

# High Frequency of Persisting or Increasing Islet-Specific Autoantibody Levels After Diagnosis of Type 1 Diabetes Presenting Before 40 Years of Age

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**OBJECTIVE** — To study the presence and levels of GAD65 antibodies (GADA), IA-2 antibodies (IA-2-A), and islet cell antibodies (ICA) during the first years after clinical onset of type 1 diabetes in relation to age at diagnosis.

**RESEARCH DESIGN AND METHODS** — Type 1 diabetic patients ( $n = 194$ ) <40 years of age were consecutively recruited at the time of diagnosis by the Belgian Diabetes Registry and followed during the first 4 years of insulin treatment. ICA were determined by indirect immunofluorescence assay and IA-2-A, GADA, and insulin autoantibodies by a radioligand assay.

**RESULTS** — Overall, 94% of initially antibody-positive patients ( $n = 180$ ) remained positive for at least 1 antibody type 4 years after diagnosis. In the case of diagnosis after 7 years of age, GADA, IA-2-A, and ICA persisted in 91, 88, and 71%, respectively, of the initially antibody-positive patients. Antibody persistence was lower in those diagnosed at <7 years of age, amounting to 60% for GADA, 71% for IA-2-A, and 39% for ICA. In 57% of the initially antibody-positive patients, at least 1 type of autoantibody reached peak values after diagnosis. This occurred more frequently for clinical onset after 7 years of age and more often for GADA (49%) than for IA-2-A (29%) or ICA (19%). Of the patients, 24% that were negative for GADA at onset became GADA-positive during the following 4 years. Among the 7% initially antibody-negative patients, 2 of 14 subjects developed antibodies after clinical onset.

**CONCLUSIONS** — In particular, for diagnosis after 7 years of age, islet cell-specific autoantibodies generally persist for many years after diagnosis. There is also a high frequency of increasing antibody levels and of conversion to antibody positivity in the first 4 years after diagnosis and start of insulin treatment. Thus, determination of antibodies at diagnosis can underestimate the number of cases with autoimmune type 1 diabetes, in particular with assays of lower sensitivity. The divergent temporal patterns of ICA, GADA, and IA-2-A suggest that the ICA test recognizes other antibody specificities besides GADA and IA-2-A and reflects other autoimmune processes; it also indicates that GADA assays have a higher diagnostic sensitivity in the period after clinical onset.

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**Abbreviations:** GADA, GAD antibodies; IAA, insulin autoantibodies; IA-2-A, IA-2 antibodies; ICA, islet cell antibodies; JDF, Juvenile Diabetes Foundation.

A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

Autoantibodies against as yet incompletely identified cytoplasmic islet cell antigens (islet cell antibodies [ICA]) or against molecularly defined antigens, such as insulin (insulin autoantibodies [IAA]), GAD65 (GAD antibodies [GADA]), or IA-2 protein (IA-2 antibodies [IA-2-A]), almost invariably accompany and precede the clinical onset of type 1 diabetes (1–5). Their presence, alone or in combination, and their levels are important for disease classification and prediction (1–6). Whether and how these antibodies relate to the underlying disease process, histopathological lesions in the pancreas (e.g., insulinitis and  $\beta$ -cell aggression or death), defense mechanisms (e.g., cellular repair and regeneration), or the effects of insulin treatment remain largely unknown (7,8). Although islet cell antibodies (ICA) have been claimed to consist mainly of IA-2-A and GADA (9), we have recently observed that ICA, at diagnosis, but not GADA or IA-2-A, are associated with a rapid decline in random C-peptide levels within the first 2 years of clinical diabetes. This suggests the presence of at least 1 additional clinically relevant component of ICA (10).

