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Analysis of pathogenesis of juvenile new-onset diabetes

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Abstract

Background—Measurement of anti-islet autoantibodies at the time of disease onset contributes greatly to the differentiation of Type 1A diabetes with HLA Class II subtyping also contributing.

Methods—Blood samples were obtained from 900 patients with age from 1 month to 25 years (median age 11.1 years) within 2 weeks of diabetes onset to test anti-islet autoantibodies to insulin (IAA), glutamic acid decarboxylase (GADA), insulinoma antigen (IA-2AA), the zinc transporter-8 (ZnT8AA), and islet-cell antibodies (ICA). Polymorphisms of the HLA Class II gene were typed in 547 randomly selected patients.

Results—Of the 900 subjects analyzed, 145 (16.1%) were negative for all five anti-islet autoantibodies, and autoantibody negativity significantly increased with age: 10.2% (38/372) among children <10 years of age, 14.2% (46/325) in those 10–14 years of age, and 30.1% (61/203) in those >14 years of age ($P < 0.001$). The prevalence of IA-2AA was the highest among young children. The prevalence of GADA increased with age while the prevalence of IAA was inversely correlated with age. At diagnosis, the subjects with negative antibodies had a higher body mass index ($P < 0.001$) and less high risk HLA genotype *DR3-DQ2/DR4-DQ8* ($P < 0.01$).

Conclusion—A large percentage of children and youths negative for all anti-islet autoantibodies at the onset of diabetes are likely to have the non-immune form, especially those without *DR3/DR4* and obese patients. Among autoantibody-positive Type 1A patients, IAA and GADA showed a reciprocal prevalence, suggesting differential disease pathogenesis.

Keywords

autoantibody; human leukocyte antigen (HLA) Class II genotype; juvenile diabetes

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Introduction

Until recently, most patients with childhood-onset diabetes have been diagnosed as having Type 1 diabetes in Western countries, with only a small proportion considered to have Type 2 or other types of diabetes, despite the increasing prevalence of Type 2 diabetes in children worldwide. It is believed that the destruction of β -cells in Type 1A diabetes is T cell mediated; however, anti-islet autoantibodies are the earliest detectable markers of autoimmunity towards β -cells. These antibodies are directed against a variety of antigens, including insulin (IAA), glutamic acid decarboxylase (GAD) 65, insulinoma antigen (IA)-2 (ICA512), and the recently defined zinc transporter ZnT8. Recent literature shows that using combinatorial analysis for antibodies, approximately 3.7–7.3% of Caucasians with newly diagnosed Type 1 diabetes were found to be islet autoantibody negative.^{1–3} However, checking these autoantibodies is the best way to differentiate autoimmune or Type 1A diabetes from the other types of juvenile diabetes in clinical practice. Because there is definitely a proportion of autoantibody-negative patients with Type 1 diabetes, HLA typing may be helpful in the classification of autoantibody-negative Type 1A diabetes.

In the present study, we analyzed all current available islet autoantibodies in different age groups from a large cohort of 900 children and young adults with newly diagnosed diabetes and evaluated the relationships of these autoantibodies with the human leukocyte antigen (HLA) genotype, body mass index (BMI) at diagnosis and the age at onset of the disease to collect evidence for differential disease pathogenesis of juvenile diabetes.

Methods

Subjects

From October 1992 to October 2004, the Barbara Davis Center cared for the majority of children with diabetes in Colorado (USA). In total, 900 (458 male and 442 female) patients with newly diagnosed diabetes were enrolled in the study. Patient ages ranged from 1 month to 25 years, with a median age of 11.1 years. Blood samples were collected from the patients within 2 weeks of disease diagnosis and insulin therapy. The samples were tested for anti-islet autoantibodies and the BMI and HbA1c were determined for most patients at the time of diagnosis. In addition, 547 patients were randomly selected and typed for *HLA DQA1*, *DQB1* and *DRB1*.

