Genetic Effects on Age-Dependent Onset and Islet Cell Autoantibody Markers in Type 1 Diabetes

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Age-dependent associations between type 1 diabetes risk genes HLA, INS VNTR, and CTLA-4 and autoantibodies to GAD65 (GADAs), ICA512/IA-2, insulin, and islet cells were determined by logistic regression analysis in 971 incident patients with type 1 diabetes and 702 control subjects aged 0–34 years. GADAs were associated with HLA-DQ2 in young but not in older patients ($P = 0.009$). Autoantibodies to insulin were negatively associated with age ($P < 0.0001$) but positively associated with DQ8 ($P = 0.03$) and with INS VNTR ($P = 0.04$), supporting possible immune tolerance induction. ICA512/IA-2 were negatively associated with age ($P < 0.0001$) and with DQ2 ($P < 0.0001$) but positively associated with DQ8 ($P = 0.04$). Males were more likely than females to be negative for GADA ($P < 0.0001$), autoantibodies to islet cells ($P = 0.04$), and all four autoantibody markers ($P = 0.004$). The CTLA-4 3′ end microsatellite marker was not associated with any of the autoantibodies. We conclude that age and genetic factors such as HLA-DQ and INS VNTR need to be combined with islet autoantibody markers when evaluating the risk for type 1 diabetes development. *Diabetes* 51:1346–1355, 2002

T he incidence of type 1 diabetes varies both by age and by sex, with males predominant in postpubertal young adults (1,2). Such age- and sex-dependent variation may in part be determined by markers for islet autoimmunity, such as autoantibodies to insulin (IAAs), GAD65 (GADAs), and ICA512/IA-2 (IA-2As) and their associations with age, sex, and genetic factors such as HLA (rev. in 3,4), the insulin gene on chromosome 11 (5,6), and the CTLA-4 gene on chromosome 2 (7,8). Previous studies have established that the frequency of immune markers, such as IAAs, GADAs, and IA-2As, at clinical onset varies with age (9,10). Autoantibodies to the ICA512/IA-2 autoantigen (11,12) are associated with HLA-DQ8 in patients (13) and are of particular interest because they may better predict type 1 diabetes in first-degree relatives (14,15). IAAs, widely known to be markedly affected by age at clinical onset (10,16), have been reported to be associated with HLA-DQ8, but only in patients who are younger than 10 years (9). Studies that combine incident children and young adults with type 1 diabetes are therefore needed to test the effects of genetic factors on autoantibody markers of β-cell autoimmunity and to dissect the age-dependent risk of type 1 diabetes. Association studies with a large number of incident patients can be a useful complement to genetic linkage studies (17).

The aim of the present investigation was to analyze the change in prevalence of IAAs, GADAs, IA-2As, and islet cell autoantibodies (ICAs) with age at clinical onset according to sex and genetic factors in patients aged 0–34 years. All available incident patients with type 1 diabetes diagnosed at 0–34 years of age (18) were therefore typed for HLA-DQ and for polymorphic genetic markers (INS VNTR) next to the insulin gene (IDDM2) on chromosome 11 (6,19) and for the 3′ end noncoding region AT repeat within the gene for CTLA-4 (IDDM12) on chromosome 2 (7,8). One means of controlling for the effects of potential confounding variables is to stratify the incident patient population on the basis of combinations of levels of these variables. However, the number of possible variables leads to a large number of strata in the data, making a conventional analysis based on contingency tables unwieldy. In addition, age at clinical onset takes on a wide range of values from 0 to 34 years, and so loss of information is expected from grouping ages into discrete categories, as is typically done in a contingency table analysis. To control simultaneously for confounding variables and to avoid loss of information from discretizing age at clinical onset, we used a logistic regression analysis (e.g., Breslow and Day [20]). Multiple logistic regression analysis permits efficient

TABLE 1
Number (%) of incident patients with type 1 diabetes and control subjects in relation to age, sex, and geographic location

	Control subjects	Patients
Total number of subjects	702 (100)	971 (100)
Age distribution (years)		
0–6	54 (7.7)	131 (13.5)
7–13	310 (44.2)	315 (32.4)
14–20	154 (21.9)	217 (22.3)
21–27	74 (10.5)	169 (17.4)
28–34	109 (15.5)	136 (14.0)
Missing	1 (0.1)	3 (0.3)
Sex distribution		
Female	339 (48.3)	399 (41.1)
Male	363 (51.7)	572 (58.9)
Geographic distribution (north to south)		
Umeå	102 (14.5)	123 (12.7)
Uppsala	152 (21.7)	197 (20.3)
Stockholm	105 (15.0)	203 (20.9)
Linköping	118 (16.8)	138 (14.2)
Göteborg	129 (18.4)	186 (19.2)
Lund	96 (13.7)	124 (12.8)

The distributions of age, sex, and geographic region differ between patients and control subjects, according to χ^2 tests on 4 ($P < 0.0001$), 1 ($P = 0.004$), and 5 ($P = 0.04$) degrees of freedom, respectively.

estimation and evaluation of measures of association and effect modification, which are adjusted for confounding factors.

RESEARCH DESIGN AND METHODS

Subjects. The two population-based matched case-control studies merged in the present study have been described elsewhere (18). Patients received diagnoses and classifications according to the World Health Organization (WHO) criteria. In the first study, all incident patients with type 1 diabetes who were younger than 15 years and received a diagnosis anywhere in Sweden between 1 September 1986 and 31 December 1987 were asked to participate. Matched control subjects were selected as described (21). The second study comprised incident patients with diabetes who were aged 15–34 years and received their diagnosis anywhere in Sweden between 1 January 1987 and 31 December 1988; matched control subjects were also ascertained (10). The two studies administered over overlapping years were combined for a total of 971 incident patients with type 1 diabetes and 702 control subjects. The distributions of age, sex, and place of residence for incident patients with type 1 diabetes and control subjects are summarized in Table 1.

Genetic markers

HLA (IDDM1). HLA typing of DQA1 and DQB1 was carried out by PCR amplification of the second exon of the genes followed by dot blot hybridizations of sequence-specific oligo probes and by restriction fragment–length polymorphism using DR- and DQ-based probes to establish haplotypes (22,23). HLA-DQ genotypes were available on 832 of the 971 patients (85.7%) and 618 of the 702 control subjects (88.0%).

Insulin gene VNTR (IDDM2). Whole genomic DNA (25 ng) was amplified with primers flanking the polymorphic *Hph*I site in the insulin gene upstream sequence, as described (6). The presence or absence of the *Hph*I site (−23) is linked to the upstream variable number of tandem repeats (VNTR) flanking the insulin gene (INS). The PCR product of 50 cycles was digested at 37°C overnight with *Hph*I and separated by agarose gel electrophoresis in parallel with a 100-bp ladder, were and previously genotyped individuals were included as control subjects (6). The gels were photographed, and the banding patterns of samples were scored independently by two investigators as either class I (short VNTR or lower band) or class III (long VNTR or upper band) alleles (5). No class II alleles were present in these Swedish white patients. INS VNTR genotypes were available on 729 of the 971 (75.1%) patients and 522 of the 702 (74.4%) control subjects (Table 2).

