2	18	3,3	0501,0501	GL, GL	35	38
3	20	3,3	0501,0501	GL, GL	50	53
4	22	3,3	0501,0501	GL, GL	—	18
5	24	3,**5**	0501,**0501**	GL, GL	—	14
6	40	3,3	0501,0501	GL, GL	—	20
7	43	3,**8**	0501,**0401**	GL, GL	—	31
8	49	3,3	0501,0501	GL, GL	13	14
9	52	3,3	0501,0501	GL, GL	—	41
10	97	3,3	0501,0501	GL, GL	48	61
11	101	3,**5**	0501,**0501**	GL, GL	—	25
12	193	3,3	0501,0501	GL, GL	10	16

IAA levels are expressed as the mean of multiple measurements for each individual. All 12 relatives homozygous for GL alleles carried at least one DQA1*0501 haplotype, and 8 out of 12 were DR3, DQA1*0501 homozygous. Among the four remaining relatives, two were DQA1*0501 homozygous but DR3/DR5 heterozygous, and two were DQA1*0501/DQA1*0401 (DR3/DR8) heterozygous.

had IAA levels > 39 nU/ml (P = 0.004, 7/12 vs 65/70, 95% C.I. = 0.07–0.49, Fisher's exact test; mean = 55±15 SEM nU/ml, $P < 0.0001$, rank sum test). Even excluding DR4-positive relatives from the calculation, those individuals with DQA1 EF alleles (n = 14) had remarkably higher levels than those (n = 12) with GL alleles (mean = 321±147 SEM nU/ml vs mean = 55±15 SEM nU/ml, P = 0.02, rank sum test). As shown in Fig. 1, 90.2% (65/72) of IAA-positive relatives carried a DQA1 EF allele (including DR4-positive relatives), while only 75% (54/72) had a DR4 haplotype (P = 0.01, 95% C.I. = 1.11–8.89, chi square). Moreover, only 5 of 10 (50%) IAA-negative relatives had DQA1 EF alleles in contrast to the majority (90.2%) of IAA-positive relatives (65/72, P = 0.004, 95% C.I. = 0.05–0.63, Fisher's exact test). Although the frequency of DQA1 EF alleles was not different between the 72 IAA-positive relatives and a group of 57 control subjects (data not shown), DQA1 EF alleles were found with a significantly lower frequency among IAA-negative relatives (50%, 5/10) than in controls (92.9%, 53/57; P = 0.002, 95% C.I. = −0.8–−0.14, Fisher's exact test).

Table IV. Mean Levels of IAA in 82 at Risk First Degree Relatives Subdivided by DQA1 Subtypes and the Three Criteria for at Risk Definition

Relatives	DQA1 EF/X* IAA levels	DQA1 GL/GL IAA levels	Rank sum test
	mean±SEM	*mean±SEM*	
Prediabetic‡	265±60 (24/27)§	63±27 (4/7)§	P = 0.01
ICA-positive	333±81 (34/39)§	60±16 (6/10)§	P = 0.001
ICA−/IAA+	393±98 (31/31)§	52 (1/1)§	

Criteria are detailed in Methods. * X = EF or GL. ‡ Followed to the development of overt diabetes. § Number of IAA+/total number. Note that categories are not all mutually exclusive. For example, of the 34 prediabetics, 26 were ICA positive, and 8 were ICA negative.

To more precisely evaluate the correlation of DQA1 subtypes with IAA levels, we subdivided our relatives into three not mutually exclusive groups according to the criteria used for the at risk definition detailed in Methods. As Table IV illustrates, the mean IAA level of relatives with DQA1 EF alleles was significantly higher than that of relatives homozygous for DQA1 GL alleles in all groups considered (relatives followed to overt IDDM, ICA-positive, and IAA-positive relatives).

In that IAA levels are inversely associated with age of diabetes diagnosis (6), we plotted the IAA levels of our 82 relatives, subdividing them by DQA1 subtypes and age (Fig. 3). The markedly lower levels of IAA in those relatives homozygous for DQA1 GL alleles are apparent, consistent with the hypothesis that DQA1 GL alleles (or any gene on the haplotypes carrying such alleles) confer recessive low responsiveness to insulin.