Islet autoantibody measurements

Autoantibodies to insulin (IAA), GAD65 (GADA), and IA-2 (IA-2AA) were tested in all patients as the first step in the measurement of anti-islet autoimmunity. The IA-2AA assay was performed with both ICA512bdc and IA-2ic constructs. As a second step in measurement, all patients who were negative for all three autoantibodies were tested for autoantibodies to ZnT8 (ZnT8AA) and cytoplasmic islet cell autoantibodies (ICA).

GADA and IA-2AA were measured in a combined assay, as described previously.⁴ Briefly, ³H-labeled GAD65 and ³⁵S-labeled CA512bdc were incubated with patient sera, immune complexes were precipitated with protein A–Sepharose on a 96-well filtration plate (Fisher Scientific, Pittsburgh, PA, USA), and radioactivity was counted on a TopCount 96-

well plate β -counter (PerkinElmer, Shelton, CT, USA). Levels were expressed as an index of a standard control. The interassay coefficients of variation (CV) for GADA and IA-2AA were 10% and 7.7%, respectively ($n = 35$). The assay cut-off values (0.032 for GADA; 0.049 for IA-2AA) were established as the 99th percentile of 198 healthy controls. The cut-off for IA-2AA with the alternative construct IA-2ic was 0.015. In the most recent Diabetes Autoantibody Standardization Program (DASP) workshop, the sensitivity and specificity for our assays were 72% and 99%, respectively, for GADA, 64% and 99%, respectively, for IA-2AA.

IAA was determined using a microradioassay, as described previously.⁵ Briefly, patient sera and ^{125}I -human insulin (GE HealthCare, Piscataway, NJ, USA) were incubated together with and without cold human insulin (Humulin; Eli Lilly, Indianapolis, IN, USA) and precipitated with protein A/G–Sepharose. The results are expressed as an index based on the difference between wells with and without cold insulin. The upper limit of normal with index 0.010 was the 99th percentile of 106 healthy controls. The interassay CV was 16% ($n = 35$) at low positive levels. In the most recent DASP workshop in 2009, the sensitivity and specificity for our microinsulin autoantibody (mIAA) assays were 48% and 100%, respectively.

The ZnT8Ab assay used the same format as the GAD65Ab or IA-2Ab assay. Briefly, ^{35}S -labeled ZnT8 was incubated with patient sera, followed by precipitation with protein A–Sepharose. Results are expressed as an index of a standard control and the upper limit of normal (0.040) was based on the 99th percentile of 100 healthy controls. The interassay CV was 10.4% ($n = 15$). In the most recent DASP workshop, the sensitivity and specificity for our ZnT8Ab assays were 62% and 99%, respectively.

ICA was measured by indirect immunofluorescence tissue staining on a cryostat-cut frozen section of human blood Type O pancreas in Dr William Winter's laboratory (Department of Pathology, University of Florida, Gainesville, FL, USA). Results 10 Juvenile Diabetes Foundation (JDF) units were considered positive.^{6,7}

HLA typing

In total, 547 subjects were typed for their HLA Class II alleles (*DQA*, *DQB*, and *DRB*). *DQA* and *DQB* were typed with polymerase chain reaction (PCR) amplification followed by hybridization using specific oligonucleotide probes (Applied Biosystems, Foster City, CA, USA). *DRB1* typing was performed by sequencing of the PCR-amplified exon 2 with alleles called by Matchmaker (Celera Genomics, Alameda, CA, USA).

Statistical analysis

Fisher's exact test was used for the comparison of categorical variables, whereas continuous variables were compared using the Wilcoxon rank-sum test. Pearson correlations were used to test the relationships among continuous variables. The Cochran–Amitage trend test was used to test for an age trend. Logistic regression was performed to test the relationships between antibody positivity and age, BMI, and high-risk HLA type. Statistical analyses were performed using PRISM or SAS software (GraphPad Software, San Diego, CA, USA).

