

Genetic Markers, Humoral Autoimmunity, and Prediction of Type 1 Diabetes in Siblings of Affected Children

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The relationships between genetic markers and disease-associated autoantibodies were studied in an unselected population of 701 siblings of children with type 1 diabetes, and the predictive characteristics of these markers over a period of 9 years were determined. Increased prevalences of all the antibodies were closely associated with HLA identity to the index case, the DR4 and DQB1*0302 alleles, and the DR3/4 phenotype and the DQB1*02/0302 genotype. Antibodies to GAD (GADA) were also associated with the DR3 and DQB1*02 alleles. Siblings carrying the protective DR2 and DQB1*0602-3 alleles were characterized by lower frequencies of islet cell antibodies (ICA), antibodies to IA-2 (IA-2A), and GADA. Higher levels of ICA were related to HLA identity, the DR4 and DQB1*0302 alleles, and the susceptible DQB1 genotypes, while no significant differences were observed in the levels of IA-2A, GADA, or insulin autoantibodies among siblings with different HLA risk markers. The DR2 or DQB1*0602-3 alleles were not related to the levels of any antibody specificity. A combination of the genetic markers and autoantibodies increased the positive predictive values of all autoantibodies substantially, which may have clinical implications when evaluating the risk of developing type 1 diabetes at the individual level or when recruiting high-risk individuals for intervention trials. However, because such combinations also resulted in reduced sensitivity, autoantibodies alone rather than in combination with genetic markers are recommended as the first-line screening in siblings. Finally, not all siblings with a broad humoral autoimmune response or high-risk genetic markers present with type 1 diabetes, while some with a low genetic risk and weak initial signs of humoral autoimmunity may progress to disease. *Diabetes* 49:48–58, 2000

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DiMe, Childhood Diabetes in Finland; GADA, antibodies to GAD; IAA, insulin autoantibodies; IA-2A, antibodies to IA-2; ICA, islet cell antibodies; JDF U, Juvenile Diabetes Foundation units; MHC, major histocompatibility complex; RU, relative units.

Prediction of type 1 diabetes (i.e., assessment of the risk for future progression to the disease) has been a major target of diabetes research over the last decade. The aims have been to find a way of identifying individuals at risk and to accurately define their degrees of risk. The long subclinical prodromal period offers an opportunity to identify these individuals before clinical manifestation of the disease or even before the autoimmune process has started. Data on the prediction of type 1 diabetes have an indisputable clinical impact, because they are currently used in the counseling of families with diabetic children. In addition, the identification of high-risk individuals is a prerequisite for intervention trials. An accurate risk assessment will be even more important in the future, when the pathomechanisms of type 1 diabetes have been resolved and methods are available for preventing the disease.

The prediction of type 1 diabetes is currently based mainly on the presence of humoral immune markers (i.e., autoantibodies). There are several reports that describe the predictive characteristics of various autoantibodies (1–6). We have previously shown that islet cell antibodies (ICA), antibodies to IA-2 (IA-2A), antibodies to GAD (GADA), and insulin autoantibodies (IAA) are all useful predictive markers of type 1 diabetes in siblings initially tested at or close to the time of diagnosis of the first affected child in the family (7). In addition, the presence of multiple antibodies has proved to indicate a higher risk for type 1 diabetes than does single antibody positivity (1–3,7). On the other hand, our results also showed that it is difficult to discriminate between progressors to clinical type 1 diabetes and nonprogressors merely on the basis of autoantibodies (7).

The major genetic determinants of type 1 diabetes reside in the HLA region within the major histocompatibility complex (MHC) on the short arm of chromosome 6. An association between HLA class I alleles and type 1 diabetes was first described in the early 1970s (8,9). Subsequently, a closer association was demonstrated between HLA-DR alleles and type 1 diabetes (10), and DR3 and DR4 were shown to be the major alleles associated with the disease. A protective effect of the DR2 antigen has also been shown consistently (11–13). More recent observations have indicated that the genes in the HLA-DQ region are even more closely associated with type 1 diabetes than the DR genes (14–17). Several studies have confirmed the association of DQB1 genes with type 1 diabetes

and have demonstrated that the DQB1*02/0302 combination is the genotype most closely associated with the disease. Nevertheless, only a few studies have been published that describe the relationships between autoantibodies and genetic risk markers in first-degree relatives of children with diabetes (2,18–20), and little is known about how genetic susceptibility modifies the predictive characteristics of various antibodies and their combinations (21–27). Thus, the relationships of these markers and their utility for the prediction of type 1 diabetes in siblings remain to be defined.

In this population-based prospective study, we define the relationships between HLA identity to the diabetic proband, HLA-DR and HLA-DQ alleles and genotypes, and type 1 diabetes-associated humoral autoimmunity. In addition, we assess the role of these genetic markers in the prediction of type 1 diabetes both individually and in combination with ICA, IA-2A, GADA, and IAA. This is the first article that evaluates both HLA risk markers and all of these autoantibodies in a large cohort of unselected siblings of children with type 1 diabetes.

RESEARCH DESIGN AND METHODS

Subjects. The study population comprised the siblings from the Childhood Diabetes in Finland (DiMe) study (28), a population-based nationwide study initiated at the beginning of September 1986 to investigate the role of genetic, immunological, and environmental factors in the development of type 1 diabetes. All newly diagnosed cases of diabetes in children <15 years of age, their siblings <20 years of age, and their parents were invited to take part. Informed consent was obtained from the subjects and/or their parents. The study design was approved by the ethical committees of all 31 participating hospitals. When recruitment terminated at the end of April 1989, 801 eligible index cases had been diagnosed with a total of 977 unaffected siblings <20 years of age. The initial blood sample from each sibling was obtained at or close to the time of diagnosis of the index case. Subsequent blood samples were taken at intervals of 3–6 months over the first 2 years and 12 months thereafter up to 4 years. Serial samples continued to be taken from the siblings who had tested positive for ICA and/or IAA on at least one occasion at intervals of 12 months or less. All the siblings were observed up to the end of August 1998 unless type 1 diabetes was diagnosed before that date. Observation of the siblings who had progressed to type 1 diabetes ended at diagnosis. The diagnosis was based on clinical symptoms and an increased random blood glucose concentration (>10 mmol/l) or on elevated fasting (>6.7 mmol/l) or random (>10 mmol/l) blood glucose concentration on two occasions in the absence of symptoms (29).

The study population comprised 701 siblings in whose initial blood samples HLA-A, -B, -C, and -DR typing was performed and autoantibodies (ICA, IA-2A, GADA, and IAA) were measured. HLA-DQB1 genotyping was performed on 565 (80.6%) of these subjects. Half-siblings were not included in the study population. The series included 324 boys (46%) and had a mean age of 9.9 years (range 0.8–19.7) at the time of the first sample.