Although the prevalence of diabetes was not significantly different in relatives with DQA1 EF or GL alleles (36 vs 50%), the mean age of diabetes diagnosis was somewhat higher in relatives with DQA1 GL alleles (mean age = 29.9±4.6 SEM yr) than in those with DQA1 EF alleles (mean age = 18.3±3.2 SEM yr, P = 0.07, rank sum test).

Discussion

Among other autoantibodies detectable in the serum of prediabetics or newly diagnosed patients, IAA precede IDDM onset and correlate with rate of progression to diabetes (1–5). IAA levels are usually stable during the prediabetic period (5, 6), suggesting that they may be genetically determined. Although DR4 haplotypes are strongly associated with higher levels of IAA (8), these haplotypes were not found in all relatives with high IAA levels since 18/26 (69.2%) DR4-negative relatives also had IAA levels exceeding our normal range of 39 nU/ml (Fig. 1 *B* and Table II). Based on this observation and on our analysis of DR4 DRB1 subtypes (we could observe no difference in IAA levels among DRB1 subtypes), we suspected that DRB1 alleles on DR4 haplotypes were not primarily responsible for elevated IAA levels. Instead, our data suggested the hypothesis that genes other than DRB1 alleles on DR4 haplotypes were responsible for the association with high IAA levels and that such alleles should be common to DR4 and non-DR4 haplotypes. A candidate to improve the correlation with IAA levels was the HLA-DQ loci. These loci have been closely associated with susceptibility (15–19) and protection (18) from IDDM and are close to the DRB1 locus on chromosome 6.

Therefore, to further investigate the genetic determinants of IAA, we HLA-typed 82 at risk first degree relatives of IDDM patients (of whom 72 were IAA positive) using PCR/SSO methods for DQA1 and DQB1 alleles. Analysis of DQB1 subtypes did not show any better association with IAA than DR4 positivity, and there was no statistical difference between the IAA levels found in individuals with DQB1*0302 or DQB1*0301 among our DR4-positive relatives.

All DR4 haplotypes carry the same allele at the DQA1 locus (DQA1*0301). Analysis of DQA1 alleles in those 18 IAA-positive relatives lacking a DR4 haplotype (Table II) showed that 11 individuals (among whom were 7 with the highest IAA levels) carried at least one DQA1 allele sharing sequences at posi-

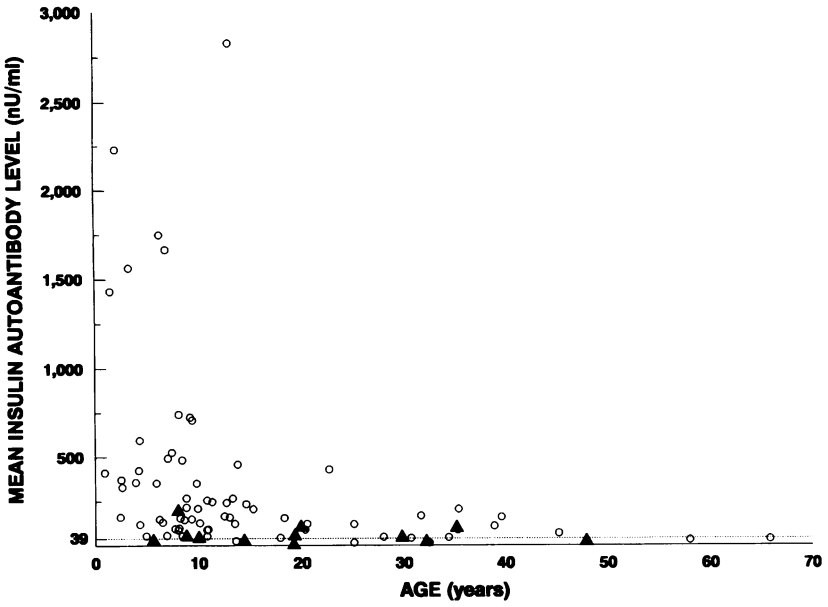

Figure 3. Mean IAA levels versus age for relatives with DQA1 EF or DQA1 GL alleles. Relatives with DQA1 GL (*solid triangles*) alleles are characterized by low IAA levels and do not show a clear influence of age on IAA levels. In contrast, relatives with DQA1 EF alleles (*open circles*) have higher IAA levels particularly apparent in children.