Results

To evaluate autoantibody positivity in general, we tested three major anti-islet autoantibodies, including mIAA, GADA, and IA-2AA (both ICA512bdc and IA-2ic constructs), in 900 patients. As a second step, we tested conventional ICA with indirect immunofluorescence staining and for recently discovered ZnT8AA in samples negative for all three major anti-islet autoantibodies. Of the 900 patients, 145 (16.1%) were negative for all five anti-islet autoantibodies tested, equally distributed across both genders (73/458 male; 72/442 female). The percentage of autoantibody-negative patients increased significantly with age ($P < 0.001$, Cochran–Amitage trend test), especially after 14 years of age, with 30% of these patients negative for islet autoantibodies. However, overall, as illustrated in Fig. 1, a significant percentage of autoantibody-negative cases appeared in each age group (10.2% (38/372) for those <10 years; 14.2% (46/325) in those 10–14 years; and 30.1% (61/203) in those >14 years).

The autoantibody with the highest prevalence among young children aged <10 years was IA-2AA (256/372; 69%), greater than the prevalence of GADA (197/372; 53%), whereas this trend was reversed in patients aged ≥18 years (50% for IA-2AA and 63% for GADA). The prevalence of GADA increased with age (Fig. 2), whereas the prevalence of mIAA was inversely correlated with age. Positivity to IAA declined from a prevalence of 80% in those <3 of age to only 22% in those ≥18 years. Of the 177 patients negative for all three major autoantibodies, a significant number (32/177; 18.1%) was identified as anti-islet autoantibody positive by the recently developed ZnT8AA assay, whereas the conventional ICA assay detected only eight (4.5%) positive samples.

HLA-DQA, *DQB*, and *DRB* were subtyped in 547 randomly selected subjects. The highest-risk HLA genotype, namely the heterozygous allelic combination *DR3-DQ2/DR4-DQ8*, was associated with the presence of autoantibodies. Among the patients who carried this highest-risk HLA genotype, only 9.9% (12/121) were autoantibody negative, compared with 27.2% (116/426) among the other HLA Class II genotypes ($P < 0.01$). Of the subjects negative to all antibodies, only 9.4% (12/128) had the *DR3-DQ2/DR4-DQ8* heterozygous genotype, compared with 26.0% (109/419) among antibody-positive patients ($P < 0.001$). Furthermore, the rate of this highest-risk HLA genotype increased with the number of autoantibodies (11% for none; 19% for one; 30% for two; and 32% for three). In contrast, the HLA Class II protective allele *DQB1*0602* was negatively correlated with the presence of autoantibodies: only 1.6% (6/384) of autoantibody-positive individuals had this allele, compared with 12.5% (10/80) of autoantibody-negative individuals. This frequency of the *DQB1*0602* allele in antibody-negative subjects is still lower than that in the general population (approximately 20%–25%;^{8,9} see Fig. 3).

BMI data were available for 818 patients. The mean BMI of antibody-negative patients was significantly higher than that of antibody-positive patients (23.9 vs 19.2 kg/m², respectively; $P < 0.001$). Analysis of BMI Standard Deviation (SD) Scores 1 (85th percentile), 2 (93rd percentile), and 3 (96th percentile) showed that BMI at all three thresholds was inversely correlated with autoantibody positivity ($P < 0.0001$ for all three cutoffs). Nearly 21% (26/124) of autoantibody-negative patients were found to have a high BMI (>30 kg/m²)

compared with only 3.3% (25/754; $P < 0.001$) among antibody-positive patients. As shown in Fig. 4, the percentage of patients with a BMI $>30 \text{ kg/m}^2$ (more than a BMI + 2SD score) was significantly higher in autoantibody-negative patients both >14 and <14 years than autoantibody-positive patients ($P < 0.001$ for both groups). Of those with a BMI $>30 \text{ kg/m}^2$, 51% (26/51) of patients were negative for all autoantibodies tested, compared with 12.8% (98/767) of patients with a BMI $\leq 30 \text{ kg/m}^2$ ($P < 0.001$).