CTLA-4 microsatellite (IDDM12). The CTLA-4 3′ end microsatellite was typed using two standard methods of PCR and PAGE. In the first assay, described in detail elsewhere (8), oligonucleotides with sequences flanking the 3′ UTR region containing the AT repeats were used as primers in a PCR to

TABLE 2
Distribution of INS VNTR genotypes in incident patients with type 1 diabetes and control subjects aged 0–34 years

	Number (%) of INS VNTR genotypes			
	I/I	III/I	III/III	Total
Control subjects	270 (51.7)	203 (38.9)	49 (9.4)	522 (100.0)
Patients	513 (70.4)	190 (26.0)	26 (3.6)	729 (100.0)

The distribution of III/III, III/I, and I/I genotypes differs between patients and control subjects, according to a χ^2 test on 2 degrees of freedom ($P < 0.0001$).

amplify selectively the polymorphic region. The first assay was used to genotype 616 patients and 500 control subjects for CTLA-4. In the second assay, we used a high-throughput genotyping method with infrared dyes (IRDs) at two wavelengths to label PCR products. DNA was dispensed into 96-well plates using a Hydra robot and vacuum-dried before the PCR mixture was added and the samples cycled (35 times at 94°C for 15 s, 50–58°C for 15 s, and 72°C for 15 s) using a Peltier Thermal Cycler (MJ Research). Each reaction contained 50 ng of DNA, primers (0.2 μmol/l each), 50 μmol/l KCl, 10 mmol/l Tris, 1–2.5 mmol/l MgCl₂, 0.5 units of *Taq* polymerase, and 0.15–0.70 μmol/l IRD label. Products were labeled during strand synthesis with the incorporation of the IRD-labeled dATP. A LI-COR 4200 sequencing machine was used for electrophoresis. Samples were loaded with a multichannel pipette onto 18-cm 6% polyacrylamide gels; after electrophoresis, the gel image was analyzed by the genotyping software (SAGA) that was used to call alleles at a sizing accuracy of 0.2 bp. The CTLA-4 alleles of the 135 patients were calibrated to the first assay using a regression equation based on 94 control subjects with complete genotypes from both assays. Parameter estimates for the calibration equation were robust to outlying assay measurements (24). The resulting prediction equation was used to impute missing alleles for the first assay based on the LI-COR genotypes from the second assay. All alleles with resulting relative length longer than 1.0 (10 AT repeats) were classified as long (L), whereas alleles with relative length of 1.0 or shorter were classified as short (S). The classification was based on the relative allele length distribution of control subjects shown in Fig. 1; for comparison, the distribution of patients is also shown. CTLA-4 genotypes were available on 751 of the 971 patients (77.3%) and 502 of the 702 control subjects (71.5%) (Table 3).

Immune markers

Insulin antibodies. Autoantibodies (IAAs) as well as antibodies to insulin were measured by radiobinding assay using acid-charcoal extraction and cold insulin displacement as described (25–27). IAA-Δ percent measurements were available on 929 of the 971 (95.7%) patients and 668 of the 702 control subjects (95.2%). However, in 201 patients with IAA measurements, sera were sampled >3 weeks after beginning insulin therapy, precluding reliable IAA testing (10). These patients were therefore excluded from the analysis of IAA.

Glutamate decarboxylase autoantibodies. Antibodies to radiolabeled human Mr 65,000 glutamate decarboxylase (GADA) were quantified by fluid-phase immunoprecipitation assay using fluorographic densitometry as described (13,28) except that for 15- to 35-year-olds, GAD65 were radiolabeled by coupled in vitro transcription and translation, as described (29,30). The two assay formats correlate well, and autoantibody levels are expressed as GADA index as described elsewhere (29), using the WHO–Juvenile Diabetes Foundation (JDF) standard (31) as the positive control. GADA index measurements were available on 938 of the 971 patients (96.6%) and 682 of the 702 control subjects (97.2%).

Islet antigen-2 or ICA512 autoantibodies. Antibodies to ICA512/IA-2 (ICA512) (12,32) were measured by radiobinding immunoassay (33). The 3′ portion of the ICA512cDNA (32) corresponding to the cytoplasmic portion of the protein (residues 602–979) was amplified by RT–PCR from human HTB-14 glioblastoma cells (12). After expression in DH10B *Escherichia coli*, sequenced plasmid DNA was identical to Genbank sequence L18983. In vitro translation with ³⁵S-methionine (29) yielded a 46-kDa polypeptide highly precipitable by diabetes sera. Radiobinding assays used scintillation counting of protein A-Sepharose pellets, and autoantibody levels are expressed as an ICA512/IA-2A index as described (29), using the WHO-JDF standard as a positive control (31). ICA512/IA-2A index measurements were available on 937 of the 971 patients (96.5%) and 671 of the 702 control subjects (95.6%).

