Contribution of Antibodies Against IA-2β and Zinc Transporter 8 to Classification of Diabetes Diagnosed Under 40 Years of Age

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OBJECTIVE—We investigated whether measuring autoantibodies against zinc transporter 8 (ZnT8A) and IA-2β (IA-2βA) may improve classification of new-onset type 1 diabetic patients based on detection of autoantibodies against insulin (IAA), GAD (GADA), and IA-2 (IA-2A). In addition, we studied the correlation of IA-2βA and ZnT8A with other biological and demographic variables.

RESEARCH DESIGN AND METHODS—Circulating autoantibodies were determined by liquid-phase radiobinding assays from 761 healthy control subjects and 655 new-onset (<1 week insulin) diabetic patients (aged 0–39 years) with clinical type 1 diabetes phenotype consecutively recruited by the Belgian Diabetes Registry.

RESULTS—At diagnosis, IA-2βA and ZnT8A prevalences were 41 and 58%, respectively. In IAA-negative, GADA-negative, and IA-2A–negative patients, one IA-2βA–positive and eleven ZnT8A-positive individuals were identified at the expense of eight and seven additional positive control subjects (1%), respectively, for each test. ZnT8A or IA-2βA screening increased ($P < 0.001$; McNemar) the number of patients with ≥2 antibodies both under (from 78 to 87% for ZnT8A and 82% for IA-2βA) and above age 15 (from 51 to 63% for ZnT8A and 56% for IA-2βA) versus 0% in control subjects. IA-2βA and ZnT8A were preferentially associated with IA-2A, and with younger age at diagnosis. Unlike ZnT8A, IA-2βA levels were positively correlated with *HLA-DQ8* and negatively with *HLA-DQ2*. ZnT8A could replace IAA for classification of patients above age 10 without loss of sensitivity or specificity.

CONCLUSIONS—ZnT8A, and to a lesser degree IA-2βA, may usefully complement GADA, IA-2A, and IAA for classifying insulin-treated diabetes under age 40 years.

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It is sometimes difficult to distinguish type 1 diabetes from other forms of the disease solely on clinical grounds—especially in adults—because of the large age-dependent heterogeneity in terms of severity of the initial clinical phenotype and the underlying insulitis and β-cell loss (1–3). The final classification of an individual as a type 1 diabetic patient relies heavily on the detection of antibodies against islet cell autoantigens (1). To this end, antibodies against insulin (IAA), the 65 kDa isoform of glutamate decarboxylase (GADA), insulinoma-associated antigen 2 (IA-2A), and as yet incompletely identified cytoplasmic antigens (ICA) have been widely used (4–8). About 10% of patients presenting with clinical features of type 1 diabetes are scored negative for these four types of antibodies, but the overrepresentation of the *HLA-DQ2/DQ8* high-risk genotype in these individuals with idiopathic type 1 diabetes suggests that at least some of them have an immune-mediated disease process (1,9). Recently, antibodies against IA-2β/phogrin (IA-2βA; a protein with 79% homology to IA-2 in the protein tyrosine phosphatase domain [10–12]) and against zinc transporter 8 (ZnT8A; an isoform largely confined to pancreatic β-cells [8,13]) have been proposed as independent immune markers of type 1 diabetes (13–15).

The aim of the current study was to measure IA-2βA and ZnT8A in a registry-based representative group of type 1 diabetic patients diagnosed under age 40 years and in healthy control subjects, with the following aims: *1*) to improve the diagnosis of immune-mediated type 1 diabetes by increasing the number of autoantibody-positive patients (higher diagnostic sensitivity) and/or the number of patients with at least two different autoantibody specificities, a condition that is extremely rare in absence of diabetes (3) (higher diagnostic specificity); *2*) to investigate associations of these additional autoantibodies with established antibody markers and with demographic (age and sex) and genetic (*HLA-DQ*) characteristics

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that have previously been correlated to some extent with differences in prevalence or levels of autoantibodies, diabetes incidence, or clinical severity of diabetes (1–4,9,16–19) to further document disease heterogeneity and patient subcategories; and 3) to search for markers that may advantageously replace IAA, an autoantibody test influenced by insulin treatment, with low sensitivity for onset after age 15 years and with generally only modestly elevated levels in case of positivity (3,7,19). These investigations are also relevant for the identification of preclinical subjects who may be enrolled in prevention studies in the future (13–15).