There was no significant difference in HbA1c between antibody-positive and antibody-negative patients (11.17% vs 11.29%, respectively; $P > 0.05$), but HbA1c at diagnosis increased with the age at onset in both the antibody-positive and antibody-negative groups (both $P < 0.01$).

Discussion

Recent reports indicate that Type 2 diabetes accounts for 8%–45% of all newly diagnosed diabetes among children and adolescents in the US, whereas before the 1990s this percentage was $<4\%$.^{1,10–12} Obesity is a strong risk factor for Type 2 diabetes in children and youth,^{2,3} and it may also increase the prevalence of Type 1 diabetes in children.¹⁰ Type 1A diabetes, the major subtype of Type 1 diabetes, is characterized by immune-mediated destruction of pancreatic β -cells, and susceptibility to this subtype is influenced both by genetics and environmental factors.

Juvenile diabetes is a cluster of different types of diabetes occurring in children and young adults. In addition to Type 1A diabetes, the second most common childhood diabetes in Western countries is Type 2 diabetes, with the others being Type 1B, maturity-onset diabetes of the young as subset of “monogenic diabetes” (MODY), secondary and endocrine forms, and some rare diabetic syndromes. Measurement of anti-islet autoantibodies contributes considerably to the differentiation of Type 1A diabetes from the other types, especially at the time of disease onset. Insulin resistance factors, such as obesity, are considered to be somewhat helpful in distinguishing Type 2 diabetes from other types.^{13,14}

In general, anti-islet autoantibodies may be expressed many years before diabetes onset¹⁵ and continue to be detectable for years after the onset of disease.¹⁶ The focus of the present study was the stage of disease onset to exclude the possibility of a decrease in autoantibody positivity over time after diagnosis. The present data show that $>16\%$ of newly diagnosed juvenile diabetic patients were negative for all anti-islet autoantibodies, $>10\%$ even in young children <10 years of age, with negativity increasing significantly with age, which is coincidentally correlated with an increased prevalence of Type 2 diabetes from early childhood to young adults. IA-2AA was shown to be the most common autoantibody during childhood, whereas the prevalence of GADA increased with age and it became the most common autoantibody in adults. In contrast, mIAA was significantly inversely correlated with age.

The difference in BMI was remarkable between antibody-negative and antibody-positive patients, with a high BMI mainly seen in older patients and those negative for antibodies. The data suggest that these antibody-negative youths with new-onset diabetes who have a

higher BMI probably have Type 2 diabetes. In Austria, of patients with newly diagnosed diabetes mellitus who were <15 years of age, 2% were diagnosed with Type 2 diabetes and, of these, 90% were <11 years of age and 45% had a BMI >30 kg/m².¹⁷ However, in North America, Type 2 diabetes seems more common in youths and there is a close linkage between Type 2 diabetes and obesity in children or adolescents.^{18,19}