Islet cell (cytoplasmic) antibodies. Islet cell cytoplasmic antibodies (ICAs) were measured by indirect two-color immunofluorescence using blood group O frozen human pancreas as described (34). The same pancreas was used throughout the study. Coded slides were evaluated by two independent
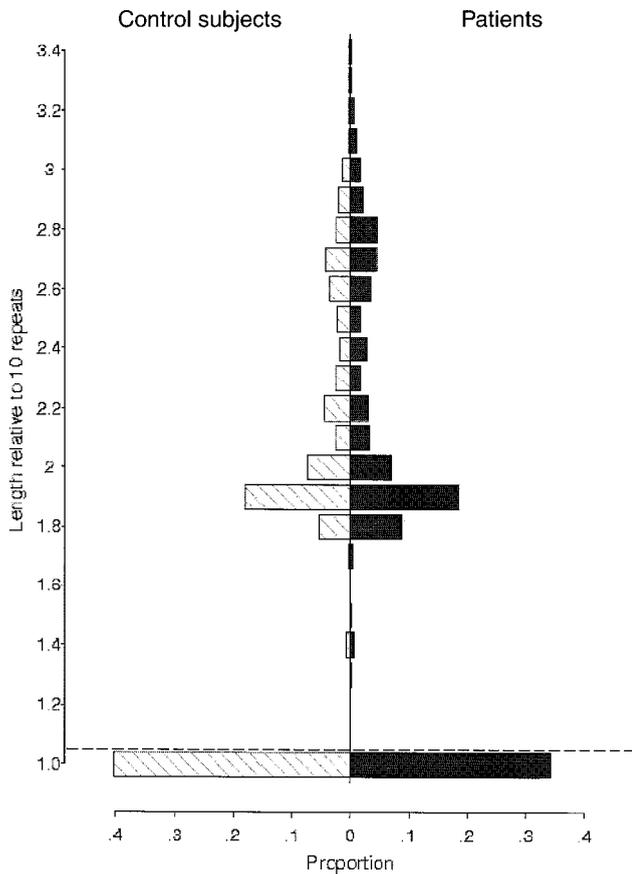
FIG. 1. Distribution of CTLA-4 AT repeat polymorphisms in 751 of the 971 incident patients with type 1 diabetes (77.3%) and 502 of the 702 control subjects (71.5%) with available CTLA-4 data. The distribution of patients and control subjects differs according to a Wilcoxon rank-sum test ($P = 0.0001$).
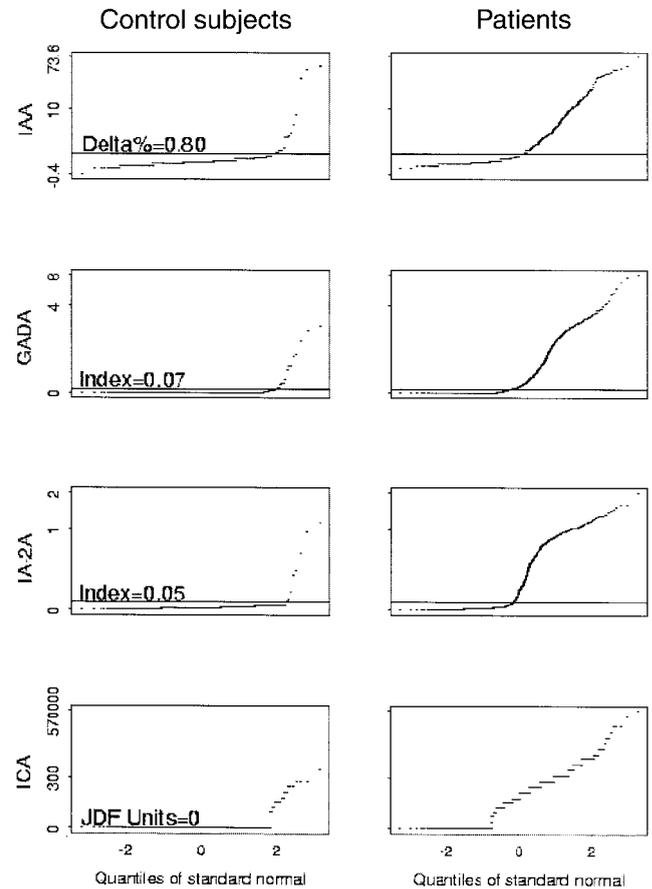

FIG. 2. Quantile-quantile plots of antibody measurements in incident patients with type 1 diabetes and control subjects. Plots for control subjects are in the left column and are based on the following numbers of the original 702 control subjects having antibody measurements: IAA, 668 (95.2%); GADA, 682 (97.2%); ICA512/IA-2A, 671 (95.6%); and ICA, 700 (99.7%). Plots for patients are in the right column and are based on the following numbers of the original 971 patients having antibody measurements: IAA, 929 (95.5%); GADA, 938 (96.6%); ICA512/IA-2A, 937 (96.5%); and ICA, 969 (99.8%). Vertical axes, quantiles of the natural logarithm of shifted antibody measurements; horizontal axes, quantiles of the standard normal probability distribution. For each antibody, patients and control subjects have the same range on the vertical axis. Vertical axes are in the logarithmic scale but are labeled according to the original scale of measurement so that the labels of the axes end points reflect the range of antibody measurements observed. Cutoffs for declaring antibody positivity are indicated in the original scale of measurement and are IAA Δ percent >0.80, GADA index >0.07, ICA512/IA-2A index >0.05, and ICA JDF units >0. In this large cohort of 971 patients, 42 of 882 (5%) with complete data on all antibodies were ICA-positive but negative for IAA, GADA, and ICA512/IA-2A. Hence, ICA are also analyzed.

observers. Samples were titered in doubling dilutions to determine end points for conversion to JDF units (35) as described (34). ICA measurements of 0 JDF units corresponded to no detectable antibody. ICA JDF unit measurements were available on 969 of the 971 patients (99.8%) and 700 of the 702 control subjects (99.7%).

Statistical methods

Antibody status. For IAA, GADA, ICA512/IA-2A, and ICA, the smallest and largest measurements differed by at least an order of magnitude, both in patients and control subjects. To normalize values and bring them onto a comparable scale, antibody measurements were shifted and then transformed by taking the natural logarithm (36). For each antibody population in both patients and control subjects, a mixture of at least two distributions was expected, corresponding to a subpopulation of antibody-negative individuals and at least one subpopulation of antibody-positive individuals. This was confirmed by plotting the ordered transformed antibody measurements against the quantiles of a standard normal distribution in both patients and control subjects. Such quantile-quantile plots are expected to show data from a single normally distributed subpopulation falling along a straight line, with

slope equal to the standard deviation of the distribution (36). Data from multiple normally distributed subpopulations with different means are expected to fall along different lines. Cutoffs for antibody-positive status were determined graphically from these quantile-quantile plots for patients and control subjects (Fig. 2). The cutoff was defined by inspecting the vertical axis describing the ordered log-transformed antibody measurements for the lowest value at which the slope of the line within the plot changed in both patients and control subjects. After determination of cutoff values, control individuals were excluded because they were no longer necessary for analysis of patient antibody frequencies.

Genetic markers. The extended DQ-DR haplotype DQ2-DR3 has been observed to be positively associated with type 1 diabetes, whereas the haplotype DQ2-DR7 is neutral (37). Consistent with these observations, only 4 of the 395 DQ2-positive patients with an available DQ-DR haplotype were non–DQ2-DR3, and none carried DR7 (2 were DR4 and 2 were DR11). All DQ8-positive patients with an available DQ-DR haplotype were DQ8-DR4. Patient HLA genotypes were therefore coded according to the number of DQ2, DQ8, and DQ6.2 haplotypes, respectively. CTLA-4 and INS VNTR genotypes were coded according to the number of long CTLA-4 alleles and class I INS

TABLE 3
Distribution of CTLA-4 genotypes in incident patients with type 1 diabetes and control subjects aged 0–34 years

	Number (%) of CTLA-4 genotypes			
	L/L	S/L	S/S	Total
Control subjects	71 (14.1)	263 (52.4)	168 (33.5)	502 (100.0)
Patients	89 (11.9)	335 (44.6)	327 (43.5)	751 (100.0)

The distribution of S/S, S/L, and L/L genotypes differs between patients and control subjects, according to a χ^2 test on 2 degrees of freedom ($P = 0.002$).