ICA and GADA were also determined in 372 and IA-2A in 374 healthy control children and adolescents (age range 0–19 years) without a family history of type 1 diabetes. IAA were analyzed in 105 healthy control children (age range 0–18 years).

HLA-A, -B, -C, and -DR typing was performed by conventional HLA serology as described previously (30). All HLA-A, -B, -C, and -DR specificities that were recognized by the Nomenclature Committee of the World Health Organization in 1984 were included in the test panel (31).

HLA-DQB1 typing was performed by a previously described method based on time-resolved fluorescence (32). We used four sequence-specific oligonucleotide probes to identify the following DQB1 alleles known to be associated with either susceptibility to or protection against type 1 diabetes in the Finnish population: DQB1*0302, DQB1*02, DQB1*0602 or 0603, and DQB1*0301 (33). The recently described simplified classification of DQB1 genotypes into high-risk (DQB1*0302/02), moderate-risk (DQB1*0302/x, where x stands for 0302 or a non-defined allele), low-risk (DQB1*0301/0302, DQB1*02/0301, DQB1*02/x, DQB1*0302/0602-3, where x stands for 02 or a non-defined allele), and decreased-risk (DQB1*x/x, DQB1*0301/x, DQB1*02/0602-3, DQB1*0301/0602-3, where x indicates a non-defined allele) genotypes was used (33).

Islet cell antibodies. ICA were determined by a standard immunofluorescence method using sections of frozen human group O pancreas (34). All sera with detectable ICA were titrated to endpoint dilution, and the results were expressed in Juvenile Diabetes Foundation units (JDF U) by comparison with an international

standard reference serum (35). The detection limit for ICA was 2.5 JDF U. Our laboratory had a sensitivity of 100%, a specificity of 98%, a validity of 98%, and a consistency of 98% in the fourth round of the international workshops on the standardization of ICA assay (35). Interassay variation of the present assay was observed to be 22.4% for samples with low titers (10 JDF U) and 26% for samples with high titers (128 JDF U).

Antibodies to IA-2. IA-2A were analyzed with a radiobinding assay modified from that reported by Bonifacio et al. (36), as described previously (37). Briefly, the intracellular fragment of the IA-2 protein, including amino acids 605–979, was produced by *in vitro* transcription and translation in the presence of [³⁵S]methionine. Serum samples were incubated overnight with labeled IA-2 protein. Immunocomplexes were isolated by using protein A-Sepharose. All the samples were tested in duplicate. A standard curve was constructed for each plate. The results are expressed in relative units (RU), and the cut-off limit for positivity (0.43 RU) was set at the 99th percentile for 374 non-diabetic Finnish children and adolescents. The disease sensitivity of our assay was 62%, and the disease specificity was 97%, based on 140 samples included in the Multiple Autoantibody Workshop (38).

Antibodies to GAD. GADA were quantified with the radiobinding assay reported by Petersen et al. (39), as described previously (7). Briefly, serum samples were incubated overnight with [³⁵S]methionine-labeled *in vitro* transcribed and translated human recombinant GAD65 protein. All the samples were analyzed in quadruplicate with and without an excess of unlabeled GAD65. Immunocomplexes were isolated by using protein A-Sepharose. The results are expressed in RU, and the cut-off limit for GADA positivity was defined as 6.5 RU, which represents the 99th percentile in a series of 372 healthy control children. All samples exceeding 2.9 RU (mean + 1 SD in 372 control children) were retested to confirm GADA positivity or negativity. The disease sensitivity of the present assay was 80%, and the disease specificity was 94%, based on the 101 samples included in the Second International GAD Antibody Workshop (40).

Insulin autoantibodies. IAA were analyzed with a competitive radiobinding assay modified from that described by Palmer et al. (41). Endogenous insulin was removed with acid charcoal before the assay, and free and bound insulin were separated after incubation with mono-¹²⁵I(Tyr A 14)-labeled human insulin (Novo Research Institute, Bagsvaerd, Denmark) in the absence or presence of an excess of unlabeled insulin. IAA levels were expressed in nanounits per milliliter, where 1 nU/ml corresponds to a specific binding of 0.01% of the total counts. The cut-off limit for IAA positivity was defined as 54 nU/ml, which represents the 99th percentile in a series of 105 nondiabetic children and adolescents. The disease sensitivity of the IAA assay was 26%, and the disease specificity was 97%, based on 140 samples included in the Multiple Autoantibody Workshop (38).

Statistical analyses. Student's *t* test was used to analyze normally distributed continuous variables, and either the Mann-Whitney *U* test or Kruskal-Wallis test was used in the case of skewed distributions. Differences in the distribution of individuals between groups were tested with χ^2 statistics with Yates' correction unless any expected value was <5, in which case Fisher's exact test was used (42). The Kaplan-Meier method (43) was used to construct life tables of the likelihood of developing type 1 diabetes. The follow-up time for each subject was calculated from the date when the initial blood sample had been obtained to clinical diagnosis or for 8.9 years, which was the minimum follow-up time in all cases. The equality of the survival distributions was tested with the log-rank test (44). Sensitivity was defined as the percentage of those who have the disease in whom the test value is positive; specificity was defined as the proportion of those without the disease correctly identified by a negative test value; positive predictive value was defined as the likelihood that a sibling with a positive test will become diabetic. A two-tailed *P* value 0.05 was considered to indicate statistical significance. All the statistical analyses were performed using the SPSS statistical software package for Windows, version 8.0 (SPSS, Chicago).

RESULTS

Autoantibodies. Of the 701 siblings, 57 (8.1%) had detectable levels of ICA in their initial blood sample (median level of 34 JDF U, range 3–640 JDF U). Of the 372 healthy control subjects, 6 (1.6%) tested positive for ICA (median level 6 JDF U, range 4–34 JDF U); all of the healthy control subjects were negative for IA-2A, GADA, and IAA. IA-2A were detected in the initial sample from 37 siblings (5.3%), GADA in 50 (7.1%), IAA in 26 (3.7%), any antibody (i.e., one or more of the antibody specificities analyzed) in 89 (12.7%), and multiple antibodies (i.e., at least two of the antibody specificities analyzed) in 44 (6.3%). The median level of IA-2A was 18.9 RU (range 0.49–277.1), that of GADA was 55.3 RU (range 6.8–129.8), and that of IAA was 78 nU/ml (range 55–1,238).