RESEARCH DESIGN AND METHODS

—Between 5 June 1996 and 4 July 2006, the Belgian Diabetes Registry (BDR) consecutively recruited 655 diabetic patients who fulfilled the following criteria: *1*) were diagnosed with diabetes before age 40 years according to American Diabetes Association criteria (1), *2*) were classified as type 1 diabetic patients by their treating physician on clinical grounds and treated with insulin within 7 days after diagnosis, and *3*) had blood sampled within 7 days after initiation of insulin treatment. This patient group is considered representative of the Belgian population of type 1 diabetic patients in that age category (3). The group consisted of 383 males and 272 females (male-to-female ratio: 1.4) with a median age (interquartile range) of 15 (9–26) years. The study was conducted in accordance with the guidelines in the Declaration of Helsinki as revised in 2008 (http://www.wma.net/en/30publications/10policies/b3/index.html, accessed 10 February 2011) and approved by the ethics committees of the BDR and the participating university hospitals. Blood was sampled at random, divided into aliquots, and stored at −80°C until analyzed for diabetes-associated autoantibodies and *HLA-DQ* genotype. Sex-matched nondiabetic control subjects aged 0–39 years (n = 761; median age [interquartile range], 18 [5–26]) were recruited among blood donors, laboratory personnel, and children attending wards for minor surgery, including correction of phimosis. None of the control subjects' relatives had type 1 diabetes (20).

Analytical methods

Diabetes autoantibodies were determined by liquid-phase radiobinding assay (IAA, GADA, IA-2A, IA-2βA, and ZnT8A) (15) or indirect immunofluorescence assay (ICA) (9) and *HLA-DQ* polymorphisms by allele-specific oligonucleotide genotyping (20) as described previously. cDNAs for the preparation of radioligands by in vitro transcription-translation were kind gifts of Drs. Å. Lernmark (when at University of Washington, Seattle, WA) for full length 65 kDa GAD, M. Christie (King's College School of Medicine and Dentistry, London, U.K.) for IA-2 (cytosolic domain), V. Lampasona (Instituto San Raffaele, Milan, Italy) for IA-2β (cytosolic domain; amino acids 662–1033), and J.C. Hutton (Barbara Davis Center for Childhood Diabetes, Aurora, CO) for the dimeric CW-CR ZnT8 construct incorporating the carboxyterminal cytosolic domains (aa 268–369) of both the Arg 325 (CR) and Trp 325 (CW) allelic variants. In the Diabetes Antibodies Standardization Program (DASP) 2009 workshop, diagnostic sensitivity and specificity were respectively 74 and 97% for GADA, 40 and 98% for IAA, 66 and 99% for IA-2A, 53 and 98% for IA-2βA, and 68 and 100% for ZnT8A (CW-CR). Cutoff values for antibody positivity were determined as percentile 99 of antibody levels in 761 nondiabetic control subjects and corresponded to ≥0.6% tracer binding for IAA, ≥2.6% for GADA, ≥0.44% for IA-2A, ≥0.39% for IA-2βA. As ZnT8A levels tended to slightly decrease with age in control subjects (r^2 = 0.04; $P <$ 0.001 by linear regression), cutoff values (percentile 99) were calculated separately for the age groups 0–14 years (≥1.28%) and 15–39 years (≥1.02%) for ZnT8A. Between-day coefficients of variation determined for quality control sera at low and medium levels were respectively 12 and 9% for IAA (n = 413), 10 and 9% for GADA (n = 427), 11 and 9% for IA-2A (n = 474), 15 and 10% for IA-2βA (n = 156), and 8 and 13% for ZnT8A (n = 115). Random C-peptide levels were determined on serum samples collected for analysis of diabetes-associated autoantibodies. The C-peptide assay was performed with a commercial kit (^{125}I-human C-peptide and guinea pig anti-human C-peptide serum; LINCO, St. Charles, MO; lower detection limit 20 pmol/L) (9).