Several studies have investigated the evolution of autoantibodies during the first years of treatment in type 1 diabetic patients. Contradictory results have been obtained regarding antibody persistence, with some studies claiming rapid disappearance after diagnosis and others describing longer persistence (11–13). These conflicting results may derive from differences in the patient selection criteria (e.g., age at diagnosis) and in the type and methodology of the antibody markers used. Recent-onset type 1 diabetes displays a striking age-dependent heterogeneity in terms of clinical, biological, and histopathological findings (7,14–18). In particular, residual  $\beta$ -cells have only been found to disappear consistently after diagnosis in cases of clinical onset before 7 years of age (14). Therefore, it is indicated to conduct follow-up studies on immune markers in representative patient populations with proper age stratification and selected through diabetes registries (7).

**Table 1—Characteristics of type 1 diabetic patients diagnosed before 40 years of age**

Characteristics	Antibody positive*† at diagnosis	Antibody negative‡ at diagnosis
<i>n</i>	180	14
Men/women; <i>n/n</i> (ratio)	98/82 (1.2)	10/4 (2.5)
Age at diagnosis (years)		
Median (range)	18 (0–38)	21 (5–31)
Random C-peptide at diagnosis§ (pmol/l)		
Median (interquartile range)	175 (88–252)	128 (51–249)
Ketonuria at diagnosis; <i>n/n</i> (%)	137/169 (81)	11/14 (79)
Insulin dose (U/kg)		
Median (interquartile range)		
At diagnosis	0.55 (0.36–0.80)	0.49 (0.34–0.70)
After 2 years	0.59 (0.40–0.80)	0.44 (0.33–0.55)
Fructosamine (μmol/l)		
Median (interquartile range)		
After 2 years	391 (338–466)	405 (352–533)

Data are *n*, medians (ranges), medians (interquartile ranges), *n/n* (ratio), or *n/n* (%). \*Positive for ICA, GADA, IA-2-A, and/or IAA; †because 6 patients were exclusively IAA positive, 174 patients were positive at diagnosis for ICA, GADA, and/or IA-2-A (see Table 2); ‡negative for ICA, GADA, IA-2-A, and IAA; §reference range (percentiles 10–90) for fasting C-peptide: 300–700 pmol/l.

In the present study, diabetes-associated antibodies (ICA, IA-2-A, and GADA) were measured in type 1 diabetic patients from the Belgian Diabetes Registry during the first 4 years after diagnosis. The presence and levels of these antibodies were monitored in relation to demographic, clinical, and other biological data. In particular, we asked the question whether the time course of ICA diverged from that of IA-2-A and GADA. Such observations would provide further support to the different nature of ICA compared with currently known molecular antibodies. Moreover, we investigated the possibility that autoimmune markers could appear or increase after diagnosis; such a finding could influence the classification and management of diabetic patients. Our results indicate that increasing antibody levels or the appearance of antibodies after onset are by no means exceptional events.

## RESEARCH DESIGN AND METHODS

### Subjects

The Belgian Diabetes Registry consecutively recruited a group of 194 diabetic patients with the following characteristics: 1) Belgian residents ( $\geq 6$  months before onset) of Caucasian ethnicity; 2) clinical onset of type 1 diabetes before 40 years of age according to the criteria of the National Diabetes Data group (19); 3) insulin treatment at diagnosis and  $\geq 2$  years later; and

4) availability of blood samples at the start of insulin treatment (0–7 days on insulin) and after a follow-up time of  $\sim 1$  year (median [interquartile range] 12 months [12–13]) and 2 years (24 months [23–26]). For 173 of the 194 patients (89%), blood samples were also available 4 years (49 months [47–54]) after diagnosis. Demographic characteristics of the patients are given in Table 1 and are in agreement with findings in age-matched patient groups in previous studies (7). The study was carried out according to the Helsinki Declaration and informed consent was obtained from the participants or their parents. The protocol was approved by the ethics committees of the universities participating in the Belgian Diabetes Registry.