TABLE 1
Relation between HLA identity with the affected proband and autoantibodies in 701 siblings of children with newly diagnosed type 1 diabetes

	HLA-identical	HLA-haploidentical	HLA-nonidentical	Statistics	P
n	177	354	170	—	—
Antibody positivity					
ICA	23 (13.0)	24 (6.8)	10 (5.9)	$\chi^2_{df2} = 7.6$	0.021
IA-2A	19 (10.7)	12 (3.4)	6 (3.5)	$\chi^2_{df2} = 14.1$	0.001
GADA	25 (14.1)	21 (5.9)	4 (2.4)	$\chi^2_{df2} = 19.7$	<0.001
IAA	12 (6.8)	10 (2.8)	4 (2.4)	$\chi^2_{df2} = 6.3$	0.040
Any antibody	33 (18.6)	38 (10.7)	18 (10.6)	$\chi^2_{df2} = 7.5$	0.024
Multiple antibodies	25 (14.1)	16 (4.5)	3 (1.8)	$\chi^2_{df2} = 26.3$	<0.001
Antibody levels					
ICA (JDF U)	80*‡ (6–640)	19* (3–320)	10‡ (3–320)	df ₂	0.019; 0.035*, 0.014‡
IA-2A (RU)	38.2 (0.49–277.1)	18.1 (0.62–161.1)	12.3 (0.58–115.3)	df ₂	NS
GADA (RU)	47.0 (6.8–108.4)	79.0 (16.1–129.8)	60.0 (30.1–98.1)	df ₂	NS
IAA (nU/ml)	102 (55–317)	78 (56–1,238)	58 (57–61)	df ₂	NS

Data are n (%) for antibody positivity and median (range) for antibody levels. Antibody-negative subjects were excluded from the analysis of antibody levels.

Relation between HLA identity and the affected proband and autoantibodies. Of the 701 siblings, 177 (25.2%) were HLA-identical to the proband with type 1 diabetes, 354 (50.5%) were haploidentical, and 170 were (24.3%) non-identical. The frequencies of ICA, IA-2A, GADA, and IAA in the initial blood samples were significantly higher among the HLA-identical siblings than among the haplo- and nonidentical siblings (Table 1). The HLA-identical siblings were also more often positive for any antibody and for multiple antibodies than the haplo- and nonidentical siblings (Table 1 and Fig. 1). When siblings were categorized into two age-groups (<10 years and ≥10 years of age), all differences in autoantibody frequencies in relation to the degree of HLA identity were still significant in the younger age-group except that for ICA, while only the differences in the prevalence of IA-2A and multiple antibodies remained significant in the older age-group.

The levels of ICA were higher in the HLA-identical siblings than in haplo- or nonidentical siblings (Table 1). No significant differences in the levels of IA-2A, GADA, or IAA were observed among the three groups of siblings who were classified according to their degree of HLA identity with the proband in the family (Table 1).

Relation among HLA-DR alleles, phenotypes, and autoantibodies. Of the 701 siblings, 150 (21.4%) carried the DR3 allele, 387 (55.2%) carried the DR4 allele, and 118 (16.8%) carried the protective DR2 allele. The background frequency of the DR2 allele is ~21% in the general Finnish population (45). Each of the four analyzed antibody specificities was found more frequently in the siblings with the DR4 allele than in those without it (Table 2). Siblings carrying the DR4 allele were also more often positive for any antibody than those without DR4, while no difference was found in this respect between those with and without the DR3 allele. ICA and GADA but not IA-2A and IAA were more frequent in the siblings with the DR3 allele than in those without it (Table 2). Multiple antibodies were more frequent in siblings carrying the DR3 allele and in siblings with the DR4 allele than in those lacking such alleles. The siblings carrying the DR2 allele tested positive for ICA, IA-2A, GADA, any antibody, and multiple antibodies less often than the other siblings

(Table 2). When comparing the mean ages of antibody-positive and antibody-negative subjects among the siblings carrying the DR4, DR3, or DR2 alleles, no significant differences were observed.

The levels of ICA were higher in the DR4-positive siblings than in the DR4-negative siblings (median 40 JDF U [range 3–640] vs. 10 JDF U [range 5–80], $P = 0.032$), whereas no significant differences in ICA levels were observed between those who were positive and negative for DR3 or DR2 (data not shown). The levels of IA-2A, GADA, and IAA did not differ significantly among the siblings who were positive and negative for DR3, DR4, or DR2 (data not shown).

Of the 701 siblings, 53 (7.6%) carried the HLA DR3/4 phenotype, 334 (47.6%) carried the DR4/x, 97 (13.8%) carried the DR3/y, and 217 (31.0%) carried other DR phenotypes. The fre-

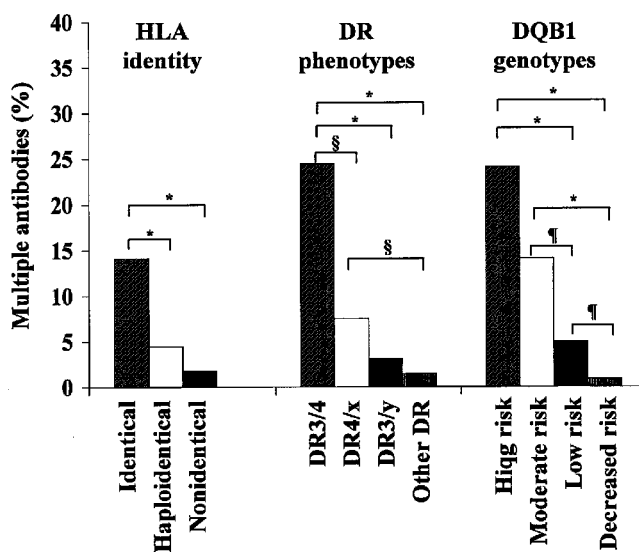


FIG. 1. Relation among the presence of multiple antibodies (≥2 antibodies), HLA identity to the diabetic proband, HLA-DR phenotype, and HLA-DQB1 genotype in siblings. * $P < 0.001$; § $P = 0.001$; ¶ $P < 0.05$.

TABLE 2

Relation between presence of HLA-DR and HLA-DQB1 risk alleles and positivity for autoantibodies in siblings of children with newly diagnosed type 1 diabetes