Statistical analysis

Statistical differences between groups were assessed by means of the Mann-Whitney U test for two groups or the Kruskall-Wallis test for more than two groups for continuous variables and by the χ^2 test using Yates correction or Fisher exact test for categorical variables. The McNemar test was used to assess differences between paired proportions. Considering log ZnT8A or log IA-2βA as dependent variables, parameters that came out with $P < 0.1$ in univariate analysis were further tested in a multivariate stepwise forward linear regression model. All statistical tests were performed two-tailed by PASW statistics for Windows 17.0 (SPSS, Chicago, IL), by EpiInfo version 6 (USD, Stone Mountain, GA), or by GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA) and considered significant at $P <$ 0.05 or, in case of k comparisons, whenever $P < 0.05/k$ (Bonferroni correction).

RESULTS

Impact of testing for IA-2βA and ZnT8A on sensitivity and specificity of detecting immune-mediated diabetes

In the present registry-based group of recent-onset patients with type 1 diabetes phenotype diagnosed before age 40 years (n = 655), the prevalences of IAA, GADA, IA-2A, IA-2βA, and ZnT8A were respectively 51, 75, 57, 41, and 58% when using percentile 99 of control subjects as cutoff for positivity. The vast majority of patients (88%) were positive for at least one antibody marker among IAA, GADA, and IA-2A (302/317 or 95% under age 15 years; 276/338 or 82% above age 15 years). Among the 77 patients that tested negative for IAA, GADA, and IA-2A, only 1 (1%) was IA-2βA positive (also ZnT8A positive and under age 15 years). Among the control subjects, 24 individuals were positive for IAA, GADA, or IA-2A (none were double positive). Testing for IA-2βA identified 8 (1%) additional antibody-positive control subjects as expected from the definition of cutoff levels (percentile 99). The ZnT8A assay detected 11 additional antibody-positive patients (14%; not significant by McNemar test), also at the expense of an extra 7 (1%) positive control subjects. Most (8/11) solitary ZnT8A–positive patients were diagnosed after age 15 years but the prevalence of ZnT8A in absence of IAA, GADA, and IA-2A—if anything—tended to be higher in the age group 0–14 years (3/15 or 20% vs. 8/62 or 13%).

Four of the eleven patients with solitary ZnT8A positivity also tested positive for ICA, and one of these four was also IA-2βA positive. All but one carried

HLA-DQ2 and/or *DQ8*. In contrast, none of the seven ZnT8A-positive control subjects were positive for any other antibody including ICA, and overall their ZnT8A levels were significantly lower (median [interquartile range]: 1.3% [1.1–1.4] tracer binding) than in patients with solitary ZnT8A positivity (6.7% [2.2–21.3] tracer binding; $P < 0.001$).

Screening for IA-2βA or ZnT8A identified respectively 27 and 70 additional patients with ≥2 antibody types among individuals with solitary positivity for IAA, GADA, or IA-2A, whereas the control subjects remained without double positivity (Supplementary Table A1). The majority of the additional IA-2βA–positive patients were also ZnT8A positive (19/27), and ZnT8A testing increased the fraction of double antibody-positive individuals more than IA-2βA, both for onset under and above age 15 ($P < 0.001$ by McNemar test; Supplementary Table A1). Overall, the total fraction of individuals positive for ≥2 autoantibodies increased from 78% under age 15 years and 51% above age 15 years to 82 and 56%, respectively, when testing for IA-2βA in addition to IAA, GADA, and IA-2A, to 87 and 63%, respectively, when testing for ZnT8A and to 88 and 65%, respectively, when including both (McNemar test; all $P < 0.001$ vs. testing for IAA, GADA, and IA-2A only; data not shown).