tions 40 and 51 with DQA1*0301 (*0101, *0102, *0201, *0301). As shown in Fig. 2, analysis of the amino acid sequences of the above DQA1 alleles revealed that all alleles were from the evolutionary lineages 1–3 (9, 10) and share glutamic acid (E) at position 40 and phenylalanine (F) at position 51 (EF alleles: DQA1*0101, *0102, *0103, *0201, *0301). DQA1 EF alleles are found on DR1, DR2, DR4, DR6, and DR7 haplotypes (Fig. 2). Most relatives (70/82, 85%) carried at least one DQA1 EF allele, and the great majority of EF relatives (93%, 65/70) had IAA levels above normal (mean = 360±63 SEM nU/ml). Most of our IAA-positive relatives (90%, 65/72) carried a DQA1 EF allele, while only 75% (54/72) of them were DR4 positive ($P = 0.01$, Fig. 1). Of note, 5/70 relatives with a DQA1 EF allele lacked IAA, suggesting that other genes or environmental factors may influence the appearance of IAA.

In contrast to DQA1 EF alleles, other DQA1 alleles (GL alleles: DQA1*0401, *0501, *0601) were not associated with high IAA levels and have glycine and leucine at positions 40 and 51 of the second exon (Fig. 2). As Table III illustrates, only 7 of 12 relatives homozygous for GL alleles were IAA positive ($P = 0.004$, 7/12 vs 65/70 relatives with EF alleles), and only 2 of them had IAA levels > 100 nU/ml. The mean IAA level for this group was significantly lower than in relatives with EF alleles (mean = 55±15 SEM nU/ml vs mean = 360±63 SEM nU/ml, $P < 0.0001$). Therefore, homozygosity for DQA1 GL alleles appears to be associated with a "recessive" lack of humoral response to insulin. Moreover, relatives homozygous for DQA1 GL alleles had lower levels of IAA at all ages in contrast to relatives with DQA1 EF alleles (Fig. 3). The two DQA1 subtypes showed a similar correlation with IAA levels, even when we subdivided our at risk relatives according to the individual criteria used for entering the study (Table IV). Although the frequency of DQA1 EF alleles was not different between IAA-positive relatives and normal controls, DQA1 EF alleles were found with a significantly lower frequency among IAA-negative relatives (50%, 5/10) than in controls (92.9%, 53/57; $P = 0.002$), and conversely DQA1 GL alleles have a higher frequency among IAA-negative relatives.

Among DQA1 GL alleles, DQA1*0601 is not found in Caucasians, and DQA1*0401 is carried only on rare DR8 haplotypes. As a consequence, the great majority of GL alleles in our relatives is represented by DQA1*0501 found on DR3 haplotypes. Indeed (Table III), all 12 relatives homozygous for GL alleles carried a DR3, DQA1*0501 haplotype, and 8 out of 12 were DR3, DQA1*0501 homozygous. However, among the four remaining relatives, two were DQA1*0501 homozygous but DR3/DR5 heterozygous, and two were DQA1*0501/DQA1*0401 (DR3/DR8) heterozygous.

A similar association of low levels of insulin antibodies with DR3 homozygosity has been reported previously (20) following insulin administration after diabetes onset. In agreement with the above study in IDDM patients, our findings in relatives at risk for diabetes support the hypothesis that DR3 haplotypes contain genes associated with a lack of humoral immune response to endogenous insulin and suggest an association with DQA1 sequences. The finding that a significant portion (4/12, 33%) of relatives homozygous for DQA1 GL alleles carried such alleles also on DR5 (DQA1*0501) and DR8 (DQA1*0401) haplotypes supports the hypothesis that DQA1 GL alleles (and not only DQA1*0501, DR3 haplotypes) are associated with a lack of humoral response to insulin.