### Autoantibodies

All available samples were tested for ICA, IA-2-A, and GADA. ICA were determined by indirect immunofluorescence and endpoint titers expressed as Juvenile Diabetes Foundation (JDF) units (18). IA-2-A, GADA, and IAA were determined by liquid phase radiobinding assays, as previously described, and expressed as percent tracer bound (20). Strongly IA-2-A- or GADA-positive samples were diluted until the measured signal fell into the linear portion of the dilution curve. Calculated antibody levels could thus exceed 100% tracer bound in some instances. Cutoff values for antibody positivity were determined as the 99th percentile of antibody levels obtained

in 783 nondiabetic control subjects after the omission of outlying values, and amounted to  $\geq 12$  JDF U for ICA,  $\geq 0.6\%$  for IAA,  $\geq 2.6\%$  for GADA, and  $\geq 0.4\%$  for IA-2-A (21). The antibody assays performed repeatedly well in successive external quality control programs (Immunology of Diabetes Workshops, proficiency testing of the University of Florida, Gainesville, FL, and of Louisiana State University, New Orleans, LA). In the latter program, our 4 assays achieved 100% diagnostic sensitivity, specificity, consistency, and validity. In the combinatorial islet autoantibody workshop (22), assay sensitivity adjusted for 99% specificity amounted to 73% for ICA, 85% for GADA, and 36% for IAA (IA-2-A was not yet available in our lab at the time of the workshop, i.e., 1995). Over the study period, the interassay coefficient of variation was  $\leq 10.5\%$  for GADA and IA-2-A for clearly elevated levels and was  $\leq 16.0\%$  at decision levels. During the follow-up, an increase in autoantibody level was considered significant if it exceeded 2.8 times the coefficient of variation at the level of the first measurement (i.e., 45% above the cutoff for conversion to positivity and a 30% increase for already positive patients) (23). The control sera for ICA did not vary more than 1 titer step (2-fold dilution). Therefore, an increase of 2 titer steps (from 0 to 12 JDF U or a 4-fold increase in end titer for initially positive patients) was considered significant.

### Other biological parameters

C-peptide was determined with a sensitive competitive radioimmunoassay, using  $^{125}\text{I}$ -C-peptide for tracer, overnight incubation, and polyethylene glycol precipitation of antibody-bound hormone. The detection limit of the assay was 20 pmol/l (24). The fructosamine assay consisted of a colorimetric method adapted to a Cobas Mira S analyzer (Roche, Mannheim, Germany) (18). HLA *DQA1-DQB1* genotypes were determined by amplifying the second exons of the *DQA1* and *DQB1* genes from leukocytic DNA by a polymerase chain reaction and hybridizing them to a panel of labeled allele-specific probes in dot-blot experiments (25).

### Statistical analysis

Statistical differences between prevalences were assessed by means of  $\chi^2$  test, using Yates' correction or Fisher's exact test whenever appropriate. Differences in median values were determined by the Mann-Whitney *U* test for unpaired values and by the

Table 2—Evolution of autoantibody positivity in initially antibody-positive type 1 diabetic patients after clinical diagnosis

Antibody type	Initially positive patients, n/n (%)	Time since diagnosis* (months)	Prevalence of positivity; n/n (%)		
			0–6 years‡	7–39 years‡	P†
ICA	149/194 (77)	0 (0–0)	18/18 (100)	131/131 (100)	—
		12 (12–13)	10/18 (56)	119/131 (91)	<0.001
		24 (23–26)	8/18 (44)	107/131 (82)	<0.001
		49 (47–54)	7/18 (39)	82/116 (71)	<0.01
IA-2-A	112/194 (58)	0 (0–0)	14/14 (100)	98/98 (100)	—
		12 (12–13)	11/14 (79)	91/98 (93)	>0.05
		24 (23–26)	10/14 (71)	88/98 (90)	>0.05
		49 (47–54)	10/14 (71)	78/89 (88)	>0.05
GADA	148/194 (76)	0 (0–0)	15/15 (100)	133/133 (100)	—
		12 (12–13)	11/15 (73)	129/133 (97)	<0.004
		24 (23–26)	11/15 (73)	124/133 (93)	<0.03
		49 (47–54)	9/15 (60)	107/117 (91)	<0.004
ICA, IA-2-A, or GADA	174/194 (90)	0 (0–0)	18/18 (100)	156/156 (100)	—
		12 (12–13)	14/18 (78)	154/156 (99)	0.001
		24 (23–26)	15/18 (83)	151/156 (97)	<0.04
		49 (47–54)	14/18 (78)	129/137 (94)	<0.04

