Autoantibodies to Sympathetic Ganglia, GAD, or Tyrosine Phosphatase in Long-Term IDDM With and Without ECG-Based Cardiac Autonomic Neuropathy

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OBJECTIVE — To evaluate the association of autoantibodies to complement-fixing sympathetic ganglia (CF-SG), GAD, and tyrosine phosphatase (IA-2) with electrocardiogram (ECG)-based cardiac autonomic neuropathy (CAN) in long-term IDDM.

RESEARCH DESIGN AND METHODS — We examined the prevalence of autoantibodies to CF-SG (by complement-fixing indirect immunofluorescence), GAD, and IA-2 (by radioligand assay) and islet cells (by indirect immunofluorescence) in 96 long-term IDDM patients (41 with ECG-based CAN, ≥2 of 5 cardiac reflex tests abnormal; 55 without ECG-based CAN). As a control group, 89 healthy nondiabetic subjects were investigated.

RESULTS — CF-SG autoantibodies were observed more frequently in long-term IDDM patients than in the control group (25 vs. 4%, $P = 0.0001$). Of the IDDM patients, 14 (34%) with CAN and 10 (18%) without CAN presented with CF-SG autoantibodies ($P = 0.06$). GAD or IA-2 autoantibodies were detected in 14 (34%) and 17 (41%) IDDM patients with CAN, compared with 24 (44%) and 29 (53%) IDDM patients without CAN ($P = 0.2$, $P = 0.2$). Islet cell antibodies were observed in 6 (15%) IDDM patients with and in 9 (16%) IDDM patients without CAN ($P = 0.5$).

CONCLUSIONS — In long-term IDDM, the role of CF-SG autoantibodies, which tend to be more frequent in patients with ECG-based CAN, requires further investigations. The persistence of GAD and IA-2 autoantibodies is not related to ECG-based CAN.

At present, it seems well established that metabolic and microvascular factors are major contributors to the pathogenesis of diabetic autonomic neuropathy (1,2). Besides this notion, the association of diabetic autonomic neuropathy with iritis (3), circulating immune complexes (4), activated T-cells (5,6), and autoantibodies against nervous tissues (7–9) also suggests that autoimmune damage is involved in the pathogenesis of this diabetic complication.

However, studies investigating autoantibodies against sympathetic nervous tissue and their association with electrocardiogram (ECG)-based cardiac autonomic neuropathy (CAN) have demonstrated controversial results (9–11). GAD and tyrosine phosphatase (IA-2/ICA512) are considered to be diabetes-specific antigens that are linked to the pathogenesis of IDDM (12–17). Both antigens have been reported to be expressed in nervous tissue and islets (18,19), but association of diabetic neuropathy and GAD autoantibodies (20) was not confirmed in subsequent studies (21–23). At the second international GAD autoantibody workshop, animal tissue previously used for preparing tracer GAD65 (20–23) was demonstrated to be inferior to the use of recombinant human GAD65 (24). In a pilot study on autoantibodies against human GAD65 and diabetic autonomic neuropathy, no association was observed (25). The recently identified diabetes-specific autoantigen IA-2 (19) has not been investigated with regard to diabetic neuropathy.

Therefore, the aim of the present study was to assess CF-SG, GAD, and IA-2 autoantibodies and islet cell autoantibodies (ICAs) in long-term IDDM patients with and without well-defined ECG-based CAN.

RESEARCH DESIGN AND METHODS

Subjects

The entire study group consisted of 96 long-term IDDM patients (41 IDDM patients with and 55 without ECG-based CAN) and 89 healthy nondiabetic subjects. Diagnosis of IDDM was based on the World Health Organization (WHO) criteria (26). The clinical characteristics of the study groups are shown in Table 1. ECG-based CAN was diagnosed with five cardiac reflex tests (see below). All IDDM patients were on intensified insulin therapy, which included four or more daily subcutaneous insulin injections or insulin administration by an external pump. Patients with a history of coronary heart disease, myocardial infarction, arrhythmias, or CAN of other than diabetic origin were excluded from the study. The diabetic patients and the control subjects were taking no medication known to interfere with cardiac function, such as calcium channel-blocking agents, β-agonist drugs, digitalis, or tranquilizing agents (27).

