Associations of GAD65- and IA-2-Autoantibodies With Genetic Risk Markers in New-Onset IDDM Patients and Their Siblings

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OBJECTIVE — To investigate the association of GAD (65-kDa) autoantibodies (GAD65-Abs) and IA-2 autoantibodies (IA-2-Abs) with human leukocyte antigen (HLA)-*DQ* and insulin gene (*INS*) risk markers in patients with recent-onset IDDM and their siblings.

RESEARCH DESIGN AND METHODS — Blood was sampled from 608 recent-onset IDDM patients and 480 siblings, aged 0–39 years and consecutively recruited by the Belgian Diabetes Registry, to determine GAD65- and IA-2-Ab (radiobinding assay), HLA-*DQ*- (allele-specific oligonucleotyping), and *INS*-genotypes (restriction fragment length polymorphism analysis; siblings, $n = 439$).

RESULTS — At the onset of IDDM, GAD65-Abs were preferentially associated with two populations at genetic risk but only in the 20- to 39-year age-group: *1*) their prevalence was higher in carriers of *DQA1*0301-DQB1*0302* (88 vs. 73% in non[*DQA1*0301-DQB1*0302*], $P = 0.001$), and *2*) an association was found in patients lacking this haplotype but carrying *DQA1*0501-DQB1*0201*, together with *INS* I/I (87 vs. 54% vs. non[*INS* I/I], $P = 0.003$). Siblings of IDDM patients also presented the association of GAD65-Abs with *DQA1*0301-DQB1*0302* (13 vs. 2% non[*DQA1*0301-DQB1*0302*], $P < 0.001$), while associations with the second genetic risk group could not yet be assessed. At the onset of IDDM, IA-2-Ab prevalence was higher in carriers of *DQA1*0301-DQB1*0302* (69 vs. 39% non[*DQA1*0301-DQB1*0302*], $P < 0.001$) but not of *DQA1*0501-DQB1*0201* or *INS* I/I. This association was present in both the 0- to 19- and the 20- to 39-year age-groups. It was also found in siblings of IDDM patients (4 vs. 0% non[*DQA1*0301-DQB1*0302*], $P < 0.001$).

CONCLUSIONS — Both GAD65- and IA-2-Abs exhibit higher prevalences in presence of HLA-*DQ*- and/or *INS*-genetic risk markers. Their respective associations differ with age at clinical onset, suggesting a possible usefulness in the identification of subgroups in this heterogeneous disease.

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Abbreviations: GAD65-Ab, GAD autoantibody; HLA, human leukocyte antigen; IA-2-Ab, IA-2 autoantibody; IAA, insulin autoantibody; ICA, islet cell autoantibody; *INS*, insulin gene; PCR, polymerase chain reaction; ROC, receiver-operating characteristic; VNTR, variable number of tandem repeats.

The preclinical phase and the clinical onset of IDDM are often accompanied by the presence of autoantibodies against insulin (IAAs), the 65-kDa isoform of GAD (GAD65-Abs), the IA-2 and IA-2β protein tyrosine phosphatases (IA-2- and IA-2β-Abs), and the as-yet incompletely identified islet cell cytoplasmic antigens (ICAs) (1–7).

The systematic collection of clinical, biological, and epidemiological data in subjects recruited through diabetes registries has revealed that IDDM is a heterogeneous disease in terms of its clinical presentation and associated biological markers, thus emphasizing the need to conduct studies in representative patient groups recruited preferably through diabetes registries (8–14). Although mostly studied in children (15,16), IDDM becomes predominantly clinically overt in adulthood (2,8,10). With increasing age at onset, clinical symptoms tend to become less pronounced and the prevalence of some but not all biological markers appears to vary (8–14). The prevalence of the HLA-*DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201* high-risk genotype, IAAs, ICAs, and IA-2-Abs decreases with age (11,17,18), whereas that of GAD65-Abs increases (8). IAAs and ICAs are preferentially associated with the HLA-*DQA1*0301-DQB1*0302* risk haplotype (11). A second genetic IDDM risk marker, the I/I genotype at the variable-number-of-tandem-repeats (VNTR) locus of the insulin gene (*INS*) is not related to age and occurs preferentially in patients without the *DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201* high-risk genotype. Overall, its presence is not associated with a higher frequency of IDDM-specific autoantibodies (14).

In the present study, we investigated whether GAD65- or IA-2-Abs are associated with any of these genetic markers in patients with recent-onset IDDM and their siblings. This analysis was carried out in two age-groups (0–19 and 20–39 years), since age-dependent differences have been noticed in the biological markers and pan-

creatic histopathology around the time of clinical onset (8,11,14,18,19).