	n	Antibody positivity					
		ICA	IA-2A	GADA	IAA	Any antibody	Multiple antibodies
Positivity for HLA allele							
DR4							
+	387	42 (10.9)	30 (7.8)	40 (10.3)	21 (5.4)	63 (16.3)	38 (9.8)
-	314	15 (4.8)	7 (2.2)	10 (3.2)	5 (1.6)	26 (8.3)	6 (1.9)
P		0.003	0.001	<0.001	0.008	0.002	<0.001
DR3							
+	150	19 (12.7)	11 (7.3)	18 (12.0)	7 (4.7)	25 (16.7)	16 (10.7)
-	551	38 (6.9)	26 (4.7)	32 (5.8)	19 (3.4)	64 (11.6)	28 (5.1)
P		0.028	NS	0.012	NS	NS	0.015
DR2							
+	118	4 (3.4)	1 (0.8)	1 (0.8)	3 (2.5)	7 (5.9)	1 (0.8)
-	583	53 (9.1)	36 (6.2)	49 (8.4)	23 (3.9)	82 (14.1)	43 (7.4)
P		0.041	0.021	0.005	NS	0.022	0.011
DQB1*0302							
+	257	36 (14.0)	25 (9.7)	34 (13.2)	17 (6.6)	51 (19.8)	34 (13.2)
-	308	19 (6.2)	9 (2.9)	11 (3.6)	7 (2.3)	30 (9.7)	8 (2.6)
P		0.003	0.001	<0.001	0.012	0.001	<0.001
DQB1*02							
+	173	23 (13.3)	13 (7.5)	20 (11.6)	8 (4.6)	29 (16.8)	19 (11.0)
-	392	32 (8.2)	21 (5.4)	25 (6.4)	16 (4.1)	52 (13.3)	23 (5.9)
P		NS	NS	0.043	NS	NS	0.037
DQB1*0602-3							
+	147	8 (5.4)	3 (2.0)	2 (1.4)	5 (3.4)	15 (10.2)	2 (1.4)
-	418	47 (11.2)	31 (7.4)	43 (10.3)	19 (4.5)	66 (15.8)	40 (9.6)
P		0.051	0.024	0.001	NS	NS	0.002

Data are n (%).

frequencies of single antibody specificities, any antibody, and multiple antibodies were significantly higher in DR3/4 heterozygous siblings than in the siblings with any other DR phenotypes (Table 3 and Fig. 1). Only one of the 97 siblings (1.0%) carrying the DR3/y phenotype tested positive for IA-2A, but the difference was not significant in relation to either those with the DR4/x phenotype (6.0%, $P = 0.06$) or those with the non-DR3/non-DR4 phenotype (2.8%, $P = 0.44$) (Table 3). All differences in autoantibody prevalences among siblings with various DR phenotypes remained significant when siblings <10 years of age and those ≥10 years of age were analyzed separately, with the exception of IAA in both age-groups. The levels of ICA, IA-2A, GADA, and IAA did not differ significantly among subjects with different DR phenotypes among the antibody-positive siblings (Table 3).

Relation among HLA-DQB1 alleles, genotypes, and autoantibodies. The DQB1*0302 allele was present in 257 siblings (45.5%), the DQB1*02 allele was present in 173 siblings (30.6%), and the DQB1*0602-3 was present in 147 siblings (26.0%). Siblings carrying the DQB1*0302 allele had ICA, IA-2A, GADA, IAA, any antibody, and multiple antibodies more often than those without this allele (Table 2). The siblings with the DQB1*02 allele tested positive for GADA and multiple antibodies significantly more often than those without the DQB1*02 allele but not for ICA, IAA, or IA-2A. The frequencies of ICA, IA-2A, GADA, and multiple antibodies (but not IAA) were significantly lower in the siblings carrying the DQB1*0602-3 allele than in the other siblings (Table 2). No significant differences in mean age were found between the

antibody-positive and antibody-negative siblings carrying the DQB1*0302, DQB1*02, or DQB1*0602-3 alleles.

The levels of ICA were higher in the siblings carrying the DQB1*0302 allele than in those without this allele (median 40 JDF U [range 3–640] vs. 10 JDF U [range 3–80], $P = 0.010$), but no significant differences in ICA levels were found between those who were positive and negative for DQB1*02 or DQB1*0602-3 (data not shown). There was a tendency for higher levels of IA-2A in the siblings with the DQB1*0302 allele than in those without this allele (median 44.9 RU [range 0.49–277.1] vs. 5.7 RU [range 0.58–61.9], $P = 0.055$). There was no difference in the levels of IA-2A between the siblings with and without the DQB1*02 or DQB1*0602-3 alleles (data not shown). The levels of GADA and IAA did not differ significantly between the siblings who were positive and negative for the DQB1*0302, DQB1*02, or DQB1*0602-3 alleles (data not shown).

The prevalence of antibodies in siblings with specific DQB1 genotypes is shown in Table 4. The highest frequency of all antibodies was seen in the siblings with DQB1*02/0302, and the second highest prevalence in those with the DQB1*0302/x genotype, although antibodies (one or more) were also present in siblings with other specific genotypes. According to the simplified classification of DQB1 conferred genetic risk, the high-risk genotype was observed in 54 siblings (9.6%), the moderate-risk genotype in 135 siblings (23.9%), the low-risk genotype in 160 siblings (28.3%), and the decreased-risk genotype in 216 siblings (38.2%). The proportions of subjects testing positive for ICA, IA-2A, GADA, IAA, any antibody, and

TABLE 3
Relation between HLA-DR phenotypes and autoantibodies in siblings of children with newly diagnosed type 1 diabetes

	DR3/4	DR4/x	DR3/y	Other DR combinations	Statistics	P
n	53	334	97	217		
Antibody positivity						
ICA	12 (22.6)	30 (9.0)	7 (7.2)	8 (3.7)	$\chi^2_{df3} = 21.1$	<0.001
IA-2A	10 (18.9)	20 (6.0)	1 (1.0)	6 (2.8)	$\chi^2_{df3} = 20.2$	<0.001
GADA	14 (26.4)	26 (7.8)	4 (4.1)	6 (2.8)	$\chi^2_{df3} = 28.1$	<0.001
IAA	4 (7.5)	17 (5.1)	3 (3.1)	2 (0.9)	$\chi^2_{df3} = 9.7$	0.014
Any antibody	15 (28.3)	48 (14.4)	10 (10.3)	16 (7.4)	$\chi^2_{df3} = 18.5$	<0.001
Multiple antibodies	13 (24.5)	25 (7.5)	3 (3.1)	3 (1.4)	$\chi^2_{df3} = 32.9$	<0.001
Antibody levels						
ICA (JDF U)	60 (3–320)	40 (3–640)	10 (5–80)	10 (5–40)	df ₃	NS
IA-2A (RU)	30.6 (0.49–138.1)	42.7 (0.79–277.1)	10.2	5.6 (0.58–61.9)	df ₃	NS
GADA (RU)	65.9 (7.6–113.0)	63.6 (6.8–129.8)	62.7 (18.4–100.0)	25.6 (8.9–87.0)	df ₃	NS
IAA (nU/ml)	184 (56–1238)	76 (55–259)	71 (61–317)	68 (57–79)	df ₃	NS

Data are n (%) for antibody positivity and median (range) for antibody levels. Antibody-negative subjects are excluded from the analysis of antibody levels. x, Non-DR3 alleles; y, non-DR4 alleles.

multiple antibodies were higher among the siblings with the high-risk genotype than among those with the other DQB1 genotypes (Table 5 and Fig. 1), although the differences were not significant between those with the high-risk genotype and those with the moderate-risk genotype. There were no significant differences in the frequencies of antibodies between any specific genotypes within the moderate-, low-, or decreased-risk groups. When the siblings were analyzed in two subgroups based on age (<10 and 10 years of age), all of the observed differences in antibody frequencies among various risk genotypes remained significant, with the exception of IAA in the older age-group.