Cardiac reflex tests

As previously published (28), the following five cardiac reflex tests were performed in diabetic patients to test for ECG-based CAN: the heart rate variation at rest and during deep breathing with calculation of coefficient

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CAN, cardiac autonomic neuropathy; CF-SG, complement-fixing sympathetic ganglia; ECG, electrocardiogram; IA-2, tyrosine phosphatase; ICA, islet cell autoantibody; 123-I-MIBG, 123-I-metaiodobenzylguanidine.

Table 1—*Clinical characteristics of study subjects*

	Long-term IDDM patients with ECG-based CAN	Long-term IDDM patients without ECG-based CAN	Control subjects	*P* value
n	41	55	89	
Sex (M/F)	22/19	33/22	42/47	NS
Age (years)	41 ± 13	35 ± 12	28 ± 5	$P < 0.05$
BMI (kg/m²)	23.8 ± 2.8	23.1 ± 1.9	21.6 ± 1.9	NS
Duration of diabetes (years)	24 ± 13	17 ± 9		$P < 0.05$
HbA_{1c} (%)	8.8 ± 1.6	8.8 ± 2.2	—	NS
Background/proliferative retinopathy	22/10	15/4	—	$P < 0.01$
Micro-/macroalbuminuria	14/94	6/2	—	$P < 0.01$

Data are means ± SD or *n*. *P* value refers to long-term IDDM patients with and without ECG-based CAN.

of variation, the immediate heart rate response to standing expressed by maximum/minimum 30:15 ratio, the Valsalva maneuver with calculation of the ratio of longest RR interval after maneuver to the shortest RR interval during maneuver, and the difference between lying and standing systolic blood pressure. Each diabetic patient was classified according to the number of age-related (29) abnormal cardiac reflex tests: less than two abnormal cardiac reflex tests equaled no ECG-based CAN, and two or more abnormal cardiac reflex tests equaled ECG-based CAN.

Assay for sympathetic ganglia autoantibodies

A previously described indirect immunofluorescence complement-fixation technique (30) was applied to test the sera for CF-SG autoantibodies. Cryostat sections of snap-frozen rabbit superior cervical sympathetic ganglia were used as substrates (7). In our assay, we used four dilutions of a positive serum and one negative control serum as a standard. Cryostat sections were tested in a blinded fashion, and intensity of fluorescence was scored by two independent observers on a scale from 0 to 3. Each serum with an immunofluorescent reaction was tested again using a cryostat section of a different ganglion.

Assay for GAD and IA-2 autoantibodies

Antibodies to GAD and IA-2 were analyzed by immunoprecipitation radioligand assays using recombinant human GAD65 and IA-2ic, as previously described (31). Radioactivity was expressed relative to an antibody-positive serum and arbitrarily assigned a value of 100 units (GAD, IA-2 index). The upper limit of normal range for GAD autoantibodies was 13 units and for IA-2 was 5 units (99th percentile of antibody levels in sera of 137 children and adults of nondiabetic parents, respectively [32]). The results of the GAD autoantibody assay in the second GAD autoantibody proficiency test were 92% consistency, 100% specificity, 94% sensitivity, and 96% validity. The assay of IA-2 autoantibodies in the first IA-2A autoantibody proficiency test achieved 100% consistency, 100% specificity, 100% sensitivity, and 100% validity.

Assay for ICAs

ICAs were detected in patient sera with indirect immunofluorescence on cryostat sections of blood group O human pancreas, as previously described (33). The results of the assay in the ninth ICA proficiency test were 78% consistency, 100% specificity, 67% sensitivity, and 78% validity.

Statistical analysis

Data are expressed as means ± SD. Differences in prevalence were compared using χ^2 test or Fisher's exact test, corrected according to the Bonferroni method. The parameter-free Spearman's rank test was used for correlation coefficients; $P < 0.05$ was considered significant.

RESULTS — A CF-SG autoantibody score of 0 or 1 was observed in 85 of 89 control subjects (95%). Because using the 95th percentile as a cutoff gave the best differentiation between control subjects and diabetic patients, a score of ≥2 was defined to represent a positive immunofluorescent reaction.