TABLE 4
Ascertainment of 971 incident patients with type 1 diabetes aged 0–34 years by different variables

Variable	n (%)
Age at onset	968 (99.7)
Sex	971 (100.0)
HLA-DQ	832 (85.7)
CTLA-4	751 (77.3)
INS VNTR	729 (75.1)
All of the above and	
IAA*	491 (51.0)
GADA	652 (67.1)
ICA512/IA-2A	651 (67.0)
ICA	669 (68.9)

*Excludes 201 patients with sera sampled >21 days after beginning insulin therapy.

VNTR alleles. The odds of positive antibody status in patients heterozygous for the CTLA-4 long allele, the class I INS VNTR allele, or DQ2, DQ8, or DQ6.2 haplotypes were modeled initially as the geometric mean of the odds in patients homozygous for these alleles or haplotypes, who were otherwise similar with respect to sex, age, and the other genetic factors.

Exploratory analysis. Each antibody marker was examined separately; patients whose data were missing were excluded from analysis. The number of patients used in each antibody analysis is summarized in Table 4 and represents those who had measurements on the antibody as well as information for the explanatory variables age at clinical onset, sex, HLA-DQ, CTLA-4, and INS VNTR. Before the logistic regression modeling, variation in antibody status according to age, sex, and genetic markers was explored by use of summary tables. Selected summary tables report the proportion of individuals who are antibody-positive and the associated 95% (CIs) in patient subgroups. We stress that the purpose of these summary tables is descriptive rather than inferential, and they are meant to introduce the data before logistic regression modeling and hypothesis testing. Formal statistical inference, such as tests of hypotheses regarding specific effects of age at clinical onset, sex, and genetic factors, was therefore deferred to the logistic regression analysis.

Logistic regression. In patients with complete data, a logistic regression model was used to relate age, sex, and genetic marker status to the odds of positive antibody status. To gain power and resolution in patterns of age dependence, we avoided discretization of age at onset. Let $P = \Pr(\text{Ab}+ | \text{sex}, \text{age}, \text{HLA}, \text{CTLA-4}, \text{INS VNTR})$ denote the conditional probability of positive antibody status in incident patients with type 1 diabetes, given information on sex, age, and genotypes for HLA, CTLA-4, and INS VNTR. The probability P is the expected proportion of incident patients with type 1 diabetes of a given sex, age, and set of HLA, CTLA-4, and INS VNTR genotypes who are antibody-positive. The ratio $P/(1 - P)$ is known as the odds of positive antibody status. For example, consider a comparison between male and female patients who are similar with respect to age at clinical onset and genetic markers, and suppose that the male subjects have an antibody prevalence of 0.69 and the female subjects have a prevalence of 0.45. Then, the male odds of positive antibody status are $0.69/(1 - 0.69) = 2.2$, whereas the female odds are $0.45/(1 - 0.45) = 0.82$. The male odds of being positive for the antibody are therefore $2.7 = 2.2/0.82$ times the female odds within the subgroup defined by the specific age at clinical onset and genetic markers. Logistic regression models the natural logarithm of the odds, or the log odds, which is referred to as logit (p). Our initial model was as follows:

logit (p) = $\beta_0 + \beta_1 \times \text{sex} + \beta_2 \times \text{DQ2} + \beta_3 \times \text{DQ8} + \beta_4 \times \text{DQ6.2} + \beta_5 \times \text{CTLA-4} + \beta_6 \times \text{INS VNTR} + g(\text{age})$.

On the right side of the equation, each regression coefficient β_1–β_6 is interpreted by holding fixed the explanatory variables other than the variable corresponding to the regression coefficient. For example, "sex" is coded as 1 if the patient is male and 0 if the patient is female, and β_1 is the difference between males and females in the log odds of positive antibody status, holding fixed the values of the other explanatory variables. The variables "DQ2," "DQ8," and "DQ6.2" are, respectively, the number of HLA-DQ2, -DQ8, and -DQ6.2 haplotypes; β_2, β_3, and β_4 are, respectively, the change in the log odds of positive antibody status corresponding to an increase of one DQ2, DQ8, or DQ6.2 haplotype; "CTLA-4" is the number of long (L) CTLA-4 alleles; β_5 is the change in the log odds of positive antibody status corresponding to an increase of one long allele in the CTLA-4 genotype; "INS VNTR" is the number of class I alleles of INS VNTR; β_6 is the change in the log odds of positive antibody status corresponding to an increase of one class I allele in the INS VNTR genotype; "age" is age at onset in years, centered by the mean age of

16.5 years in patients; g(age) is a natural cubic spline (38) relating age at clinical onset to the log odds of positive antibody status, with interior knots placed at ages 7, 14, 21, and 28 years; and β_0 is the log odds of positive antibody status in the baseline group composed of female patients aged 16.5 years, who carry genotypes X/X at HLA-DQ, S/S at CTLA-4, and III/III at INS VNTR, where DQX is any HLA-DQ haplotype other than DQ2, DQ8, or DQ6.2.

Backward elimination (e.g., Weisberg [36]) of sex and genetic markers was used to simplify the initial model. When possible, the modeled antibody response in age was then reduced to a simple linear or quadratic function. All model reductions were based on likelihood ratio tests (e.g., Breslow and Day [20]) with significance level 10% or more for exclusion of variables. A relatively liberal significance level was chosen to yield an expanded set of possible explanatory variables for a second phase of model building. In the second model-building stage, terms were added to quantify two-way interactions between remaining explanatory variables and departures from a multiplicative model in the number of alleles or haplotypes for genetic factors. Terms were added by forward selection (e.g., Weisberg [36]) with significance level ≤5% for inclusion. Higher-order interactions were fit and tested only in the presence of lower-order terms, with the exception of the interaction term for the potentiating effect of DQ2 and DQ8 in DQ2/8 heterozygotes and the interaction term for DQ8 and age at clinical onset. The final model thus obtained was used to describe the change with age at onset in antibody prevalence among subsets of incident patients, with subsets defined by sex and by the genetic factors HLA, INS VNTR, and CTLA-4. Parameter estimates from the final model were used to calculate fitted antibody prevalences, odds ratios, and approximate 95% CIs.

RESULTS

In the following, the data are introduced by way of an exploratory descriptive analysis based on unadjusted frequencies from contingency tables, and then results of hypothesis tests (i.e., P values) are presented from a logistic regression analysis that adjusts for confounding variables.