The levels of ICA were significantly lower in the siblings with the decreased-risk genotype than in those with the high-risk, moderate-risk, or low-risk genotypes (Table 5). The levels of IA-2A, GADA, and IAA did not differ significantly among these risk groups (Table 5). No significant differences were observed in mean age between the antibody-positive and antibody-negative siblings carrying different DQB1 genotypes. Genetic markers, autoantibodies, and progression to type 1 diabetes. Of the 701 siblings, 33 (4.7%) progressed to clinical type 1 diabetes during the follow-up period. No

HLA-DQB1 data were available for two of these siblings. The median follow-up time between the first blood sample and the diagnosis of type 1 diabetes was 3.3 years (range 0.01–7.7), while the median follow-up time for the nonprogressors was 10.3 years (range 8.9–12.0).

The siblings with antibodies had a higher risk for progression to type 1 diabetes than those without antibodies, and the proportions of siblings testing positive for ICA, IA-2A, GADA, IAA, any antibody, or multiple antibodies were significantly higher among the progressors than among the nonprogressors (data not shown). Survival analysis showed the HLA-identical siblings, the DR3/4 heterozygous siblings, and those carrying the HLA-DQB1*02/0302 genotype to have a significantly higher risk of contracting clinical disease over the follow-up period of 8.9 years than the haplo- and nonidentical siblings, those with other DR phenotypes, and those with other DQB1 genotypes, respectively (Table 6). The HLA-identical siblings who were positive for ICA, IA-2A, GADA, IAA, any antibody, or multiple antibodies progressed to type 1 diabetes more often than the haplo- and nonidentical subjects. The risk of clinical disease was also higher in the DR3/4 heterozygous siblings who were positive for any antibody than it was in those

TABLE 4
Relation between specific HLA-DQB1 genotypes and autoantibodies in siblings of children with newly diagnosed type 1 diabetes

	n	ICA	IA-2A	GADA	IAA	Any antibody	Multiple antibodies
DQB1 genotype	565						
*02/0302	54	12 (22.2)	9 (16.7)	13 (24.1)	5 (9.3)	16 (29.6)	13 (24.1)
*0302/x	135	21 (15.6)	15 (11.1)	18 (13.3)	10 (7.4)	29 (21.5)	19 (14.1)
*02/x	71	8 (11.3)	2 (2.8)	5 (7.0)	1 (1.4)	9 (12.7)	4 (5.6)
*0302/0301	30	1 (3.3)	0	2 (6.7)	0	2 (6.7)	1 (3.3)
*02/0301	21	2 (9.5)	2 (9.5)	2 (9.5)	1 (4.8)	2 (9.5)	2 (9.5)
*0301/x	38	1 (2.6)	2 (5.3)	2 (5.3)	0	5 (13.2)	0
*0302/0602 or 0603	38	2 (5.3)	1 (2.6)	1 (2.6)	2 (5.3)	4 (10.5)	1 (2.6)
*02/0602 or 0603	27	1 (3.7)	0	0	1 (3.7)	2 (7.4)	0
*0301/0602 or 0603	18	0	1 (5.6)	0	0	1 (5.6)	0
*0602 or 0603/x	64	5 (7.8)	1 (1.6)	1 (1.6)	2 (3.1)	8 (12.5)	1 (1.6)
*x/x	69	2 (2.9)	1 (1.4)	1 (1.4)	2 (2.9)	3 (4.3)	1 (1.4)

Data are n (%). x, Unrecognized allele or homozygosity for the marked allele.

TABLE 5
Relation between HLA-DQB1 genotypes and autoantibodies in siblings of children with newly diagnosed type 1 diabetes

	High risk	Moderate risk	Low risk	Decreased risk	Statistics	P
n	54	135	160	216		
Antibody positivity						
ICA	12 (22.2)	21 (15.6)	13 (8.1)	9 (4.2)	$\chi^2_{df3} = 22.9$	<0.001
IA-2A	9 (16.7)	15 (11.1)	5 (3.1)	5 (2.3)	$\chi^2_{df3} = 22.1$	<0.001
GADA	13 (24.1)	18 (13.3)	10 (6.3)	4 (1.9)	$\chi^2_{df3} = 33.6$	<0.001
IAA	5 (9.3)	10 (7.4)	4 (2.5)	5 (2.3)	$\chi^2_{df3} = 9.3$	0.019
Any antibody	16 (29.6)	29 (21.5)	17 (10.6)	19 (8.8)	$\chi^2_{df3} = 21.5$	<0.001
Multiple antibodies	13 (24.1)	19 (14.1)	8 (5.0)	2 (0.9)	$\chi^2_{df3} = 42.9$	<0.001
Antibody levels						
ICA (JDF U)	27* (3–320)	40‡ (5–640)	40§ (5–320)	5* † § (3–40)	df ₃	0.012; 0.030*, 0.001‡, 0.016§
IA-2A (RU)	44.9 (0.49–138.1)	38.2 (0.79–277.1)	44.8 (0.62–161.1)	1.1 (0.58–13.9)	df ₃	NS
GADA (RU)	76.1 (7.6–113.0)	48.4 (6.8–129.8)	57.8 (16.1–100.0)	53.5 (8.9–100.1)	df ₃	NS
IAA (nU/ml)	144 (55–1238)	92 (57–181)	152 (62–317)	61 (57–79)	df ₃	NS

Data are n (%) for antibody positivity and median (range) for antibody levels. Antibody-negative subjects are excluded from analysis of antibody levels. High risk = DQB1*02/0302; moderate risk = DQB1*0302/x; low risk = DQB1*0301/0302, 02/0301, 02/x, or 0302/0602-3; decreased risk = DQB1*0602-3/x, 0301/0602-3, 0301/x, x/x. x, Unrecognized allele or homozygosity for the marked allele.

with other DR genotypes, but no significant differences were observed between the DR3/4 heterozygous and other siblings who were positive for any single antibody specificity or multiple antibodies (Table 6). Similarly, the siblings who tested positive for ICA or any antibody in combination with the DQB1*02/0302 genotype had higher risk of type 1 diabetes than those with other DQB1 genotypes, whereas no differences were found among siblings positive for other antibody specificities or multiple antibodies.