The HLA Class II heterozygous genotype *DR3-DQ2/DR4-DQ8* is known to be the most susceptible gene for Type 1A diabetes.²⁰ In the present study, the antibody-negative patients had a lower probability of carrying this highest-risk genotype compared with antibody-positive patients; however, the frequency of the highest-risk genotype among antibody-negative patients (9.9%) was still greater than the frequency found in the general population (2.4%; $P < 0.001$),²¹ suggesting that a subset of these patients has Type 1A diabetes but islet autoantibodies were not detectable using currently available autoantibody screening. Autoantibody-negative patients had a higher frequency of the *DQB1*0602* allele than antibody-positive patients, but it was still lower than that in the normal control population (approximately 20% in the healthy Caucasian population).⁸ It has been considered that *DQB1*0602* has a natural protective effect against Type 1A diabetes and that it can even prevent progression to overt diabetes in ICA-positive relatives of patients with Type 1A diabetes.²² However, some new-onset patients in the present study carried the *DQB1*0602* allele and were positive for anti-islet autoantibodies; this has been observed in other studies, suggesting the protective effect associated with *DQ*0602* is not absolute.²³ From the present data, we conclude that there is a large percentage of children and youth who are anti-islet autoantibody negative at the time of diabetes onset, suggesting that a significant percentage of children, even those <10 years of age, may have a non-immune form of diabetes, with a marked increase in non-immune form of diabetes after 10 years of age. Autoantibody-negative patients without *DR3/DR4*, >10 years of age and who are obese are likely to have Type 2 diabetes. Antibody-negative children who are younger, do not have *DR3/DR4* and are not obese are likely to form a subset of patients that do not have either Type 1A or Type 2 diabetes and further examination should be undertaken in these children to exclude Type 1B or other monogenic types of diabetes. Those antibody-negative patients who carry the *DR3/DR4* allele, but lack *DQ*0602*, probably do have Type 1A diabetes but do not share the autoimmune markers detectable at present. With the discovery of new islet autoantigens and the development of new autoantibody assays, we believe more cases of Type 1A diabetes will be identified. In addition, a small percentage of antibody-positive patients in the present study carried the *DQB1*0602* allele, confirming that the protection afforded by the *DQ0602* molecule is not complete. In addition, among autoantibody-positive Type 1A children, mIAA and GADA showed a reciprocal prevalence, suggesting differential disease pathogenesis.

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References

1. Bingley PJ, Bonifacio E, Williams AJK, Genovese S, Bottazzo GF, Gale EAM. Prediction on IDDM in the general population; strategies based on combinations of autoantibody markers. *Diabetes*. 1997; 46:1701–10. [PubMed: 9356015]
2. Hagopian WA, Sanjeevi CB, Kockum I, et al. Glutamate decarboxylase-, insulin-, and islet cell-antibodies and HLA typing to detect diabetes in a general population-based study of Swedish children. *J Clin Invest*. 1995; 95:1505–11. [PubMed: 7706455]
3. Tiberti C, Buzzetti R, Anastasi E, et al. Autoantibody negative new onset Type 1 diabetic patients lacking high risk HLA alleles in a Caucasian population: are these Type 1b diabetes cases? *Diabetes Metab Res Rev*. 2000; 16:8–14. [PubMed: 10707033]
4. Yu L, Rewers M, Gianani R, et al. Antiislet auto-antibodies usually develop sequentially rather than simultaneously. *J Clin Endocrinol Metab*. 1996; 81:4264–7. [PubMed: 8954025]
5. Yu L, Robles D, Abiru N, et al. Early expression of antiinsulin autoantibodies of human and the NOD mouse: evidence for early determination of subsequent diabetes. *Proc Natl Acad Sci USA*. 2000; 97:1701–6. [PubMed: 10677521]
6. Schatz D, Krischer J, Horne G, et al. Islet cell antibodies predict insulin-dependent diabetes in United States school age children as powerfully as in unaffected relatives. *J Clin Invest*. 1994; 93:2403–7. [PubMed: 8200974]
7. Greenbaum CJ, Schatz DA, Cuthbertson D, et al. Islet cell antibody-positive relatives with human leukocyte antigen *DQA1*0102*, *DQB1*0602*: identification by the Diabetes Prevention Trial–Type 1. *J Clin Endocrinol Metab*. 2000; 85:1255–60. [PubMed: 10720072]
8. Redondo MJ, Kawasaki E, Mulgrew CL, et al. DR- and DQ-associated protection from Type 1A diabetes: comparison of *DRB1*1401* and *DQA1*0102–DQB1*0602*. *J Clin Endocrinol Metab*. 2000; 85:3793–7. [PubMed: 11061540]
9. Greenbaum CJ, Eisenbarth G, Atkinson M, et al. High frequency of abnormal glucose tolerance in *DQA1*0102/DQB1*0602* relatives identified as part of the Diabetes Prevention Trial–Type 1 Diabetes. *Diabetologia*. 2005; 48:68–74. [PubMed: 15602651]
10. Betts P, Mulligan J, Ward P, Smith B, Wilkin T. Increasing body weight predicts the earlier onset of insulin-dependant diabetes in childhood: testing the ‘accelerator hypothesis’ (2). *Diabet Med*. 2005; 22:144–51. [PubMed: 15660730]
11. The Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. Report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care*. 1997; 20:1183–97. [PubMed: 9203460]
12. Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1. Diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998; 15:539–53. [PubMed: 9686693]
13. Gahagan S, Silverstein J, Committee on Native American Child Health and Section on Endocrinology. Prevention and treatment of Type 2 diabetes mellitus in children, with special emphasis on American Indian and Alaska native children. *Pediatrics*. 2003; 112:e328–e328. [PubMed: 14523221]
14. McMahon SK, Haynes A, Ratnam N, et al. Increase in Type 2 diabetes in children and adolescents in Western Australia. *Med J Aust*. 2004; 180:459–61. [PubMed: 15115424]
15. Kukreja A, Maclaren NK. Autoimmunity and diabetes. *J Clin Endocrinol Metab*. 1999; 84:4371–8. [PubMed: 10599690]
16. Decochez K, Tits J, Coolens JL, et al. High frequency of persisting or increasing islet-specific autoantibody levels after diagnosis of Type 1 diabetes presenting before 40 years of age. The Belgian Diabetes Registry. *Diabetes Care*. 2000; 23:838–44. [PubMed: 10841006]
17. Rami B, Schober E, Nachbauer E, Austrian Diabetes Incidence Study Group. Type 2 diabetes mellitus is rare but not absent in children under 15 years of age in Austria. *Eur J Pediatr*. 2003; 162:1323–7.
18. Young TK, Dean HJ, Flett B, Wood-Steiman P. Childhood obesity in a population at high risk for Type 2 diabetes. *J Pediatr*. 2000; 136:365–9. [PubMed: 10700694]