IAA. In the exploratory analysis, the unadjusted frequency of IAA increased with the number of class I alleles of INS VNTR: 15% of III/III patients (95% CI 0–30), 30% of I/III patients (22–38), and 40% of I/I patients (35–45) were positive for IAA. Unadjusted frequencies of IAA were higher in DQ8-positive than in DQ8-negative patients: 27% of patients with no DQ8 haplotypes (95% CI 20–34), 41% of patients with one DQ8 haplotype (35–46), and 42% of patients with two DQ8 haplotypes (26–57) were positive for IAA. Finally, the unadjusted frequencies in Table 5 support numerous observations that IAAs are more common in younger than in older patients.

Likelihood ratio tests from the logistic regression analysis confirmed the negative association between IAA and age at onset ($P < 0.0001$) and the positive associations between IAA and DQ8 ($P = 0.03$) and IAA and the class I allele of INS VNTR ($P = 0.04$). Fitted IAA prevalences are shown plotted against age at onset (Fig. 3), by number of DQ8 haplotypes in patients negative for the class I allele of INS VNTR (Fig. 3A), and by number of class I alleles of INS VNTR in DQ8-negative patients (Fig. 3B).

The logistic regression modeling allows comparison of patients in subgroups defined by different levels of an explanatory variable, such as age at clinical onset, adjusted for the effects of the other variables. With each additional class I allele of INS VNTR, the odds of IAA were estimated to increase by a factor of 1.5 (95% CI 1.0–2.2). For patients carrying two class I alleles, the odds of IAA were estimated to be 2.2 ($\sim 1.5^2$) times those for patients carrying no class I alleles (95% CI 1.0–4.8). A nonmultiplicative effect in the number of DQ8 haplotypes was not evident after adjusting for other variables ($P = 0.62$).

TABLE 5
Proportion (95% CI) of incident patients with type 1 diabetes who are antibody positive by age category

	Age at clinical onset (years)				
	0–6	7–13	14–20	21–27	28–34
IAA					
	0.69 (0.60–0.79)	0.41 (0.34–0.48)	0.24 (0.15–0.032)	0.19 (0.10–0.29)	0.13 (0.06–0.22)
GADA by number of DQ2 haplotypes					
0	0.28 (0.14–0.42)	0.44 (0.35–0.52)	0.42 (0.30–0.55)	0.62 (0.47–0.74)	0.63 (0.49–0.76)
1	0.51 (0.39–0.63)	0.63 (0.54–0.72)	0.61 (0.49–0.73)	0.69 (0.56–0.81)	0.66 (0.48–0.83)
2	0.75 (0.25–1.00)	0.75 (0.25–1.00)	0.89 (0.67–1.00)	0.62 (0.25–0.88)	0.25 (0.00–0.75)
ICA512/IA-2A					
	0.68 (0.59–0.77)	0.72 (0.67–0.78)	0.56 (0.47–0.64)	0.40 (0.31–0.50)	0.30 (0.21–0.40)
ICA					
	0.89 (0.83–0.95)	0.85 (0.81–0.90)	0.77 (0.70–0.84)	0.68 (0.59–0.77)	0.67 (0.57–0.77)

The purpose of the table is descriptive rather than inferential, and so no P values are reported. Statistical testing of hypotheses is deferred to the logistic regression analysis. Of the original 971 patients, 491 (51.0%), 652 (67.1%), 651 (67.0%), and 669 (68.9%) were analyzed for IAA, GADA, ICA512/IA-2A, and ICA, respectively (see Table 4).

GADA. In the exploratory analysis, the unadjusted frequency of GADA was higher in female (69%; 95% CI 63–74) than in male subjects (45%; 40–50). The unadjusted frequencies in Table 5 suggest that the prevalence of GADA increases with the number of DQ2 haplotypes in younger age groups but not necessarily in older age groups and that GADA are more common in older- than in younger-onset patients. Likelihood ratio tests from the logistic regression analysis confirmed the positive association between GADA and age at onset ($P = 0.001$) and between GADA and sex ($P < 0.0001$). DQ2 was positively associated with GADA ($P < 0.0001$), but a statistically significant attenuation of its effect was observed with age at onset ($P = 0.009$). Fitted GADA prevalences are shown plotted against age at onset in Fig. 4, by number of DQ2 haplotypes in female (Fig. 4A) and male (Fig. 4B) patients.

After the observed effects of age at clinical onset and DQ2 were adjusted for, the odds of GADA for female patients were estimated to be 3.1 times those for male patients (95% CI 2.2–4.3). By contrast, in patients with age at clinical onset at 34 years, the odds of GADA did not differ significantly according to the number of DQ2 haplotypes ($P = 0.55$). After other variables were adjusted for, there was insufficient statistical evidence for an interaction between DQ8 and age at clinical onset ($P = 0.08$) and for a potentiating effect of DQ2 and DQ8 on the odds of GADA in DQ2/8 heterozygotes ($P = 0.12$).

ICA512/IA-2A. In the exploratory analysis, the unadjusted frequency of ICA512/IA-2A decreased with increasing age at onset (Table 5). Unadjusted ICA512/IA-2A frequencies decreased with the number of DQ2 haplotypes and increased with the number of DQ8 haplotypes (Table 6). ICA512/IA-2A seemed to be slightly more common in male (59%; 95% CI 54–63) than in female subjects (55%; 49–61). Likelihood ratio tests from the logistic regression

FIG. 4. Fitted prevalences of GADA against age at onset, by number of DQ2 haplotypes ($P < 0.0001$) in female (A) and male (B) incident patients with type 1 diabetes. Female patients had a significantly higher prevalence of GADA than males ($P < 0.0001$), irrespective of the number of DQ2 haplotypes or of the age at clinical onset. The positive association between GADA and DQ2 in younger patients is attenuated with age at onset ($P = 0.009$). In patients with two DQ2 haplotypes, the apparent decrease with age at onset in the prevalence of GADA is not statistically significant ($P = 0.32$).
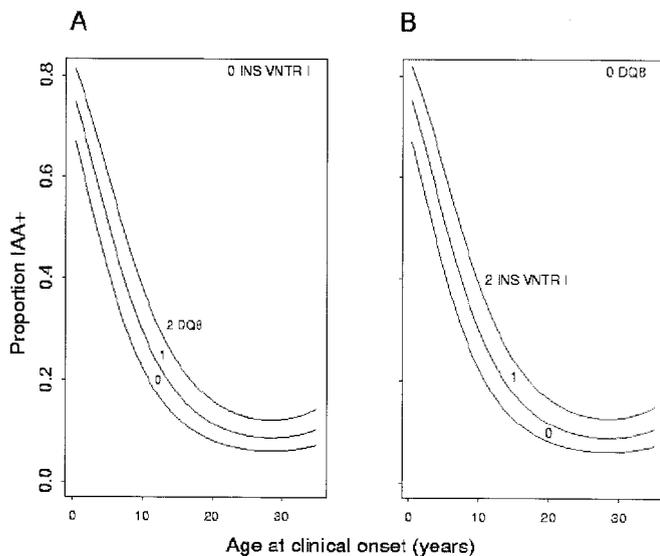