The positive predictive values and sensitivities of the autoantibodies, high-risk genetic markers, and combinations of these markers are summarized in Table 7. IA-2A and multiple antibodies had the highest positive predictive values (56.8%). As a single genetic marker, the HLA-DQB1*02/0302 genotype had the highest positive predictive value (22.2%). Positivity for any antibody, ICA, and multiple antibodies had the highest sensitivities (81.8, 78.8, and 75.8%, respectively). The sensitivity of increased DQB1-defined risk (i.e., the combination of the siblings with high, moderate, and low genetic risk) was high (96.8%) because the six antibody-negative progressors were also identified by this measure (Fig. 2). However, the positive predictive value and the specificity of this combination were low (8.6 and 40.4%, respectively) (Fig. 2).

The combination of HLA identity, DR3/4 heterozygosity, or the DQB1*02/0302 genotype and autoantibodies increased the positive predictive values of all humoral markers but reduced the sensitivities considerably at the same time. These effects are illustrated for ICA as a survival plot in Fig. 3. The DQB1*02/0302 genotype in combination with IA-2A had the highest positive predictive value (77.8%), but its sensitivity was only 22.6%.

The risk for progression to type 1 diabetes was related to the number of antibodies (Table 8). The positive predictive value was highest for the presence of three antibodies (74.1%) and was further increased by combination with HLA identity, DR 3/4 phenotype, or DQB1*02/0302 genotype (80.0, 100, and 100%, respectively). Similar to single antibody markers, the combination also reduced sensitivity at the same time. No specific antibody combination had positive predictive values that were significantly different than the others (data not shown), although all three subjects with ICA, IA-2A, and IAA and the only subject with ICA and IAA have progressed to type 1 diabetes. Of the former three subjects, two carried the HLA DR4/6-DQB1*0302/x genotype and one carried the DR4/1-DQB1*0302/x genotype, whereas the latter subject had the DR4/7–DQB1*02/0302 genotype.

TABLE 6
Survival analysis of progression to type 1 diabetes over 8.9 years of follow-up in siblings of children with type 1 diabetes

Antibody status	Progression to type 1 diabetes								
	HLA-identical	HLA-haploidentical and -nonidentical	Log-rank P value	DR3/4	Other DR phenotypes	Log-rank P value	DQB1*02/0302	Other DQB1 genotypes	Log-rank P value
All	11.9% (21/177)	2.3% (12/524)	<0.0001	18.9% (10/53)	3.5% (23/648)	<0.0001	22.2% (12/54)	3.7% (19/511)	<0.0001
ICA positive	73.9% (17/23)	26.5% (9/34)	0.0001	66.7% (8/12)	40.0% (18/45)	NS	75.0% (9/12)	34.9% (15/43)	0.036
IA-2A positive	73.7% (14/19)	38.9% (7/18)	0.031	70.0% (7/10)	51.9% (14/27)	NS	77.8% (7/9)	48.0% (12/25)	NS
GADA positive	56.0% (14/25)	28.0% (7/25)	0.041	57.1% (8/14)	36.1% (13/36)	NS	61.5% (8/13)	34.4% (11/32)	NS
IAA positive	50.0% (6/12)	14.3% (2/14)	0.036	25.0% (1/4)	31.8% (7/22)	NS	40.0% (2/5)	31.6% (6/19)	NS
Any antibody	54.5% (18/33)	16.1% (9/56)	<0.0001	53.3% (8/15)	25.7% (19/74)	0.048	56.3% (9/16)	24.6% (16/65)	0.023
Multiple antibodies	68.0% (17/25)	42.1% (8/19)	NS	61.5% (8/13)	54.8% (17/31)	NS	69.2% (9/13)	48.3% (14/29)	NS

HLA identity with the diabetic proband, HLA-DR 3/4 heterozygosity, and the HLA-DQB1*02/0302 genotype were combined with autoantibody status.

TABLE 7
Positive predictive value and sensitivity of autoantibodies and genetic markers for progression to type 1 diabetes over 8.9 years in siblings of children with type 1 diabetes

	Positive predictive value	Sensitivity (%)
ICA	45.6% (32.4–59.3)	78.8
IA-2A	56.8% (39.5–72.9)	63.6
GADA	42.0% (28.2–56.8)	63.6
IAA	30.8% (14.3–51.8)	24.2
Any antibody	30.3% (21.0–41.0)	81.8
Multiple antibodies	56.8% (41.0–71.7)	75.8
HLA-identical	11.9% (7.5–17.6)	63.6
HLA-DR3/4	18.9% (9.4–32.0)	30.3
HLA DQB1*0201/0302	22.2% (12.0–35.6)	38.7
HLA-identical + ICA	73.9% (51.6–89.9)	51.5
HLA-identical + IA-2A	73.7% (48.8–90.9)	42.4
HLA-identical + GADA	56.0% (34.9–75.6)	42.4
HLA-identical + IAA	50.0% (21.9–78.9)	18.2
HLA-identical + any antibody	54.5% (36.4–71.9)	54.5
HLA-identical + multiple antibodies	68.0% (46.5–85.1)	51.5
HLA-DR3/4 + ICA	66.7% (34.9–90.1)	24.2
HLA-DR 3/4 + IA-2A	70.0% (34.8–93.3)	21.2
HLA-DR3/4 + GADA	57.1% (28.9–82.3)	24.2
HLA-DR3/4 + IAA	25.0% (0.6–80.6)	3.0
HLA-DR3/4 + any antibody	53.3% (26.6–78.7)	24.2
HLA-DR3/4 + multiple antibodies	61.5% (31.6–86.1)	24.2
DQB1*02/0302 + ICA	75.0% (42.8–94.5)	29.0
DQB1*02/0302 + IA-2A	77.8% (40.0–97.2)	22.6
DQB1*02/0302 + GADA	61.5% (31.6–86.1)	25.8
DQB1*02/0302 + IAA	40.0% (5.3–85.3)	6.5
DQB1*02/0302 + any antibody	56.3% (29.9–80.2)	29.0
DQB1*02/0302 + multiple antibodies	69.3% (38.6–90.9)	29.0

Data in parentheses are CIs.

The characteristics of the five siblings who had all four antibody specificities in their initial blood sample are shown in Table 9. Only two of these to date have presented with type 1 diabetes. The follow-up times between the first blood sample and diagnosis were 0.2 and 2.9 years, while the follow-up times for the nonprogressors were 10.1, 10.8, and 10.8 years. Both progressors were HLA-identical to the diabetic proband in their family. One of the nonprogressors was HLA-identical, and two were HLA haploidentical. Neither of the progressors carried the high-risk DR3/4 or DQB1*02/0302 genotype (one had DR4/4 and DQB1*0302/0302 and the other DR1/5 in combination with DQB1*x/x). One sibling (no. 5) became negative for IA-2A, GADA, and IAA but is still positive for ICA. One nonprogressor (no. 3) has recently become negative for IAA but is positive for the other antibody specificities, and the remaining nonprogressor (no. 4) is still positive for all four antibody specificities.