19. Writing Group for the SEARCH for Diabetes in Youth Study Group. Incidence of diabetes in youth in the United States. *JAMA*. 2007; 297:2716–24. [PubMed: 17595272]
20. Kelly MA, Rayner ML, Mijovic CH, Barnett AH. Molecular aspects of Type 1 diabetes. *Mol Pathol*. 2003; 56:1–10. [PubMed: 12560454]
21. Rewers M, Bugawan TL, Norris JM, et al. Newborn screening for HLA markers associated with IDDM: diabetes autoimmunity study in the young (DAISY). *Diabetologia*. 1996; 39:807–12. [PubMed: 8817105]
22. Pugliese A, Gianani R, Moromisato R, et al. HLA-*DQB1*0602* is associated with dominant protection from diabetes even among islet cell antibody-positive first-degree relatives of patients with IDDM. *Diabetes*. 1995; 44:608–13. [PubMed: 7789622]
23. Rayner ML, Kelly MA, Mijovic CH, Barnett AH. Sequencing of the second exon of the MHC Class II DQ6 alleles in patients with Type 1 diabetes. *Autoimmunity*. 2002; 35:155–7. [PubMed: 12071438]

Figure 1.

The number of autoantibody (Ab)-positive patients in each age group and the number of Abs patients tested positive to (□, zero; ▨, one Ab; ■, two Abs; ▩, three Abs).

Figure 2. Prevalence of three anti-islet autoantibodies tested in each age group. (▲), microinsulin autoantibody; (■), autoantibodies to glutamic acid decarboxylase 65; (◆), ICA512AA.

Figure 3. Percentage of the human leukocyte antigen (HLA) *DR3-DQ2/DR4-DQ8* genotypes and the *DQB 0602* allele in antibody-positive (□), antibody-negative patients (▨), and in normal controls (■).

Figure 4. Percentage of patients older and younger than 14 years of age with a body mass index (BMI) >30 kg/m² who are anti-islet autoantibody negative (□) and positive (■).