Data are n/n (%) or medians (interquartile ranges), unless otherwise indicated. \*Median (interquartile range); †overall  $\chi^2$  test; threshold for significance:  $P < 0.05/12$  or  $P < 0.004$  (Bonferroni adjustment). ‡Total number of patients in the different age-groups:  $n = 21$  for 0–6 years;  $n = 173$  for 7–39 years.

Friedman's test or the Wilcoxon's rank-sum test for paired values. All tests were performed 2-tailed and considered significant when  $P < 0.05$  or, in the case of k comparisons, when  $P < 0.05/k$  (Bonferroni adjustment). Multivariate analysis was performed by linear stepwise regression analysis after log transformation of autoantibody levels. All statistical tests were calculated by the SPSS for Windows 8.0 (SPSS, Chicago) statistical software package for personal computers or by Epi Info Version 6 (USD, Stone Mountain, GA).

## RESULTS

### Persistence or increase of autoantibodies during the first years after diagnosis

In the age-group 0–39 years, 93% of type 1 diabetic patients were positive for ICA, IA-2-A, GADA, or IAA at clinical onset (Table 1). ICA or GADA was more prevalent (149 of 194 patients [77%] and 148 of 194 patients [76%], respectively) than IA-2-A (112 of 194 patients [58%]) or IAA (94 of 194 patients [49%]); 174 of 194 patients (90%) were positive for ICA, IA-2-A, and/or GADA (Table 2). For each type of antibody tested, persistence of elevated levels was assessed in initially antibody-positive patients (Table 2). For diagnosis after 7 years of age, the vast majority of initially antibody-positive patients remained positive for at least 1 type of the monitored antibodies (ICA, IA-2-A,

and GADA) during the first 4 years after diagnosis (99% after 1 year, 94% after 4 years). However, in the patient group <7 years of age, antibodies, in particular ICA and GADA, disappeared more rapidly (Table 2). In these younger patients, the loss in antibody positivity was most pronounced for ICA (56 and 39% positivity was left after 1 and 4 years vs. 91 and 71% in the older age-group, respectively;  $P < 0.001$  and  $P < 0.01$ ).