DISCUSSION

A total of 701 siblings of children with newly diagnosed type 1 diabetes <20 years of age were studied for the relationship between autoantibodies and genetic risk markers, and the utility of these markers for the prediction of type 1 diabetes was evaluated. The risk of progression to clinical disease was studied over a period of 8.9 years, which was the minimum follow-up time among the siblings. The initial blood samples were taken at or close to the time of diagnosis of the diabetic proband (i.e., at the most practical and urgent moment for evaluating the risk of type 1 diabetes in siblings). The study population is unselected and well defined, thereby allowing an unbiased view.

Higher frequencies of all antibodies were found in the siblings who were HLA-identical with the diabetic proband than in those who were HLA-haploidentical or -nonidentical. Similarly, the presence of these antibodies was closely associated with the DR4 allele, DR3/4 heterozygosity, the DQB1*0302 allele, and the DQB1*02/0302 genotype. In addition, ICA and GADA were also associated with the DR3 allele, and GADA was associated with the DQB1*02 allele. These results demonstrate the importance of DR3 and DQB1*02 alleles for

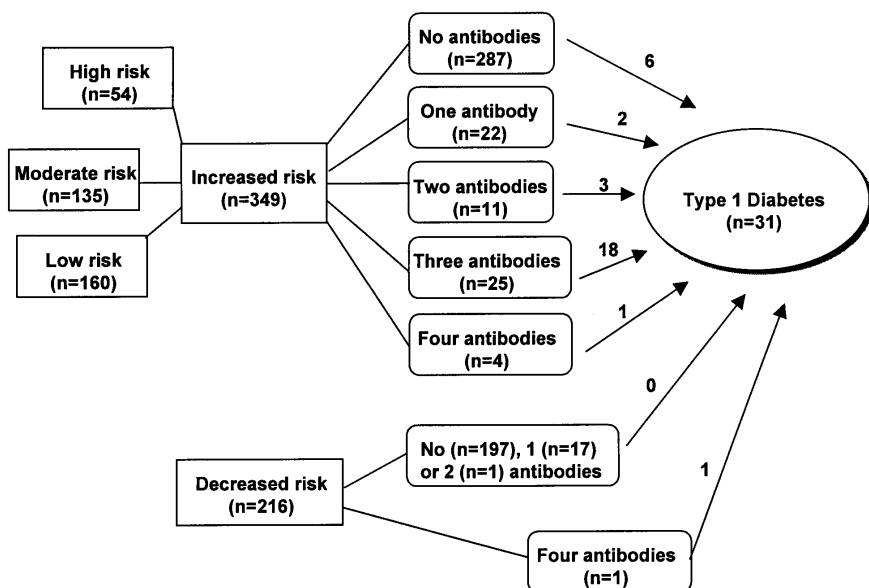


FIG. 2. DQB1-defined genetic risk, number of antibodies, and progression to type 1 diabetes in 565 siblings.

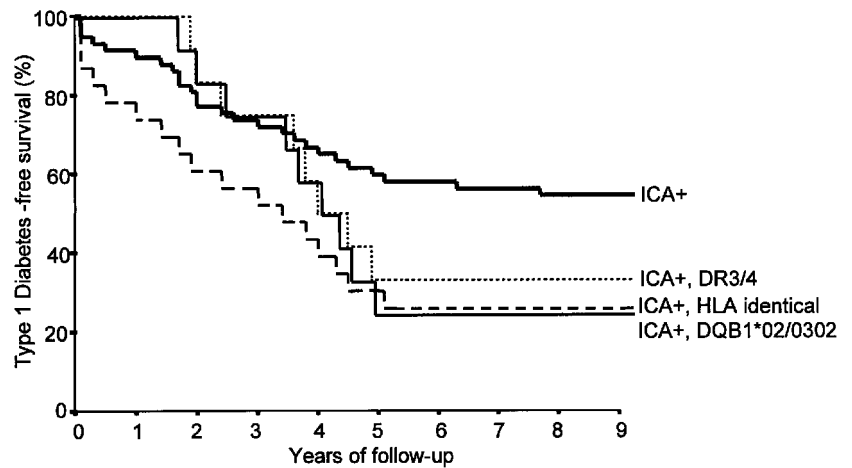


FIG. 3. Probability of remaining free of type 1 diabetes over 8.9 years of follow-up and the effect of the combination of antibody-positivity and HLA markers. Statistical analysis is not valid because the subjects in the four groups are overlapping.

n=57	51	45	41	37	34	33	32	31	31	ICA+
n=23	17	14	12	9	7	6	6	6	6	ICA+, HLA identical
n=12	12	11	9	6	4	4	4	4	4	ICA+, DR3/4
n=12	12	10	9	6	3	3	3	3	3	ICA+, DQB1*02/0302

the formation of GADA, as distinct from other antibody specificities, and are in line with the observations of Ziegler et al. (18) and Vandewalle et al. (20), whereas Verge et al. (2) did not find any correlation between GADA and the DR3-DQ2 or DR4-DQ8 haplotypes. Multiple antibodies were associated with all of these high-risk genetic markers, and positivity for any antibody (i.e., for one or more antibodies) was associated with all but the DR3 allele. Siblings carrying the protective DR2 allele had a lower frequency of ICA, IA-2A, GADA, any antibody, and multiple antibodies (but not of IAA) than the other siblings. Similar differences were observed between siblings with or without the DQB1*0602-3 allele, except that no difference was seen in the frequency of positivity for any antibody. These results suggest that both the DR2 and DQB1*0602-3 alleles are not only associated with a low risk of the clinical disease (11–13,17,46) but are also associated with a partial protection from disease-associated humoral autoimmunity.

Only a few reports have been published on the relationship between genetic markers and levels of autoantibodies in first-degree relatives. Ziegler et al. (18) reported a positive correlation between the levels of IAA and the DR4 allele in first-degree relatives but not between the levels of ICA and the DR4 allele (18). No studies are available on the association between the levels of IA-2A or GADA and genetic markers in siblings. In the present series, ICA titers were higher in siblings carrying markers of increased genetic risk, except for the DR3 and DQB1*02 alleles, than in the other siblings. A tendency for higher levels of IA-2A was found in those siblings with the DQB1*0302 allele than in those who were negative for this allele ($P = 0.055$). There were no significant differences in the levels of GADA or IAA between the siblings with and without specific genetic-risk markers. No significant differences were found in the levels of any antibody specificity between those with and without the DR2 or DQB1*0602-3 alleles, suggesting that these protective alleles do not modulate the intensity of the humoral immune response.