RESEARCH DESIGN AND METHODS

Subjects

IDDM patients. Between January 1989 and August 1995, 1,304 newly diagnosed IDDM patients, aged 0–39 years, were prospectively enrolled nationwide on a voluntary basis by physicians participating in the Belgian Diabetes Registry (11). Among these 1,304 patients, 608 fulfilled the following criteria: *1*) Caucasian ethnicity; *2*) availability of DNA and serum, taken at clinical onset (i.e., before start or within the first 7 days after start of insulin treatment) in sufficient amounts to assay the different genetic and immune markers; and *3*) availability of a standard questionnaire with clinical information. The patients' mean (± SD) age at clinical onset was 20 ± 10 years. In agreement with previous observations (11), the male-to-female ratio was 1.0 in the 0- to 19-year age-group and 2.2 in the 20- to 39-year age-group.

IDDM was diagnosed according to the criteria of the National Diabetes Data Group on the basis of random or fasting glucose concentrations and dependency on injected insulin at onset (20). Typical clinical symptoms such as polydipsia, polyuria, and weight loss were reported in all questionnaires. Abrupt clinical onset (<8 weeks' duration) was noted in 78% and ketonuria or ketoacidosis in 86% of patients. All patients for whom clinical follow-up information was available were still on insulin at the latest postdiagnostic timepoint (median follow-up time, 36 months; range, 8–85 months). Remission periods were noted in at least 4% of patients (median duration, 5 months; range, 2–29 months). Regardless of age, 91% of the subjects were positive for at least one type of autoantibody (IAAs, ICAs, GAD65-Abs, and IA-2-Abs) and 81% for at least two immune markers. In contrast, only 6% of the 789 nondiabetic control subjects, aged 0–39 years and recruited among blood donors, laboratory personnel, and children attending wards for minor surgery, were positive for at least one autoantibody (1.4% for IAAs, 1.3% for ICAs, 2.7% for GAD65-Abs, and 0.6% for IA-2-Abs). Only two healthy subjects (0.2%) were positive for at least two autoantibodies.

The demographic, clinical, and biological data of the present group of patients, recruited in a large region (Belgium) with incomplete ascertainment (presently 44%), were indistinguishable from those obtained in a smaller group recruited in a region (Antwerp) with almost complete ascertainment (92%) (21). The present study was approved by the ethical committees of the five participating universities. Informed consent was obtained from each subject. Patients were stratified according to age at onset between 0–19 and 20–39 years.

Siblings. Nondiabetic first-degree relatives were also registered on a voluntary basis by physicians participating in the Belgian Diabetes Registry (22). Between March 1989 and June 1995, 541 siblings (0–39 years) of newly diagnosed IDDM patients were reported. Among them, 480 fulfilled the following criteria: *1*) they were nondiabetic; *2*) they were of Caucasian ethnicity; and *3*) DNA and serum, taken as close as possible to the clinical onset of IDDM in the proband (median elapsed time, 1.5 months; range, 0–44 months), were available and in sufficient amounts to assay the different genetic (*INS*, n = 439) and immune markers. The siblings' mean (± SD) age was 15 ± 9 years. The male-to-female ratio was 0.9.

Autoantibody assays

GAD65- and IA-2-Abs were determined by radiobinding assay (18). Both antibody assays performed well in successive external quality control programs (18). The cutoff values for positivity for GAD65-Abs (≥2.6% tracer bound) and for IA-2-Abs (≥0.4% tracer bound) were established by receiver-operating characteristic (ROC) analysis (23) based on measurements in 693 patients with recent-onset IDDM and 789 healthy control subjects.

HLA-*DQ* and insulin genotyping

DNA was extracted from EDTA-blood that was stored frozen at −70°C (14). The second exons of the DQA1 and DQB1 genes were amplified by polymerase chain reaction (PCR) and hybridized to a panel of labeled allele-specific probes in dot-blot experiments (17). The latest nomenclature of the World Health Organization Nomenclature Committee for Factors of the HLA System was used (24).

Genotyping of the polymorphic VNTR locus of the insulin gene (*INS*) was performed by restriction fragment length polymorphism analysis (14). Subjects with two small-sized (class I) alleles were denoted as having the *INS* I/I risk genotype (14). Other *INS* genotypes with at least one large-sized (class III) allele (I/III or III/III) were less frequently observed in IDDM patients.

Statistical analysis

Differences in prevalences were tested by χ^2 test with Yates' correction or Fisher's Exact test, when appropriate. They were considered significant at $P < 0.05$ or, in case of k independent tests, at $P < 0.05/k$ (Bonferroni correction) (25). All statistical tests were performed by SPSS for Windows 7.0 (SPSS, Chicago, IL) for personal computers.