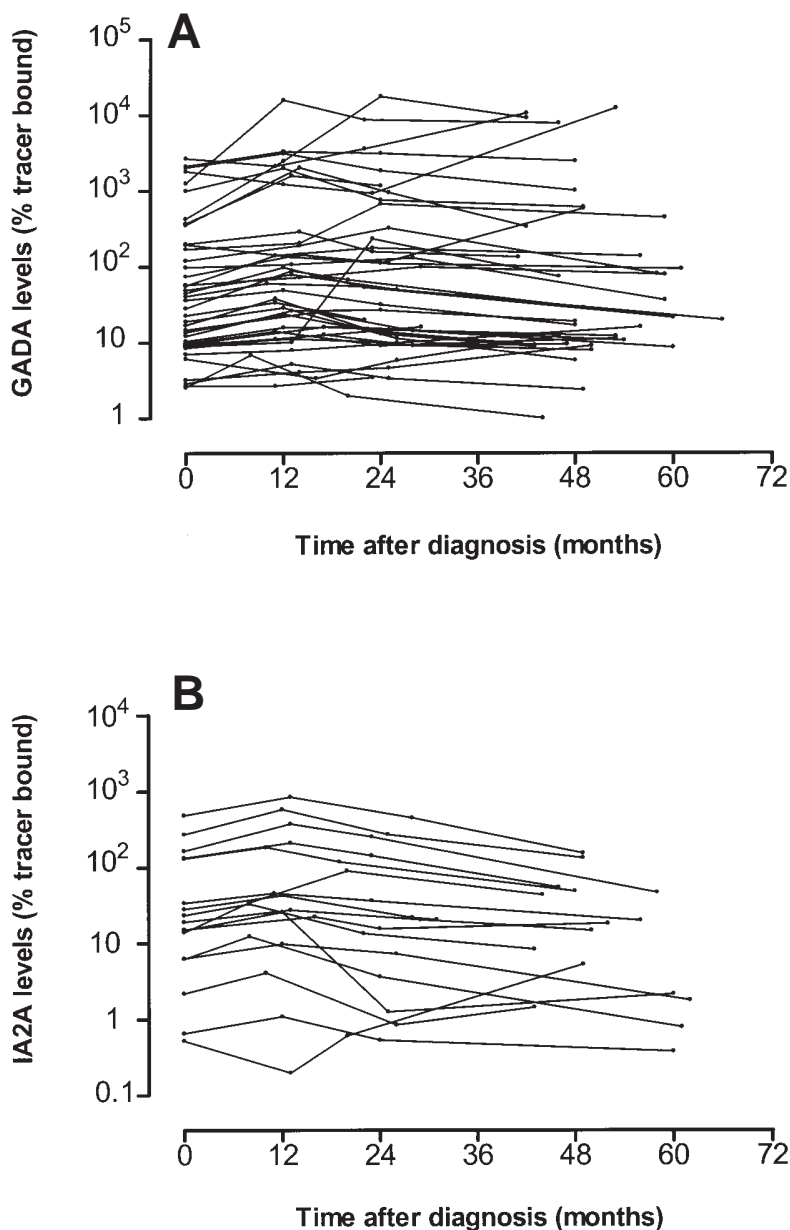
A significant number of patients initially antibody-positive for ICA, IA-2-A, or GADA (100 of 174 [57%]) reached maximal antibody levels after clinical diagnosis, in general during the first year of treatment. In more than half of these patients (58 of 100), this rise was considered significant (>30% increase versus initial antibody level for GADA and IA-2-A and at least a 4-fold increase in ICA end titer; see RESEARCH DESIGN AND METHODS). Peak values after diagnosis were observed in 28 of 149 (19%) initially ICA-positive patients (11 of 28 with significant increase), 33 of 112 (29%) in IA-2-A-positive patients (17 of 33 significant), and 72 of 148 (49%) in GADA-positive patients (41 of 72 significant). Figure 1 shows the temporal changes in GADA and IA-2-A levels for patients with significant increases ( $n = 41$  and 17, respectively). Peak values after diagnosis were more frequently observed for GADA (49%) than for IA-2-A (29%,  $P < 0.002$  by Fisher's exact test) or

ICA (19%,  $P < 0.001$ ). Its prevalence was higher with increasing age at diagnosis (2 of 15 [13%] of GADA-positive patients <7 years of age vs. 70 of 133 [53%] between 7 and 40 years of age,  $P < 0.004$  by Fisher's exact test).

Using multivariate stepwise regression analysis after log-transformation of antibody levels in initially antibody-positive patients, the circulating antibody levels 2 years after diagnosis were primarily correlated with concentrations of the same specificity at diagnosis (ICA: partial  $r = 0.37$ ,  $P < 0.001$ ; IA-2-A: partial  $r = 0.92$ ,  $P < 0.001$ ; GADA: partial  $r = 0.89$ ,  $P < 0.001$ ). In addition, 2-year ICA levels were correlated with initial levels of IA-2-A (partial  $r = 0.30$ ,  $P < 0.001$ ) and GADA (partial  $r = 0.20$ ,  $P < 0.03$ ); 2-year IA-2-A levels were inversely correlated with initial GADA levels (partial  $r = -0.30$ ,  $P < 0.005$ ) and associated with age (partial  $r = 0.27$ ,  $P < 0.009$ ) and absence of HLA *DQA1\*0501-DQB1\*0201* (partial  $r = -0.24$ ,  $P < 0.022$ ). On the other hand, 2-year GADA levels correlated with log-transformed C-peptide levels at that time point (partial  $r = 0.26$ ,  $P < 0.003$ ) and the absence of HLA *DQA1\*0301-DQB1\*0302* (partial  $r = -0.20$ ,  $P < 0.024$ ). No effect of sex was observed.