A total of 33 siblings have progressed to type 1 diabetes during the follow-up period, which lasted almost 9 years. Among the humoral markers, IA-2A and the presence of multiple antibodies had the highest positive predictive values (57%), as we

have reported previously (7). The sensitivities were highest for ICA (79%), any antibody (82%), and multiple antibodies (76%). Among the genetic markers, DQB1*02/0302 had the highest positive predictive value (22%), while HLA identity to the diabetic proband had the highest sensitivity (64%). Deschamps et al. (24) reported that the combination of ICA and the DR3/4 genotype had a high predictive value. The present results point to a marked increase in the positive predictive values of all the individual autoantibody specificities, any autoantibody, and multiple antibodies when genetic and humoral markers are combined. This effect may have clinical implications when evaluating the risk of type 1 diabetes at the individual

TABLE 8
Positive predictive value and sensitivity of the number of positive autoantibodies combined with genetic markers for progression to type 1 diabetes over 8.9 years in siblings of children with type 1 diabetes

	Positive predictive value	Sensitivity (%)
No antibodies	1.0% (6/612)	18.2
One antibody	4.4% (2/45)	6.1
Two antibodies	25.0% (3/12)	9.1
Three antibodies	74.1% (20/27)	60.6
Four antibodies	40.0% (2/5)	6.1
HLA-identical + no antibodies	2.1% (3/144)	9.1
HLA-identical + one antibody	12.5% (1/8)	3.0
HLA-identical + two antibodies	42.9% (3/7)	9.1
HLA-identical + three antibodies	80.0% (12/15)	36.4
HLA-identical + four antibodies	66.7% (2/3)	6.1
HLA-DR 3/4 + no antibodies	5.3% (2/38)	6.1
HLA-DR3/4 + one antibody	0% (0/2)	0
HLA-DR3/4 + two antibodies	0% (0/3)	0
HLA-DR3/4 + three antibodies	100% (8/8)	24.2
HLA-DR3/4 + four antibodies	0% (0/2)	0
DQB1*02/0302 + no antibodies	7.9% (3/38)	9.7
DQB1*02/0302 + one antibody	0% (0/3)	0
DQB1*02/0302 + two antibodies	25% (1/4)	3.2
DQB1*02/0302 + three antibodies	100% (8/8)	25.8
DQB1*02/0302 + four antibodies	0% (0/1)	0

TABLE 9
Characteristics of the five siblings who tested initially positive for all four antibody specificities

Sibling	Type 1 diabetes	Sex	Age at first sample (years)	Follow-up (years)	HLA identity with a diabetic proband	HLA-DR	HLA-DQB1	ICA (JDF U)	IA-2A (RU)	GADA (RU)	IAA (nU/ml)
1	+	F	5.3	0.2*	HLA-identical	DR4/4	0302/0302	160	38.2	6.8	181
2	+	M	9.9	2.9*	HLA-identical	DR1/5	X/X	20	13.9	8.9	79
3	-	M	4.8	10.8	HLA-haploidentical	DR4/8	0302/0302	40	0.79	84.0	136
4	-	M	7.6	10.8	HLA-haploidentical	DR3/4	02/0301	20	0.62	79.0	223
5	-	F	7.8	10.1	HLA-identical	DR3/4	02/0302	10	0.49	8.9	144

The limits for antibody-positivity are 2.5 JDF U for ICA, 0.43 RU for IA-2A, 6.5 RU for GADA, and 54 nU/ml for IAA. *Follow-up ended at diagnosis.

level or identifying individuals at maximal risk of progression to clinical disease for intervention purposes. Although a specific risk genotype did not increase the sensitivity of the screening, the combination of siblings with increased DQB1-defined genetic risk revealed a sensitivity of 96.8%. However, this combination had a low specificity and had a positive predictive value of <10%. From a practical point of view, such a screening strategy would require the observation of an extensive sibling population, a minority of whom would progress to type 1 diabetes. Accordingly, the use of autoantibodies alone can be recommended when aiming at a sensitive screening method with an optimal specificity for the identification of the majority of future progressors among siblings.

Some differences were observed between DR and DQB1 alleles and combinations in relation to antibody prevalences and predictive characteristics. These were probably not due to the design of the study because all of these differences still remained and many of them even increased in magnitude when the DR analyses were restricted to the 565 siblings with DQB1 data available. The observed differences were minor, however, and the combination of DR and DQB1 analysis with autoantibody tested revealed comparable results. In other words, neither DR nor DQB1 was superior to the other in terms of predictive characteristics when combined with autoantibody markers. In terms of practicality, DQB1 typing is cheaper and technically easier than DR typing and also facilitates a more detailed classification of individuals into various risk groups. Accordingly, we would like to recommend DQB1 typing over DR typing.

Only two of the five siblings with all four antibody specificities in their initial blood sample have so far progressed to clinical type 1 diabetes. Of the three nonprogressors, one has become negative for all the antibodies except ICA, while the other two are still positive for multiple antibodies. The initial levels of IA-2A were higher in the two siblings who contracted the disease than in those who remained nondiabetic, but they increased to even higher levels in the latter subjects at the later stages of follow-up (except for the sibling who became negative for IA-2A, GADA, and IAA). The fact that the initial levels of GADA were higher in the two nonprogressors who remained positive for multiple antibodies than in the two progressors supports the notion that GADA may be associated with a slower progression to type 1 diabetes. A moderate decline in the levels of GADA was seen in two nonprogressors during the follow-up, but both remained clearly positive. Both of the progressors are HLA-identical to the diabetic

proband, but neither of them had a high-risk DR3/4 or DQB1*02/0302 genotype. Interestingly, the nondiabetic sibling who became negative for all antibodies except ICA is also HLA-identical to the diabetic proband and even carries a high-risk DR3/4, DQB1*02/0302 genotype. This observation suggests that environmental factors or genetic factors, other than those conferred by the HLA class II region, may modify the autoimmune process.

In conclusion, we have shown that the presence of autoantibodies in an initial blood sample taken at or close to the time of diagnosis of a diabetic proband is closely associated with high-risk genetic markers in siblings of children with type 1 diabetes. However, the intensity of this humoral immune response (i.e., the levels of these antibodies) is not as obviously related to such genetic markers. We also observed that the combination of humoral and genetic markers substantially increases the positive predictive values of the presence of antibodies. Such a combination also results in reduced sensitivity, however, and a considerable proportion of the future patients may be lost. Accordingly, autoantibody testing alone, rather than in combination with genetic markers, is recommended for first-line screening of the siblings of children with type 1 diabetes. On the other hand, when the most susceptible subjects are to be identified (e.g., for intervention trials) the analysis of high-risk genetic markers in combination with autoantibodies offers an excellent tool for identifying these subjects. Finally, accurate assessment of the risk for type 1 diabetes in siblings is a complicated matter, because not all of those with a broad humoral autoimmune response and/or high-risk HLA markers progress to clinical disease even within a long follow-up period, while some siblings with a low genetic risk and minor initial signs of humoral autoimmunity may present with disease.

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APPENDIX

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