RESULTS

Prevalence of immune and genetic markers

IDDM patients. In agreement with previous observations (8,11,14,18), the present group of patients with recent-onset IDDM exhibited overall prevalences of GAD65-Abs (79%) and IA-2-Abs (56%) with distinct age-dependencies (increase with age for GAD65-Abs, $P < 0.05$; decrease for IA-2-Abs, $P < 0.001$) (data not shown). Its two most prevalent HLA-*DQ* haplotypes were *DQA1*0301-DQB1*0302* (58%) and *DQA1*0501-DQB1*0201* (56%). The prevalence of the heterozygous *DQA1*0301-DQB1*0302/DQA1*0501-DQB1*0201* high-risk genotype (29% overall) was also lower at a higher age of clinical onset ($P < 0.001$), whereas that of the *INS* I/I risk genotype (71% overall) did not present any age-dependency (data not shown).

Siblings. In siblings of IDDM patients, the prevalences of these markers were intermediate between those observed in patients at onset and in control subjects (8,11,14, 18), amounting to 6% for GAD65-Abs, 2% for IA-2-Abs, 12% for the heterozygous HLA-*DQ* high-risk genotype, and 65% for the *INS* I/I genotype (data not shown).

GAD65-Abs, IA-2-Abs, and HLA-*DQ* risk markers

IDDM patients. At clinical onset of IDDM, GAD65-Abs were associated with *DQA1*0301-DQB1*0302* (Table 1), but this association depended on the age at clinical onset: absent in the 0- to 19-year age-group (156/198 [79%] vs. 83/110 [76%] without), present in the 20- to 39-year age-group (134/152 [88%] vs. 108/148 [73%] without, P = 0.001). On the other hand, GAD65-Abs were also associated with *DQA1*0501-DQB1*0201* (Table 1), but this association exhibited no age-dependency (data not shown).

Table 1—*Prevalence of GAD65- and IA-2-Abs in recent-onset IDDM patients (n = 608) and siblings (n = 480) carrying HLA-DQ risk haplotypes*

Subjects	*DQA1*-DQB1**	*n*	GAD65-Abs	IA-2-Abs
IDDM patients	0301-0302	350	290 (83)	240 (69)
	Non(0301-0302)	258	191 (74)	101 (39)
	P	—	<0.006	<0.001
	0501-0201	342	279 (82)	192 (56)
	Non(0501-0201)	266	202 (76)	149 (56)
	P	—	<0.001	>0.05
Siblings	0301-0302	166	22 (13)	7 (4)
	Non(0301-0302)	314	8 (3)	0 (0)
	P	—	<0.001	<0.001
	0501-0201	215	16 (7)	4 (2)
	Non (0501-0201)	265	14 (5)	3 (1)
	P	—	>0.05	>0.05

Data are *n* (%) or *P*. To correct for multiple comparisons (Bonferroni), tests were considered significant whenever $P < 0.05/8$ or $P < 0.006$.

Like GAD65-Abs, IA-2-Abs were also associated with *DQA1*0301-DQB1*0302* (Table 1). This association was present in both age groups (0–19 years: 150/198 [76%] vs. 66/110 [60%] without, $P = 0.004$; 20–39 years: 90/152 [59%] vs. 35/148 [24%] without, $P < 0.001$). For both autoantibodies, an association with a particular genotype could not be demonstrated (Table 2).

Siblings. As in patients, GAD65- and IA-2-Abs were more prevalent in siblings carrying *DQA1*0301-DQB1*0302* (Table 1). No association was found with *DQA1*0501-DQB1*0201* (Table 1). Because of the low number of antibody positive siblings, further statistical analysis by age and HLA-*DQ*- and *INS*-genotype could not be assessed.

GAD65- and IA-2-Abs and *INS* risk markers

IDDM patients. The I/I risk genotype at the VNTR region 5′ of the insulin gene (*INS* I/I) confers susceptibility to IDDM in patients lacking the *DQA1*0301-DQB1* 0302/DQA1*0501-DQB1*0201* high-risk genotype (14). We investigated whether GAD65- and IA-2-Abs were associated with *INS* I/I in the four HLA-*DQ* genotypes studied in Table 2. In patients carrying *DQA1*0301-DQB1*0302*, GAD65- and IA-2-Abs were not associated with *INS* I/I (Table 3). On the other hand, in patients lacking *DQA1*0301-DQB1*0302*, GAD65-Abs but not IA2-Abs were associated with *INS* I/I in presence of *DQA1*0501-DQB1*0201* (Table 3). This association was only significant in patients with clinical onset after 20 years of age (Table 4).