### Appearance of autoantibodies after clinical onset

The 7% of patients who were negative for ICA, IA-2-A, GADA, and IAA at clinical



**Figure 1**—Changes in antibody levels with time after diagnosis in initially positive type 1 diabetic patients with significant increases (>30% vs. initial value) in GADA levels (A [ $n = 41$ ]) or in IA-2-A levels (B [ $n = 17$ ]) after diagnosis.

onset did not differ in terms of demographic, clinical, or biological data from subjects positive for at least 1 of these antibodies (Table 1). They all had type 1 diabetes at diagnosis and had similar fructosamine levels after 2 years of insulin treatment (Table 1). Two of the 14 patients developed IA-2-A, GADA, and/or ICA during the first 2 years after diagnosis (Table 3). One subject (patient 7) developed IA-2-A and GADA during the first year after diagnosis; another sub-

ject (patient 10) developed GADA during the first year of follow-up and ICA during the second (Table 3). Conversion to antibody positivity after diagnosis was also observed in 18 other patients who tested negative at diagnosis for a given antibody type but positive for another antibody type (Table 3). Among the patients who were initially negative for ICA, IA-2-A, or GADA, subsequent conversion to positivity for that antibody also occurred within the first 24 months, except in 1

individual (patient 8), in whom conversion to ICA occurred 5.7 years after diagnosis (Table 3; see footnote). Overall, 19% of all of the patients who were initially negative for ICA, IA-2-A, and/or GADA at diagnosis (20 of 108) became positive for 1 or more antibodies during follow-up. The highest conversion rate was noted for GADA (11 of 46 [24%]) vs. 4 of 82 (5%) for IA-2-A and 8 of 45 (18%) for ICA. In all but 4 patients (3 seroconverters for GADA and 1 for ICA), this increase was considered significant (>45% increase over the cutoff value for GADA or IA-2-A or from 0 to  $\geq 12$  JDF U steps for ICA) (Table 3). Interestingly, of the 20 patients who became positive for a particular antibody in the post-diagnosis period, only 1 (<1 year old) was <7 years of age. The prevalence of seroconversion to antibody positivity did not significantly differ according to sex, HLA *DQA1-DQB1*-linked risk, or C-peptide levels after 2 years (not shown).

**CONCLUSIONS** — In agreement with previous studies (11–13), the present results indicate that overall diabetes-associated autoantibody levels decline with time after diagnosis of type 1 diabetes. However, temporal patterns of antibody levels may vary according to the type of antibody with large interindividual differences. Of the patients who were positive at diagnosis for ICA or for 1 of its alleged constituents (i.e., GADA and IA-2-A) (9), >90% remained positive for at least 1 antibody type after 4 years of follow-up. Overall positivity for GADA and IA-2-A persisted longer than for ICA. In the case of clinical onset <7 years of age, the various types of autoantibodies, ICA in particular, disappeared more rapidly than in older patients. Overall, the present results confirm and extend previous observations in children and adolescents (11–13). The long persistence of autoantibodies after clinical onset of diabetes facilitates retrospective diagnosis of autoimmune type 1 diabetes in patients who were not tested for autoantibodies at the onset of hyperglycemia.