FIG. 3. Fitted prevalences of IAA against age at onset, by number of DQ8 haplotypes ($P = 0.03$) in incident patients with type 1 diabetes negative for the INS VNTR class I allele (A), and by number of INS VNTR class I alleles ($P = 0.04$) in incident patients with type 1 diabetes negative for the DQ8 haplotype (B).
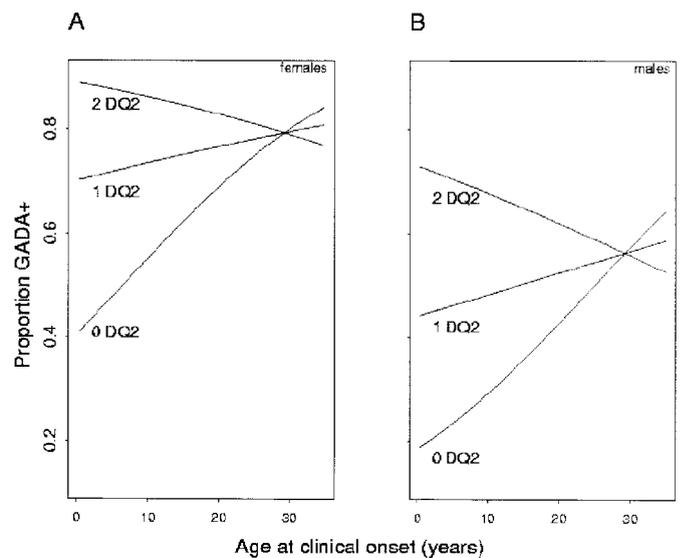
TABLE 6
Proportion (95% CI) of incident patients with type 1 diabetes who are ICA512/IA-2A–positive by number of DQ2 and DQ8 haplotypes

	Number of haplotypes		
	0	1	2
DQ2	0.65 (0.60–0.70)	0.52 (0.47–0.58)	0.24 (0.10–0.41)
DQ8	0.47 (0.40–0.54)	0.62 (0.57–0.66)	0.69 (0.56–0.80)

The purpose of the table is descriptive rather than inferential, and so no P values are reported. Statistical testing of hypotheses is deferred to the logistic regression analysis. Of the original 971 patients, 651 (67.0%) were analyzed for ICA512/IA-2A (see Table 4).

analysis confirmed the negative associations between ICA512/IA-2A and age at onset ($P < 0.0001$) and ICA512/IA-2A and DQ2 ($P < 0.0001$), as well as the positive association between ICA512/IA-2A and DQ8 ($P = 0.04$). A statistically marginal association was observed between ICA512/IA-2A and sex ($P = 0.06$). Fitted ICA512/IA-2A prevalences are shown in Fig. 5, plotted against age at onset by number of DQ2 haplotypes in female (Fig. 5*A*) and male (Fig. 5*B*) patients negative for DQ8 and by number of DQ8 haplotypes in female (Fig. 5*C*) and male (Fig. 5*D*) patients negative for DQ2.

After the observed effects of DQ2, DQ8, and age at clinical onset were controlled for, the odds of ICA512/IA-2A for male subjects were estimated to be 1.4 times those for female subjects (95% CI 0.97–1.9). The odds of ICA512/IA-2A were estimated to increase by a factor of 1.4 (1.0–1.8) with each additional DQ8 haplotype and to decrease by a factor of 0.52 (0.38–0.70) with each additional DQ2 haplotype. For patients carrying two DQ8 haplotypes, the odds of ICA512/IA-2A were estimated to be 1.8 times those for patients carrying no DQ8 haplotypes (1.0–3.3). For patients carrying two DQ2 haplotypes, the odds of ICA512/IA-2A were estimated to be 0.27 those for
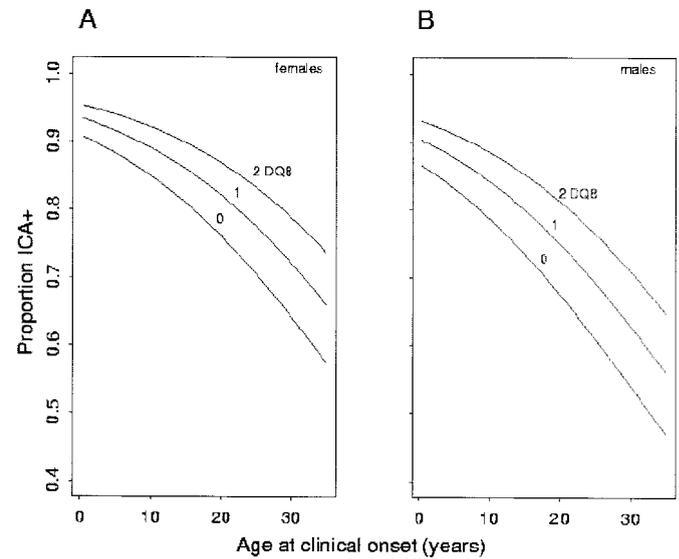
FIG. 6. Fitted prevalences of ICA against age at onset, by number of DQ8 haplotypes ($P = 0.03$) in female (*A*) and male (*B*) incident patients with type 1 diabetes. Female patients had a significantly higher prevalence of ICA than male patients ($P = 0.04$), irrespective of the number of DQ2 haplotypes or of the age at clinical onset.

patients carrying no DQ2 haplotypes (0.15-.49). The observed negative association between ICA512/IA-2A and DQ2 cannot be attributed to exclusion of DQ8 because the effect of DQ8 is adjusted for by the logistic regression.

ICA. In the exploratory analysis, the unadjusted frequency of ICA was higher in female (84%; 79–88) than in male patients (76%; 71–80) and higher in patients carrying two DQ8 haplotypes (80%; 69–89) or a single DQ8 haplotype (83%; 79–86) than in patients carrying no DQ8 haplotypes (72%; 66–78). ICA seemed to be more common in patients carrying the class I allele of INS VNTR than in patients not carrying this allele: 68% of patients with no class I alleles (48–74), 75% of patients with one class I allele (69–81), and 81% of patients with two class I alleles (77–84) were positive for ICA. The unadjusted frequency of ICA decreased with age at onset (Table 5).

Likelihood ratio tests from the logistic regression analysis confirmed the negative association between ICA and age at onset ($P < 0.0001$) and the positive association between ICA and DQ8 ($P = 0.03$). ICA and sex were also associated ($P = 0.04$). A nonmultiplicative effect in the number of DQ8 haplotypes to increase the odds of ICA was not evident after adjusting for the observed effects of sex and age at clinical onset in the logistic regression ($P = 0.18$). There was no apparent association between ICA and INS VNTR after adjusting for other variables ($P = 0.12$). Fitted ICA prevalences are shown in Fig. 6, plotted against age at onset by number of DQ8 haplotypes in female (Fig. 6*A*) and male (Fig. 6*B*) patients. In female patients, the estimated odds of ICA were 1.5 times those in male patients (95% CI 1.0–2.3).