Of the entire group of 96 diabetic patients, 24 (25%) were positive for CF-SG autoantibodies, 38 (40%) were positive for GAD autoantibodies, and 46 (48%) were positive for IA-2 autoantibodies (respectively, $P < 0.001$, $P < 0.00001$, $P < 0.00001$ vs. control subjects; Table 2). Also, 15 of 44 IDDM patients with ECG-based CAN (34%) and 10 of 55 IDDM patients without ECG-based CAN (18%) were found to have CF-SG autoantibodies ($P = 0.06$). The distribution of CF-SG autoantibody scores in IDDM patients with and without ECG-based CAN and control subjects is shown in Fig. 1. Table 2 summarizes the prevalence of CF-SG, GAD, IA-2 autoantibodies, and ICAs in long-term IDDM patients with and without ECG-based CAN and in the control subjects. No association of GAD, IA-2 autoantibodies, or ICAs with ECG-based CAN was observed. IA-2 autoantibodies, but not CF-SG or GAD autoantibodies, were associated with ICAs ($P = 0.02$).

In the study group of long-term IDDM patients, the score of CF-SG autoantibodies was not related to age, sex, duration of diabetes, or HbA_{1c}. No relationship was found between GAD autoantibodies, IA-2 autoantibodies, or ICA and age, sex, duration of diabetes, or HbA_{1c}. Titers of IA-2 autoantibodies and age correlated in long-term IDDM patients ($r = -0.24$, $P = 0.02$), in that younger patients exhibited higher titers than older patients.

CONCLUSIONS — The present study investigates three autoantibodies to antigens expressed on nervous tissues (CF-SG, GAD, IA-2) and ICAs in long-term IDDM patients with regard to well-defined ECG-based CAN.

A new, remarkable finding is the high prevalence of IA-2 autoantibodies in long-term IDDM patients. This suggests persistence of IA-2 and has also been observed for GAD autoantibodies to a higher extent than for ICAs (20,21,23). The lack of association between IA-2 autoantibodies and ECG-based CAN does not suggest a pathogenetic role of these autoantibodies in diabetic autonomic neuropathy.

We confirm the lack of association between GAD autoantibodies and diabetic autonomic neuropathy (25). Kaufman et al. (20), who reported an association, gave no information on methods evaluating autonomic neuropathy in the small group of IDDM patients.

Our observations do not confirm the results reported by Zanone et al. (10), who detected CF-SG autoantibodies in 20% of 30 long-term IDDM patients with sympto-