At variance with some reports (12), but in accordance with others (13,26), large interindividual differences in the evolution of antibody levels were observed during the first years of clinical diabetes. Our results document that ~60% of initially antibody-positive patients reach a peak level after clinical onset for at least 1 of the 3 antibodies tested; these peak values were most often measured during the first year after diagnosis, but later time points were not excep-

Table 3—Seroconversion in initially autoantibody-negative type 1 diabetic patients after clinical diagnosis

Patient ID	Age (years)	Sex	Type (U)	Late seroconversion of antibodies				Antibodies at onset† (n)
				Levels				
				0 (0–0)* months	12 (12–13)* months	24 (23–26)* months	49 (47–54)* months	
1	0	M	IA-2-A (%)	<0.1	<b>14.0</b> ‡	<0.1	<0.1	1
2	7	M	GADA (%)	1.9	2.2	<b>2.9</b>	<b>4.9</b> ‡	3
3	9	F	GADA (%)	0.7	<b>6.6</b> ‡	0.8	—	2
4	12	F	GADA (%)	2.5	<b>2.6</b>	2.0	<b>4.6</b> ‡	1
5	15	F	GADA (%)	1.6	<b>2.8</b>	<b>3.1</b>	2.4	2
6	17	M	ICA (JDF U)	0	<b>12</b> ‡	<b>12</b> ‡	0	2
7	20	M	IA-2-A (%)	0.4	<b>1.2</b> ‡	<b>1.5</b> ‡	0.3	0
			GADA (%)	0.1	<b>9.2</b> ‡	1.9	0.4	
8§	21	M	ICA (JDF U)	0	0	0	0§	2
			GADA (%)	1.4	1.8	<b>2.7</b>	1.4	
9	21	M	ICA (JDF U)	0	<b>12</b> ‡	0	6	1
10	22	M	ICA (JDF U)	0	0	<b>12</b> ‡	0	0
			GADA (%)	2.0	<b>141.2</b> ‡	<b>199.2</b> ‡	<b>97.2</b> ‡	
11	25	F	GADA (%)	0.4	<b>3.1</b>	<b>5.2</b> ‡	—	1
12	26	M	GADA (%)	1.6	<b>3.6</b>	2.1	<b>2.7</b>	2
13	27	M	GADA (%)	1.7	<b>3.5</b>	<b>4.6</b> ‡	<b>3.5</b>	2
14	27	F	IA-2-A (%)	0.2	<b>0.8</b> ‡	0.2	0.1	3
15	27	F	ICA (JDF U)	0	0	<b>12</b> ‡	—	1
16	30	M	IA-2-A (%)	0.1	<b>0.6</b> ‡	0.1	0.1	2
17	31	F	ICA (JDF U)	6	6	<b>12</b>	0	1
18	33	M	ICA (JDF U)	0	0	<b>12</b> ‡	6	1
19	34	F	ICA (JDF U)	0	<b>50</b> ‡	0	0	2
20	37	M	GADA (%)	1.9	<b>2.9</b>	2.3	<b>4.6</b> ‡	2

Data are individual antibody levels. \*Time since diagnosis expressed as median (interquartile range); antibody levels in bold are above cutoff for positivity; †IAA, ICA, GADA, or IA-2-A; ‡increase ≥45% above cutoff or from 0 to ≥12 JDF U for ICA; §this patient became ICA positive (12 JDF U) after 68 months of follow-up.

tional. This phenomenon was most prominent for GADA in patients diagnosed at an older age at diagnosis. Peak levels after diagnosis were significantly less frequent for IA-2-A and ICA. Based on the long-term analytical performance of our antibody assays, ~60% of all peak values after diagnosis should be considered significant and may correspond to increased antibody production or decreased clearance. In multivariate analysis, the persistence of antibodies was primarily related to the respective levels at diagnosis, which is in agreement with previous observations for ICA (11). The additional association of persisting GADA levels with the absence of HLA *DQA1\*0301-DQB1\*0302* and residual C-peptide levels 2 years after diagnosis is indicative of a less aggressive form of the disease (27,28). Likewise, persisting IA-2-A levels were associated with older age and the absence of HLA *DQA1\*0501-DQB1\*0201*, which is consistent with previous correlations at clinical onset (20,22,29).