Table 1—*Prevalence of IA-2βA and ZnT8A in 655 new-onset diabetic patients according to demographic data,* HLA-DQ *genotype, and conventional antibody status*

	n	Prevalence	
		IA-2βA	ZnT8A
Age at onset (years)			
0–9	170	96 (57)	109 (64)
10–19	223	105 (47)	152 (68)
20–29	149	47 (32)	76 (51)
30–39	113	21 (19)	44 (39)
Overall *P*		<0.001	<0.001
Sex			
Male	383	158 (41)	223 (58)
Female	272	111 (41)	158 (58)
P		>0.05	>0.05
HLA-DQ			
DQ2/DQ8	173	91 (53)	115 (67)
nonDQ2/DQ8	175	89 (51)	103 (59)
DQ2/nonDQ8	210	59 (28)	122 (58)
nonDQ2/nonDQ8	97	30 (31)	41 (42)
Overall *P*		<0.001	<0.001
Number of antibodies*			
0	77	1 (1)	11 (14)
1	157	27 (17)	70 (44)
2	229	97 (42)	143 (62)
3	192	144 (75)	157 (82)
Overall *P*		<0.001	<0.001
Conventional antibody status			
IAA^- and $GADA^-$ and $IA\text{-}2A^-$	77	1 (1)	11 (14)
IAA^+ only	23	2 (9)	11 (48)
$GADA^+$ only	103	4 (4)	39 (38)
$IA\text{-}2A^+$ only	31	21 (68)	20 (65)
IAA^+ and $GADA^+$	82	5 (6)	36 (44)
IAA^+ and $IA\text{-}2A^+$	35	27 (77)	25 (71)
$GADA^+$ and $IA\text{-}2A^+$	112	65 (58)	82 (73)
IAA^+ and $GADA^+$ and $IA\text{-}2A^+$	192	144 (75)	157 (82)
Overall *P*		<0.001	<0.001
All $IA\text{-}2A^+$ patients	370	257 (70)	284 (77)
All $IA\text{-}2A^-$ patients	285	12 (4)	97 (34)
P		<0.001	<0.001

Data are *n* (%) unless otherwise indicated. *IAA^+, $GADA^+$, and/or $IA\text{-}2A^+$; threshold for significance: $P < 0.05/12$ or $P < 0.004$.

Demographic and biological correlates of IA-2βA and ZnT8A positivity in new-onset patients

The prevalence of IA-2βA and ZnT8A decreased with age at diagnosis, particularly after age 20 years, but did not differ according to sex (Table 1). IA-2βA was more frequent in carriers of *HLA-DQ8* and ZnT8A in carriers of one or two *HLA-DQ* risk haplotypes. The prevalence of both IA-2βA and ZnT8A increased with the number of conventional antibodies present. In patients with the same number of conventional antibodies (either solitary positivity or two antibodies) the prevalences of IA-2βA and ZnT8A were highest when IA-2A was also present. Consequently, ZnT8A was preferentially—and IA-2βA almost exclusively—associated with IA-2A (Table 1; bottom line). These associations were observed both for onset under or above age 15 years (not shown). In an important subgroup of patients (n = 492) data on random C-peptide at diagnosis and ketonuria were available, and after stratification for age, both parameters did not vary according to ZnT8A or IA-2βA status (Supplementary Table A2). In multivariate analysis, log-transformed random C-peptide levels were only negatively correlated with the presence of ketonuria ($P < 0.001$) and positively with age ($P = 0.027$), but not significantly with *HLA-DQ* risk haplotypes, log-transformed levels of molecular autoantibodies (IAA, GADA, IA-2A, and ZnT8A), or number of antibodies present (Supplementary Table A3). In 121 patients, random C-peptide values 2 years after diagnosis were also available. Using multiple linear regression analysis, log-transformed ZnT8A or IA-2βA levels at diagnosis could not predict a rapid decrease of C-peptide within the first 2 years after onset.