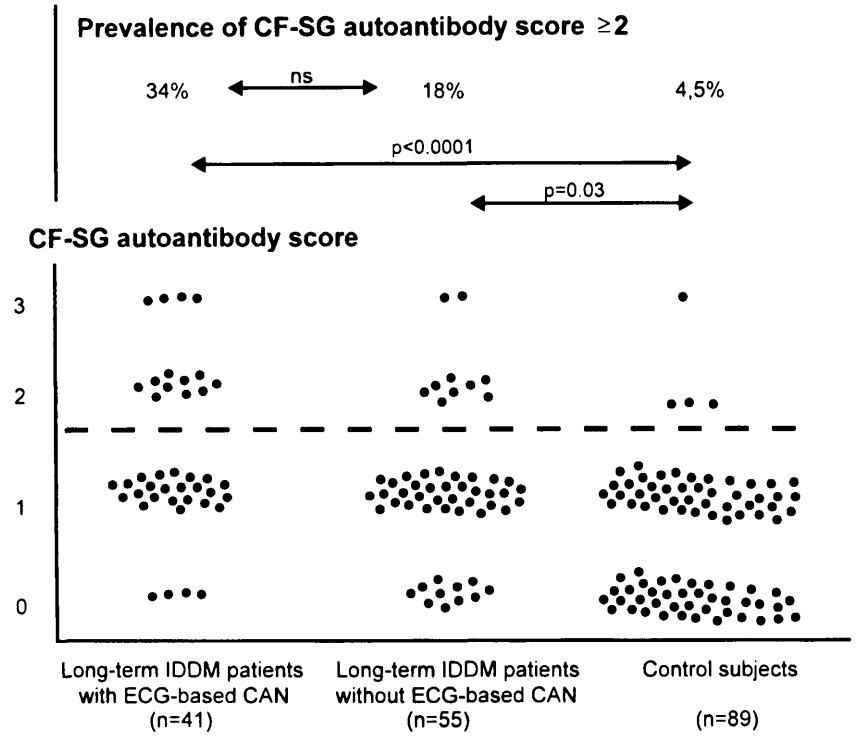

Figure 1—*Distribution of CF-SG autoantibody score and prevalence of CF-SG autoantibodies (score ≥2) in long-term IDDM patients with and without ECG-based CAN and in control subjects. Horizontal line represents the upper limit of the normal range (95th percentile of control subjects).*

matic ECG-based CAN, compared with 0% of 25 long-term IDDM patients without ECG-based CAN. In the Zanone et al. study, diagnosis of CAN was based on four cardiac reflex tests and on normal ranges not stratified for age (10). Only two cardiac reflex tests without age-related normal ranges were used for diagnosis of ECG-based CAN in a study by Sundkvist et al. (11), who reported CF-SG autoantibodies in 7% of 44 long-term IDDM patients with ECG-based CAN, compared with 37% of 30 long-term IDDM patients without ECG-based CAN. In a study on 48 long-term IDDM patients, which focused on cardiac 123-I-metaiodobenzylguanidine (MIBG) uptake and CF-SG autoantibodies, we reported an association of CF-SG autoantibodies and ECG-based CAN (30). However, in the present study on a larger group of long-term IDDM patients, the frequency of CF-SG autoantibodies tended to be higher in patients with ECG-based CAN, but did not reach statistical significance.

At present, it is unknown whether CF-SG autoantibodies precede future cardiac sympathetic dysfunction; this requires further investigation. As indicated by 123-I-MIBG scintigraphy, cardiac sympathetic denervation can frequently be observed in long-term IDDM even in the absence of ECG-based CAN (28,34–36). Therefore, it cannot be excluded that IDDM patients without ECG-based CAN and with CF-SG autoantibodies exhibit cardiac sympathetic abnormalities. In long-term IDDM patients, there is evidence for an association of CF-SG autoantibodies and scintigraphically assessed cardiac sympathetic denervation (30,37).

A limitation of the current CF-SG assay is the fact that standardization at international workshops has not become available, which may, at least in part, contribute to not univocal results of the studies (10,11).

We found that CF-SG autoantibodies and ICAs are not associated, as previously reported in IDDM patients (7,10). Furthermore, we demonstrate that CF-SG and IA-2 autoantibodies are not associated and, as found in two studies (22,25), that CF-SG and GAD autoantibodies are not related.

The results indicate different target cells for CF-SG, GAD, and IA-2 autoantibodies or for ICAs. The observation that IA-2 autoantibodies were associated with ICAs is probably explained by the common expression of their antigens in pancreatic islets.

Whereas CF-SG autoantibodies in long-term IDDM tend to be more frequent in patients with ECG-based CAN than in patients without ECG-based CAN, GAD and IA-2 autoantibodies are not related to ECG-based CAN. Further investigation is required to determine whether autoantibodies to antigens expressed in nervous tissues contribute to the initiation of ECG-based CAN in the earlier stage of IDDM.

Table 2—*Frequency of CF-SG, GAD, IA2 autoantibodies, and ICAs in long-term IDDM patients with and without ECG-based CAN and in control subjects*

	Long-term IDDM patients with ECG-based CAN	Long-term IDDM patients without ECG-based CAN	Control subjects
n	41	55	89
CF-SG autoantibodies	14 (34)*	10 (18)†	4 (4)
GAD autoantibodies	14 (34)	24 (44)	0 (0)
IA-2 autoantibodies	17 (42)	29 (53)	1 (1)
ICA	6 (15)	9 (16)	0 (0)

Data are *n* (%). *$P < 0.0001$, †$P = 0.03$ vs. control subjects.

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