With each additional DQ8 haplotype, the odds of ICA were estimated to increase by a factor of 1.4 (95% CI 1.0–2.0). In patients carrying two DQ8 haplotypes, the estimated odds of ICA were 2.1 ($\sim 1.4^2$) times those in patients carrying no DQ8 haplotypes (95% CI, 1.1–4.0).

Antibody-negative patients. As age at clinical onset increased, so did the proportion of patients negative for all
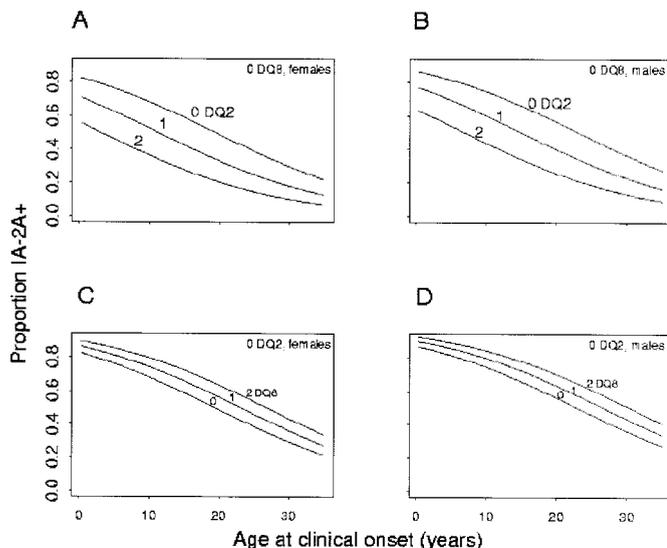
FIG. 5. Fitted prevalences of ICA512/IA-2A against age at onset by number of DQ2 haplotypes ($P < 0.0001$) in female (*A*) and male (*B*) incident patients with type 1 diabetes negative for DQ8 and by number of DQ8 haplotypes ($P = 0.04$) in female (*C*) and male (*D*) patients negative for DQ2. Male patients had a marginally significant increased prevalence of ICA512/IA-2A compared with female patients ($P = 0.06$), irrespective of the number of DQ2 or DQ8 haplotypes or of the age at clinical onset.

four of the IAA, GADA, ICA512/IA-2A, and ICA markers. Approximately 3% of patients aged 5 years at clinical onset versus 33% of patients aged 34 years at clinical onset were expected to be antibody-negative ($P < 0.0001$). Patients negative for all four antibody markers were mostly male (69%; 95% CI 59–78), and the conditional odds of being male given that an incident patient was negative for all four antibody markers were significantly higher than the overall odds of being male ($P = 0.004$), irrespective of age at clinical onset. The observed effect of sex was not modified by genetic factors, and genetic factors were not predictive.

DISCUSSION

Type 1 diabetes may occur at any age, but clinical symptoms, genetic factors, and immune phenomena vary with age at onset. To our knowledge, the present study is the first detailed analysis of age-specific associations between multiple genetic factors (HLA, INS, VNTR, and CTLA-4) and the four most commonly used autoantibody markers for type 1 diabetes. The validity of our investigation is underscored by the high ascertainment of incident patients and control subjects in the homogeneous Swedish population (18). As shown here, the data on matched control subjects and patients 0–34 years of age at clinical diagnosis (18) made it possible for us to investigate the effect of genetic factors on autoantibody markers of β-cell autoimmunity and therefore on age-dependent risk for type 1 diabetes. Taken together, our data permit a comprehensive analysis of how genetic factors are associated with autoantibody markers and contribute to the age-dependent risk of type 1 diabetes in 0- to 34-year-olds.

Our study confirms several previous findings of age dependence in immune markers among incident patients with type 1 diabetes (3). Older patients were more likely than younger patients to be negative for all four antibody markers. For example, 33% of patients aged 34 years at clinical onset were expected to be negative for all antibodies, compared with 3% of patients aged 5 years at clinical onset. Most patients who were negative for all four antibody markers were male (69%; 95% CI 59–78). The novel observation that more male than female subjects are negative for all four antibody markers may relate to the male preponderance of type 1 diabetes among young adults (1,2), particularly if the adult onset is less influenced by autoimmunity than the childhood onset form of type 1 diabetes.

Our observation that both GADAs and ICAs were more frequent in female subjects is consistent with other studies and supports the notion that autoimmunity is more common among females (for a review, see Schranz and Lernmark [3]). This is best illustrated by the estimated odds of GADA and ICA for female subjects, which were, respectively, 3.1 (95% CI 2.2–4.3) and 1.5 (95% CI 1.0–2.3) times those for male subjects. There was no evidence that the effects of any of the genetic factors were modified by sex for any of the immune markers. In contrast to the results for GADA and ICA, the estimated odds of ICA512/IA-2A for male subjects were 1.4 (95% CI 0.97–1.9) times those for female subjects. The marginally significant effect of sex on ICA512/IA-2A ($P = 0.06$) suggests that development of ICA512/IA-2A may signify a type 1 diabetes disease pro-

cess that is more common in males. Additional studies are needed to determine to what extent, if any, a possible male ICA512/IA-2A preponderance may contribute to the increased incidence of type 1 diabetes among young adult males.

Our present analysis suggests that genetic factors influence antibody status. First, the association between DQ2 and GADA shown previously (14,39) is confirmed in this group of 0- to 34-year-old incident patients with type 1 diabetes. More important, however, is the observation that the positive association between GADA and DQ2 is attenuated with age at onset suggesting that early development of GADA may be closely controlled by DQ2, DR3, or both (22). Although DQ8 heterodimers are known to bind specific GAD65 peptides (40), it is possible that disease-associated peptide binding also takes place on DQ2 or perhaps DR3 heterodimers. Again, this risk is illustrated by the odds of GADA, which are estimated to increase by a factor of 3 (95% CI 2–4) with each additional DQ2 haplotype for patients aged 5 years at clinical onset. By contrast, the odds do not differ significantly according to DQ2 for patients aged 34 years at clinical onset ($P = 0.55$). For patients carrying two HLA-DQ2 haplotypes, support for the apparent decrease of GADA prevalence with age at onset is weak ($P = 0.32$). It cannot be excluded that in GADA-negative patients with two HLA-DQ2 haplotypes, GADAs have either already disappeared or, more plausible in the older patients, appear after clinical diagnosis (41). Conversely, patients carrying no or one DQ2 haplotype have increased frequencies of GADA with increasing age, consistent with the possibility that GAD65 peptide presentations necessary for a subsequent T-cell–dependent autoantibody response can also occur on HLA class II heterodimers other than DQ2. We conclude that although there is a highly significant association between GADA and DQ2 overall, the association between type 1 diabetes and DQ2 is much stronger in younger- than in older-onset patients.