CONCLUSIONS — IDDM is a heterogeneous disease in terms of etiologic factors, progression rates, histopathological findings, severity of clinical symptoms, and responses to therapy (2,5,9,19). Its clinical onset is associated with genetic and immune markers (1–7) for which age-dependent patterns have also been recognized (8–14,18). A more detailed knowledge of such marker associations may, in the long run, help improve the prediction of the disease, thereby contributing to preventive or therapeutic strategies (7,9, 26–33). This is especially true for the numerous patients with adult-onset IDDM who are sometimes difficult to distinguish clinically from those with NIDDM (2,8,9). Since GAD65- and IA-2-Abs, respectively, are the most sensitive IDDM markers in adult-onset IDDM patients and the most specific marker in young IDDM patients, we investigated their association with genetic (HLA-*DQ* and *INS*) risk markers in a representative registry-based population, including new-onset patients and their siblings up to 40 years of age. The upper age limit of 40 years is a compromise between recruiting as many IDDM patients as possible and minimizing problems linked to patient classification at an older age at onset (especially >40 years) (2,9,14).

Our results indicate a preferential association of GAD65-Abs with the *DQA1* 0301-DQB1*0302* risk haplotype or, in its absence, with the combination of *DQA1* 0501-DQB1*0201* and the *INS* I/I genotype at clinical onset of IDDM. These associations were significant only in the 20- to 39-year age-group. Our results help reconcile seemingly contradictory reports in the literature describing an association of GAD65-Abs only with *DQA1*0501-DQB1*0201* or *DR3* in diabetic children or adolescents on the one hand (26–28) and, on the other, an increased prevalence of GAD65-Abs in adult IDDM patients carrying *DQ2* (coded for by the *DQB1*0201* allele) and/or *DQ8* (coded for by the *DQB1*0302* allele) (34). Indeed, the present study detected an association between GAD65-Abs with *DQA1*0301-DQB1*0302* but only in adult patients. When the *INS* genotype was not taken into account, there was a significant association between GAD65-Abs and *DQA1*0501-DQB1*0201* in the 0- to 39-year age-group. Our results on the association of IA-2-Abs with *DQA1*0301-DQB1*0302*, regardless of age, are in line with previous findings (18,28).

The present report is to our knowledge the first to describe an association of GAD65-Abs with *INS* I/I in a subgroup of patients. Such an association was not disclosed for IA-2-Abs. In a previous study of our group (14), we found an interaction

Table 2—*Prevalence of GAD65- and IA-2-Abs in recent-onset IDDM patients (n = 608) according to HLA-DQ genotype*

DQA1-DQB1**	*n*	GAD65-Abs	IA-2-Abs
0301-0302/0501-0201	179	148 (83)*	122 (68)*
0301-0302/non(0501-0201)	171	142 (83)*	118 (69)*
Non(0301-0302)/0501-0201	163	131 (80)†	70 (43)
Non(0301-0302)/non(0501-0201)	95	60 (63)	31 (33)
P		<0.001	<0.001

Data are *n* (%) or *P*. Overall χ^2 tests were used to compute statistical significances of differences in autoantibody prevalence according to genotype. To correct for multiple comparisons (Bonferroni), tests were considered significant whenever $P < 0.05/2$. If an overall χ^2 test was significant ($P < 0.025$), individual χ^2 tests vs. non(0301-0302)/non(0501-0201) were performed and considered significant whenever $P < 0.05/3$ or $P < 0.017$. *$P < 0.001$; †$P < 0.005$.

Table 3—*Association of GAD65- and IA-2-Abs with the* INS *I/I genotype in recent-onset IDDM patients* (n = 608)

DQA1-DQB1**	*INS*	*n*	GAD65-Abs	IA-2-Abs
0301-0302/0501-0201	I/I	126	101 (80)	86 (68)
	Non(I/I)	53	47 (89)	36 (68)
	P	—	>0.05	>0.05
0301-0302/non(0501-0201)	I/I	131	108 (82)	84 (64)
	Non(I/I)	40	34 (85)	34 (85)
	P	—	>0.05	0.021
Non(0301-0302)/0501-0201	I/I	117	101 (86)	53 (45)
	Non(I/I)	46	30 (65)	17 (37)
	P	—	0.005	>0.05
Non(0301-0302)/non(0501-0201)	I/I	58	41 (71)	18 (31)
	Non(I/I)	37	19 (51)	13 (35)
	P	—	>0.05	>0.05