In multivariate analysis, log-transformed IA-2βA and ZnT8A levels were significantly correlated, and their correlation with other antibodies was confirmed (Supplementary Table A4). This association was strongest with IA-2A, particularly in the case of IA-2βA. After adjustment for the confounding effect of age, the association of ZnT8A levels with *HLA-DQ* risk haplotypes was lost, but IA-2βA levels remained positively correlated with *HLA-DQ8* and negatively with *HLA-DQ2* (Supplementary Table A4).

Potential of ZnT8A and IA-2βA to replace IAA

When using ZnT8A instead of IAA to complement an antibody screen with GADA and IA-2A, the overall diagnostic sensitivity for positivity for ≥1 or ≥2 antibody markers remained unchanged in the age 0–39 years group (88 and 64%, respectively; not shown). For onset under age 10, the combination with IAA tended to be more sensitive than the combination with ZnT8A particularly when considering positivity for ≥2 antibodies, but after age 10 the inverse was true (Table 2). Replacing IAA by IA-2βA as a complement of GADA and IA-2A screening resulted in lower diagnostic sensitivity (not shown).

CONCLUSIONS—In line with previous reports (10–14), the current study has confirmed that ZnT8A and IA-2βA are significantly associated with clinical onset of type 1 diabetes. It has also been documented that ZnT8A could be detected in 14% of patients with a type 1 diabetes phenotype who lack IAA, GADA, and IA-2A, thereby slightly increasing the sensitivity to objectify the presence of an immune-mediated disease process. Screening for IA-2βA did not contribute further to this. In patients with solitary positivity for IAA, GADA, or IA-2A, additional testing for IA-2βA and in particular for ZnT8A increased the number of individuals positive for ≥2 autoantibodies, thus providing more certainty regarding the existence of an immune-mediated disease process in these individuals because such combined positivity had 100% diagnostic specificity for type 1 diabetes in our hands. After adjustment for age, no relation between metabolic data and antibody levels or status at diagnosis could be demonstrated. Multivariate analysis confirmed the association of IA-2βA and ZnT8A levels with those of other autoantibodies, especially IA-2A, and in case of IA-2βA also with the *HLA-DQ8* susceptibility haplotype. The antibody levels did not differ according to sex, and their univariate correlation with younger age at diagnosis disappeared after adjustment for *HLA-DQ* and antibody status. IA-2βA levels were negatively associated with *HLA-DQ2*. Our results suggest that ZnT8A but not IA-2βA may replace IAA without loss of diagnostic sensitivity or specificity for the classification of immune-mediated diabetes, but only for onset after age 10 years.

The strengths of this study are the registry-based recruitment of representative insulin-requiring new-onset diabetic patients over a large age range (3) and the use of multivariate analysis on the group of participants with a complete data set with centrally determined genetic and immune markers including a sensitive ZnT8A assay (21). We did not determine ZnT8A in patients with clinical type 2 diabetes, but others did not detect ZnT8A in such patients (13), while ZnT8A prevalence was low in noninsulin requiring patients with adult-onset autoimmune diabetes (22,23).

Our results indicate that measuring IA-2βA in addition to IAA, GADA, and IA-2A contributes little to the classification of diabetic patients because IA-2βA occurs almost exclusively together with IA-2A but at a lower prevalence (12,19,24). In contrast, additional testing for ZnT8A identified a limited number of solitary ZnT8A–positive patients, some with adult-onset and some with childhood-onset disease. The fact that several of them were also ICA positive and had on average higher ZnT8A levels than ZnT8A-positive control subjects suggests that most of these patients do not represent "statistical" positives resulting from the definition of cutoff levels (percentile 99). In line with previous findings (13), we observed that 20% of insulin-requiring children without IAA, GADA, and IA-2A tested positive for ZnT8A, but this fraction was, if anything, lower for the more numerous adult-onset patients, suggesting that classification of adult-onset diabetes may further benefit from the discovery and implementation of yet unknown additional autoantibody markers. Measurement of ZnT8A at or after clinical onset of diabetes is also useful to ascertain the classification of patients with solitary positivity for IAA, GADA, or IA-2A, as this increased the number of patients with double antibody positivity both for onset under and above age 15. The differential association of ZnT8A and IA-2βA levels with *HLA-DQ* susceptibility haplotypes (none in case of ZnT8A; positively with *DQ8* and negatively with *DQ2* for IA-2βA) suggests the existence of different subpopulations of patients (8). The 66 patients that tested negative for the five molecular autoantibodies studied should not necessarily consitute a truly antibody-negative group. Indeed, 3 of these 66 patients tested positive for ICA (12, 50, and 50 JDRF units, respectively; not shown), and several candidate autoantigens suggested in the literature were not investigated in the current study (13).