Of interest, as indicated by the recently reported three-dimensional structure of a class II molecule (21), position 51 on the α chain lies within the peptide-binding site. Besides positions 40 and 51, DQA1 EF alleles differ from GL alleles also at positions 47, 50, and 53, where GL alleles all have cysteine, valine, and glutamine, respectively, at these residues (Fig. 2). Thus, the region encompassed by amino acid residues 40–53 is dramatically different between these two DQA1 subtypes, and we hypothesize that this may influence binding and presentation of insulin peptides. A similar association has been described recently between anticentromere autoantibodies and polar amino acids at position 26 of the HLA-DQB1 first domain (second exon) in scleroderma (22).

In this study, relatives with DQA1 GL alleles developed diabetes at an older age (mean age = 29.9±4.6 SEM yr) than those with DQA1 EF alleles (mean age = 18.3±3.2 SEM yr, $P = 0.07$, rank sum test), confirming previous reports that the lack of immune response to insulin may be associated with a slower rate of the diabetogenic process (3, 6).

In conclusion, once tolerance to insulin is broken during the prediabetic phase, DQA1 EF alleles show the strongest association (stronger than DR4) with prevalence and higher levels of IAA. Further analysis is required to address the question of whether this association is the consequence of the recessive lack of response mediated by DQA1 GL alleles (and DR3, DQA1*0501 homozygosity in particular). The differences in the DQA1 amino acid sequences discussed above may be associated with the phenotypic variation observed and support the hypothesis of two functional DQA1 allele subsets which correlate with different levels of the humoral immune response to insulin. If confirmed by further studies, DQA1 typing may improve our ability to predict the humoral immune response to insulin and may explain a major form of phenotypic variation among prediabetic first degree relatives of patients with type I diabetes.

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References

1. Pugliese, A., and G. S. Eisenbarth. 1992. Type I diabetes mellitus: lessons for human autoimmunity. *J. Lab. Clin. Med.* 120:363–366.

2. Atkinson, M. A., and N. K. Maclaren. 1990. What causes diabetes? *Sci. Am.* 263:62–71.

3. Rotter, J. I., C. M. Vadheim, and D. L. Rimoin. 1992. Diabetes Mellitus. *In* The Genetic Basis of Common Diseases. R. A. King, J. I. Rotter, and A. G. Motulsky, editors. Oxford University Press, New York, Oxford. 413–481.

4. Palmer, J. P., C. M. Asplin, P. Clemons, K. Lyen, O. Tatpati, P. K. Raghu, and T. L. Paquette. 1983. Insulin antibodies in insulin-dependent diabetics before insulin treatment. *Science (Wash. DC).* 222:1337–1339.

5. Eisenbarth, G. S., R. A. Jackson, and A. Pugliese. 1992. Insulin autoimmunity: the rate limiting factor in pre-type I diabetes? *J. Autoimmun.* 5:241–246.

6. Vardi, P., S. A. Dib, M. Tuttleman, J. E. Connelly, M. Grinbergs, A. Rabizadeh, W. J. Riley, N. K. Maclaren, G. S. Eisenbarth, and J. S. Soeldner. 1987. Competitive insulin autoantibody RIA: prospective evaluation of subjects at high risk for development of type I diabetes mellitus. *Diabetes.* 36:1286–1291.

7. Ziegler, A. G., R. Ziegler, P. Vardi, R. A. Jackson, J. S. Soeldner, and G. S. Eisenbarth. 1989. Life-table analysis of progression to diabetes of anti-insulin autoantibody positive relatives of individuals with type I diabetes. *Diabetes.* 38:1320–1325.

8. Ziegler, R., C. A. Alper, Z. L. Awdeh, L. Castano, S. J. Brink, J. S. Soeldner, R. A. Jackson, and G. S. Eisenbarth. 1991. Specific association of HLA-DR4 with increased prevalence and level of insulin autoantibodies in first degree relatives of patients with type I diabetes. *Diabetes.* 40:709–714.