Data are *n* (%) or *P*. To correct for multiple comparisons (Bonferroni), tests were considered significant whenever $P < 0.05/8$ or $P < 0.006$.

between *INS* I/I and the absence of the highest HLA-*DQ* linked risk but no overall association between autoantibodies and *INS* I/I. The larger number of subjects in the present report allowed us to investigate further a possible association between autoantibodies and *INS* I/I in patients carrying HLA-*DQ* genotypes other than the heterozygous high-risk genotype. It was thus shown that GAD65-Abs, but not IA-2-Abs, are associated with *INS* I/I in adult patients lacking *DQA1*0301-DQB1*0302* but carrying *DQA1*0501-DQB1*0201*. However, it should be noted that GAD65-Abs also tended to be associated with *INS* I/I in the absence of both HLA-*DQ* risk haplotypes, but statistical significance was not reached, possibly due to the low number of patients lacking HLA-*DQ* risk markers ($P = 0.06$; Table 3).

As in IDDM patients at clinical onset, siblings also presented more frequently GAD65- and IA-2-Abs when carrying the *DQA1*0301-DQB1*0302* risk haplotype. This may indicate that the age-dependent associations between the different genetic and immune markers observed at clinical onset may also exist at the preclinical stage and thus help to identify subjects at high risk for developing diabetes. Our results are partly at variance with another report (3) describing an association between IA-2-Abs, but not GAD65-Abs, and *DQA1* 0301-DQB1*0302* in first-degree relatives of IDDM patients. Due to the lower prevalence of autoantibodies in siblings, compared with patients, no further (age-dependent) associations with HLA-*DQ* and *INS* genotypes could be assessed. Further longitudinal studies on a larger number of siblings are required to unravel the precise (age-dependent) relationships between these autoantibodies and HLA-*DQ*- and *INS*-linked risk.

Presently, we have no explanation for the observed age-dependent associations between genetic and immune markers. They may point to an age-dependent heterogeneity in etiologic factors. It is conceivable that genetic risk for IDDM is associated with a propensity to form autoantibodies, but that the type of autoantibodies produced tends to differ according to the genetic background and the age at onset, as a reflection of age-dependent differences in either environmental triggers, antigen expression, or the disease progression rate following early exposure to such triggers (8–11,18,35,36). Our results do not allow us to propose a mechanism for the association between insulin genotypes and GAD65-Abs. One could speculate, however, that different alleles at the VNTR locus of the insulin gene may lead to differences in the level of expression of insulin or products of neighboring genes (such as the tyrosine hydroxylase and insulin-like growth factor II gene) in the thymus or pancreas, which may, in combination with environmental agents and particular HLA-*DQ* genotypes containing *DQA1*0501-DQB1*0201*, trigger the slow autoimmune process leading to adult-onset IDDM (9,14,37,38).

Finally, it is unlikely that the lower frequency of GAD65-Abs in the older patients carrying only *DQA1*0501-DQB1*0201*, but not *DQA1*0301-DQB1*0302* or *INS* I/I (54%), is due to an increasing admixture of NIDDM patients mistakenly classified as IDDM with older age at onset. Indeed, all patients were classified according to the same clinical criteria and follow-up data confirmed their permanent insulin-dependency. Notwithstanding the detected statistical associations between genetic and immune markers, the observation that many patients without these genetic markers are still antibody positive indicates that the genetic markers studied are neither sufficient nor necessary to develop autoantibodies and IDDM.

In conclusion, both GAD65- and IA-2-Abs are increased in different subsets of IDDM patients with different age at onset and with different genetic risk as determined by HLA-*DQ* and *INS* genotyping. This may suggest that these associations might also exist at the preclinical stage, thereby identifying subgroups at high risk for the heterogeneous disease. Larger prospective studies in siblings are needed to unravel the exact (age-dependent) relationships between genetic and immune markers to refine the prediction of diabetes further.

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Table 4—*Age-dependent association of GAD65-Abs with the* INS *I/I genotype in recent-onset IDDM patients* (n = 608)

Age (years)	*DQA1*-DQB1**	*INS*	*n*	GAD65-Abs
0–19	Non(0301-0302)/0501-0201	I/I	49	42 (86)
		Non(I/I)	22	17 (77)
		P	—	>0.05
20–39	Non(0301-0302)/0501-0201	I/I	68	59 (87)
		Non(I/I)	24	13 (54)
		P	—	0.003

Data are *n* (%) or *P*. To correct for multiple comparisons (Bonferroni), tests were considered significant whenever $P < 0.05/2$ or $P < 0.025$.

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