Of patients who were initially negative for ICA, IA-2-A, and/or GADA, 19%

became positive for at least 1 of these antibodies in the period after diagnosis and start of insulin treatment. In the vast majority of these patients, the increase was significant on the basis of long-term assay imprecision. Three patients seroconverted for 2 antibodies, further arguing against spurious assay fluctuations. Similar to the occurrence of peak antibody levels after diagnosis, late seroconversion was most often noted for GADA, mostly in patients with clinical onset after 7 years of age. We identified 2 type 1 diabetic patients who were negative for ICA, IA-2-A, GADA, and IAA at diagnosis, but who became antibody positive during the first year of clinical diabetes. Of course it cannot be excluded that some of these individuals did present autoantibodies at a stage before clinical onset of the disease. Late appearance of autoantibodies has also been noted in >15% of initially antibody-negative subjects in a group of Swedish diabetic patients, also including type 2 patients, with clinical presentation between 15 and 35 years of age and followed for 1 year after

diagnosis (30). These findings indicate that the diagnosis of autoimmune type 1 diabetes should not be excluded on the basis of a single blood sample at clinical onset, even if multiple antibodies are being tested. Taken together with the surprisingly high prevalence of autoantibodies in recent-onset diabetic patients with clinical onset <40 years of age and initially classified as type 2 diabetes (31,32), our results suggest that the incidence and prevalence of autoimmune type 1 diabetes may be underestimated in the absence of serial autoantibody determinations in adolescence- or adult-onset diabetes.

How the observed high frequency of persisting or increasing levels of diabetes-associated antibodies for clinical onset after 7 years of age relates to the underlying process remains a matter of speculation. However, the levels are consistent with the remarkably long persistence of residual β-cells, and thus of β-cell antigens, up to 30 years after clinical onset in type 1 diabetic patients diagnosed after 7 years of age (14). For diagnosis before 7 years of age,

the more rapid disappearance of autoantibodies, and of ICA in particular, after clinical onset (11,12) coincides with the complete loss of  $\beta$ -cells observed in the pancreases of young type 1 diabetic patients who died soon after diagnosis (under 7 years of age) (14) and with the rapid decline in C-peptide levels after diagnosis described in initially ICA-positive patients in that age-group (10,33). This may suggest that the presence of  $\beta$ -cell antigens drives the ongoing production of ICA. The divergent temporal patterns of ICA compared with 2 of its alleged components (GADA and IA-2-A) (9) in terms of persistence, delayed peak levels, or late appearance provide further support to the view that other as yet unknown antibody specificities must significantly contribute to ICA reactivity (10,34). Our findings cannot be ascribed to the differences in diagnostic performance of the different tests. Indeed, the cutoff levels were determined by the receiver-operating characteristic curve analysis to secure ~99% diagnostic specificity, hereby minimizing the number of false positives. Conversely, the differences in test sensitivity could not be responsible because, at diagnosis, ICA-positive patients were more numerous than IA-2-A-positive subjects in all age-groups and than GADA-positive subjects in the age-group 0–14 years. The diagnostic performance of the assays remained stable over the observation period, as reflected by long-term internal and external quality control data.

Of the type 1 diabetic patients, ~6% ( $n = 12$ ) remained consistently negative for ICA, GADA, and IA-2-A during follow-up and did not present IAA at diagnosis. These subjects may suffer from a nonautoimmune form of type 1 diabetes; alternatively, autoantibodies may have disappeared earlier, are to appear later, or display other immunoglobulin specificities or isotypes not searched for by us.

In conclusion, islet cell-specific autoantibodies tend to persist for many years after the clinical onset of type 1 diabetes. In more than half of the patients, at least 1 type of autoantibody peaks or appears after diagnosis; this occurs more often for GADA and at diagnosis after >7 years of age. Determination of autoantibodies at clinical onset underestimates the number of cases with autoimmune diabetes. Repeated determination of various types of ICAs, especially of GADA, may help objectify the diagnosis of autoimmune diabetes.

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