Second, we confirm previous observations (42) that DQ8 is positively associated with IAA, ICA512/IA-2A, and ICA. If we speculate that HLA associations are related to antigen presentation of the HLA class II molecule, then the association between DQ8 and ICA is complicated because the ICA reaction is likely to represent numerous antigens. The possibility that the DQ8 class II molecule is important to the disease process is illustrated by the odds of IAA and ICA512/IA-2A, which are estimated to increase by a factor of 1.5 (95% CI 1.0–2.1) and 1.4 (1.0–1.8), respectively, for each additional DQ8 haplotype. DQ8 is of potential importance to both disease initiation and disease progression because the DQ 8/8 or 8/X genotype category, where "X" is any DQ haplotype other than DQ2, DQ8, or DQ6.2, was ranked behind DQ2/8 but ahead of the DQ 2/2 or 2/X genotype category in terms of risk for type 1 diabetes (18). In contrast to DQ2/8, the relative risk of type 1 diabetes associated with the DQ8/8 or 8/X genotype category was not affected by age at clinical onset (18). Therefore, DQ8 may be important to the development of IAA and ICA512/IA-2A, even though the frequencies of these two antibody markers decrease with age at clinical onset. It should be noted that DQ8 effects may be mediated through DQ8 itself

or, especially in the case of ICA512/IA-2A, via the closely linked DR4 alleles (43).

Third, our novel observation of a negative association between DQ2 and ICA512/IA-2A is potentially important and may reflect the possibility that ICA512/IA-2 peptides are poorly presented by DQ2 class II molecules. Peptide binding analyses to DQ2 or peptide elution studies from homozygous DQ2 heterodimers will be important to test the possibility of reduced binding. The alternative hypothesis is that DQ2 is associated with profound binding and induction of immunologic tolerance that is not broken by an islet autoimmune process. The negative association seems to be multiplicative by the number of DQ2 haplotypes, with the estimated odds of ICA512/IA-2A decreasing by a factor of 0.52 (95% CI 0.38–0.70) for each DQ2 haplotype. The following illustration from our data suggests that the negative association between DQ2 and ICA512/IA-2A may be explained by the DQ2 molecules themselves. After adjusting for other variables, ICA512/IA-2A frequencies for patients carrying two DQ2 haplotypes were lower than frequencies for patients carrying no or one DQ2 haplotype. Specifically, coding as Y any HLA-DQ haplotype other than DQ2 or DQ8, the estimated odds of ICA512/IA-2A relative to those for DQY/Y patients of the same age were, in order from lowest to highest, 0.27 (95% CI 0.15–0.49), 0.52 (0.38–0.70), 0.70 (0.44–1.1), 1.3 (1.0–1.8), and 1.8 (1.0–3.3) for DQ 2/2, 2/Y, 2/8, 8/Y, and 8/8 patients, respectively. We speculate, therefore, that ICA512/IA-2A affects diabetes risk primarily in non-DQ2 subjects. Because ICA512/IA-2A has been shown to predict type 1 diabetes, most likely as a marker of ongoing β-cell destruction, we speculate that this process may be decelerated in DQ2 individuals because of a reduced immune response to ICA512/IA-2. This speculation is consistent with the observation that DQ2-DR3 patients have higher C-peptide levels at the time of clinical diagnosis (44,45).

Fourth, our novel observation that IAA but not GADA and ICA512/IA-2A were associated with the (−23) polymorphism that is in linkage disequilibrium with INS VNTR alleles suggests that the contribution of the INS VNTR may be specific to insulin as an autoantigen. The suggestion is strengthened by the observation that with each additional class I allele, the odds of IAA were estimated to increase by a factor of 1.5 (95% CI 1.0–2.2). Our observation can therefore be taken as support for the hypothesis that the association between INS VNTR and type 1 diabetes is due to variable induction of immunologic tolerance to insulin because of differences in thymic insulin gene expression (46,47). However, these authors were unable to confirm the hypothesis, possibly owing to a sample size of only 53 patients. Our analysis of IAA would be expected to have increased power to detect any effect of INS VNTR because it is based on 491 patients and takes into account the age at clinical onset and other confounding variables.

Finally, that we did not observe associations between CTLA-4 and any of the immune markers is consistent with the results of a previous study in Belgian patients (48) but not the recent finding that CTLA-4 is associated with ICA512/IA-2A in Japanese patients (49). However, the Japanese patients had an increased prevalence of autoimmune thyroid disease compared with white patients, and CTLA-4 has been shown to be associated with autoimmune thyroid disease in several ethnic populations (50,51).

In conclusion, the present analysis of HLA, INS VNTR, and CTLA-4 gene polymorphisms in relation to IAA, GADA, ICA512/IA-2A, and ICA in 0- to 34-year-old Swedish incident patients with type 1 diabetes suggests that specific associations between genetic factors and autoantibodies may affect age-dependent risks for type 1 diabetes. First, GADA were less affected by age at clinical onset in patients than the other autoantibody markers. Patients with two HLA-DQ2 haplotypes had higher odds of GADA irrespective of age, whereas patients with no or one HLA-DQ2 haplotype had reduced odds of GADA in children. Female subjects had a threefold increased odds of GADA relative to male subjects. Second, the negative association between IAA and age at clinical onset was independent of the observed increased odds of IAA in patients with an increasing number of HLA-DQ8 haplotypes and of the observed increased odds of IAA in patients with an increasing number of class I alleles of the INS VNTR. Third, ICA512/IA-2A were also negatively associated with age at clinical onset. This age effect was independent of the observed increased odds of ICA512/IA-2A in patients with an increasing number of DQ8 haplotypes and of the observed increased odds of ICA512/IA-2A in patients with a decreasing number of DQ2 haplotypes. The negative association between ICA512/IA-2A and DQ2 is adjusted for the effects of confounding variables such as the number of HLA-DQ8 haplotypes, the age at onset, and sex and therefore cannot be attributed to exclusion of DQ8. Fourth, ICAs, whose reaction is explained by an immune response to multiple autoantigens, were also negatively associated with age at clinical onset in patients. This age effect was independent of the observed increased odds of ICA in patients with an increasing number of DQ8 haplotypes and of the observed increased odds of ICA in female patients. Finally, CTLA-4 was not associated with any of the autoantibody markers and may therefore confer risk for type 1 diabetes by a mechanism that is independent of the development of autoantibody markers. The present data underscore the importance of taking age and genetic factors into account when evaluating healthy subjects for type 1 diabetes risk.

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