Our data suggest that in order to limit the number of antibody tests for the classification of diabetes at diagnosis the ZnT8A assay may replace IAA as a complement to GADA and IA-2A testing without loss of diagnostic sensitivity or specificity for type 1 diabetes, but only for onset after age 10. This is compatible with the rapid decrease in IAA prevalence with age at diagnosis, particularly after age 10 years (3), while ZnT8A prevalence did not decrease before age 20 years in this study. Such replacement may be beneficial because IAA results appear to be more variable than those of other islet autoantibodies. IAA levels are often only borderline elevated, and their interpretation may be flawed by interference from hemolysis or antibodies induced by insulin treatment (7,19). Therefore ZnT8A may provide a useful addition to GADA and IA-2A for retrospective classification of insulin-treated patients, although there are reports that ZnT8A may disappear

Table 2—*Prevalence of ≥1 or ≥2 diabetes autoantibodies in 655 new-onset diabetic patients and 761 control subjects according to the antibody panel tested and to age*

	Prevalence			
Antibody status	Patients			Control subjects
Age at onset (years)	0–9	10–19	20–39	0–39
n	170	223	262	761
≥ 1 autoantibody positive				
GADA, IA-2A, or IAA	164 (96)*	207 (93)*	207 (79)*	24 (3)
GADA, IA-2A, or ZnT8A	162 (95)*	209 (94)*	206 (79)*	21 (3)
≥ 2 autoantibodies positive				
GADA, IA-2A, and/or IAA	138 (81)*†	154 (69)*	129 (49)*	0 (0)
GADA, IA-2A, and/or ZnT8A	123 (72)*	162 (73)*	139 (53)*	0 (0)

Data are *n* (%) unless otherwise indicated; threshold for significance: $P < 0.05/12$ or $P < 0.004$ (Bonferroni correction). *$P < 0.001$ vs. control subjects; threshold for significance McNemar test: $P < 0.05/8$ or $P < 0.006$. †$P = 0.011$ vs. combination with ZnT8A in same age group.

relatively soon after diagnosis (24,25). Moreover, IAA methods are less amenable to harmonization than liquid-phase radiobinding assays using ^{35}S-labeled antigens produced by in vitro transcription/translation (7,19). As IAA are often the first antibodies to appear before onset, we do not recommend replacing the IAA assay by ZnT8A testing for preclinical identification of individuals at risk (2–7). Last but not least, our results on ZnT8A in recent-onset patients are in line with our observations in first degree relatives where positivity for ZnT8A was not observed in absence of IAA, GADA, and IA-2A. Detection of ZnT8A in IAA-positive or GADA-positive individuals was associated with more rapid progression to diabetes, compatible with the generally later appearance of ZnT8A in the preclinical phase (15). Altogether, our results suggest that in the perspective of enrollment in prevention studies, ZnT8A could be used as a second-line screening parameter in individuals positive for one of the conventional autoantibodies (IAA, GADA, or IA-2A).

In conclusion, ZnT8A, and to a lesser extent IA-2βA, may usefully complement IAA, GADA, and IA-2A for the classification of both childhood-onset and adult-onset diabetes. For reasons of efficiency, measurements could be limited to patients with a type 1 diabetes phenotype and solitary positivity for, or absence of other antibody markers. ZnT8A could also replace IAA without loss of diagnostic sensitivity when used in combination with GADA and IA-2A for onset after age 10 years.

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