9. Gyllensten, U. B., and H. A. Erlich. 1989. Ancient roots for polymorphism at the HLA-DQ alpha locus in primates. *Proc. Natl. Acad. Sci. USA.* 86:9986–9990.

10. Erlich, H. A., and U. B. Gyllensten. 1991. The evolution of allelic diversity at the primate major histocompatibility complex class II loci. *Hum. Immunol.* 30:110–118.

11. Srikanta, S., A. Rabizadeh, M. A. K. Omar, and G. S. Eisenbarth. 1985. Assay for islet cell antibodies: protein A-monoclonal antibody method. *Diabetes.* 34:300–305.

12. Gianani, R., A. Pugliese, S. Bonner-Weir, A. J. Shiffrin, J. S. Soeldner, H. Erlich, Z. L. Awdeh, C. A. Alper, R. A. Jackson, and G. S. Eisenbarth. 1992. Prognostically significant heterogeneity of cytoplasmic islet cell antibodies in relatives of patients with type I diabetes. *Diabetes.* 41:347–353.

13. Ronningen, K. S., A. Spurkland, B. D. Tait, B. Drummond, C. Lopez-Larrea, F. S. Baranda, M. J. Menendez-Diaz, S. Caillat-Zucman, G. Beaurain, H. J. Garchon, et al. 1991. HLA class II associations in insulin-dependent diabetes mellitus among Blacks, Caucasoids, and Japanese. *In* HLA 1991. K. Tsuji, M. Aizawa, and T. Sasazuki, editors. Oxford University Press, Oxford/New York/Tokyo. 713–722.

14. Saiki, R. K., T. L. Bugawan, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1986. Analysis of enzymatically amplified β-globin and HLA-DQα DNA with allele specific oligonucleotide probes. *Nature (Lond.).* 324:163–166.

15. Todd, J. A., J. I. Bell, and H. O. McDevitt. 1987. HLA-DQ beta gene contribution to susceptibility and resistance to insulin dependent diabetes mellitus. *Nature (Lond.).* 329:599–604.

16. Horn, G. T., T. L. Bugawan, C. M. Long, and H. A. Erlich. 1988. Allelic sequence variation of the HLA-DQ loci: relationship to serology and to insulin-dependent diabetes susceptibility. *Proc. Natl. Acad. Sci. USA.* 85:6012–6016.

17. Nepom, G. T., and H. Erlich. 1991. MHC class-II molecules and autoimmunity. *Annu. Rev. Immunol.* 9:493–525.

18. Baisch, J. M., T. Weeks, R. Giles, M. Hoover, P. Stastny, and J. D. Capra. 1990. Analysis of HLA-DQ genotypes and susceptibility in insulin-dependent diabetes mellitus. *N. Eng. J. Med.* 322:1836–1841.

19. Erlich, H. A., R. L. Griffith, T. L. Bugawan, R. Ziegler, C. Alper, and G. S. Eisenbarth. 1991. Implication of specific DQB1 alleles in genetic susceptibility and resistance by identification of IDDM siblings with novel HLA-DQB1 allele and unusual DR1 and DR1 haplotypes. *Diabetes.* 40:478–481.

20. McEvoy, R. C., M. E. Witt, F. Ginsberg-Fellner, and P. Rubinstein. 1986. Anti-insulin antibodies in children with type I diabetes mellitus. *Diabetes.* 35:634–641.

21. Brown, J. H., T. Jardetzky, J. C. Gorga, L. J. Stern, R. G. Urban, J. L. Strominger, and D. C. Wiley. 1993. Three-dimensional structure of the human class II histocompatibility antigen HLA-DR1. *Nature (Lond.).* 364:33–39.

22. Reveille, J. D., D. Owerbach, R. Goldstein, R. Moreda, R. A. Isern, and F. C. Arnett. 1992. Association of polar amino acids at position 26 of the HLA-DQB1 first domain with the anticentromere autoantibody response in systemic sclerosis (scleroderma). *J. Clin. Invest.* 89:1208–1213.