

Proinsulin/Insulin Autoantibodies Measured With Electrochemiluminescent Assay Are the Earliest Indicator of Prediabetic Islet Autoimmunity

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OBJECTIVE—We evaluated a novel electrochemiluminescent assay for insulin/proinsulin autoantibodies (ECL-IAA) as a new marker of the onset of islet autoimmunity and as a predictor of type 1 diabetes.

RESEARCH DESIGN AND METHODS—The Diabetes Autoimmunity Study in the Young (DAISY) prospectively follows children at increased genetic risk for development of islet autoimmunity (defined as presence of autoantibodies to insulin, GAD65, IA-2, or zinc transporter 8 [ZnT8]) and type 1 diabetes (general population of children and first-degree relatives). Serial serum samples from subjects who progressed to type 1 diabetes and who had their first islet autoantibodies measured by age 18 months ($N = 47$) were tested using ECL-IAA.

RESULTS—Almost all prediabetic children tested positive for ECL-IAA (46 of 47, 98%) during follow-up. ECL-IAA was almost always the first autoantibody to appear (94% total; 21% very first [by itself]; 23% with only mIAA; 19% with another islet autoantibody [GAD or ZnT8]; and 30% with ≥ 2 other antibodies [mIAA, GAD, IA-2, or ZnT8]). Among the 46 subjects who were ECL-IAA positive, ECL-IAA antedated the onset of other islet autoantibodies by a mean of 2.3 years (range, 0.3–7.2 years). Both the age of appearance of autoantibody and IAA levels (but not GAD65, IA2, or ZnT8 levels) are major determinants of the age of diabetes onset.

CONCLUSIONS—This new ECL-IAA assay defines more precisely the onset of prediabetic autoimmunity and may help identify events triggering islet autoimmunity, as well as allow earlier intervention for type 1 diabetes. Nearly all young children progressing to diabetes are insulin autoantibody positive.

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Anti-islet autoimmunity currently detected by measurement of islet autoantibodies almost always precedes by years the development of type 1A diabetes. If the autoimmunity is triggered by time-correlated factors such as acute viral infections, then the discovery of pathogenic viruses may depend on accurate timing of the appearance of islet autoantibodies. Most of the trials to prevent type 1A diabetes target persons in the preclinical phase of the disease marked by the presence of persistent islet autoantibodies (1). Because this preclinical period is quite variable, accurate prediction of the

time to progression to overt diabetes is critical for the design and implementation of preventive trials. Although genetic markers can identify varying risk, it is only once autoimmunity has begun (marked by the presence of multiple autoantibodies to pancreatic β -cell antigens) that a high positive predictive value ($>90\%$) can be achieved. Multiple autoantibodies are present in the majority of prediabetic individuals (2–4). Screening for risk of type 1 diabetes uses “biochemical” autoantibody assays for specific islet autoantigens (1). These include insulin autoantibodies (IAA) (5), GAD65 (6), protein tyrosine IA-2 (ICA512) (7),

and, most recently, zinc transporter 8 (ZnT8) (8). Individuals having a single positive autoantibody (insulin, GAD65, IA-2, or ZnT8 autoantibodies) are at low risk for progression to diabetes, whereas individuals expressing two or more positive autoantibodies, especially on multiple tests over time, are at very high risk for progression to diabetes (9,10).

IAA are usually extremely high at the onset of diabetes in young children but usually negative in individuals first presenting with diabetes after age 12 years. There is a log-linear inverse relationship between these levels and the age of onset of diabetes (11), as well as between levels of IAA and time of progression from first appearance of islet autoantibodies to diagnosis of diabetes in prospectively followed Diabetes Autoimmunity Study in the Young (DAISY) children (10). We recently have reported (12) development of an electrochemiluminescence assay for IAA (ECL-IAA) using Meso Scale instrumentation and ruthenium-labeled proinsulin. This assay detects high-affinity IAA and is more sensitive than the micro-IAA (mIAA) radioassays in the last Diabetes Autoantibody Standardization Program (DASP) workshop, yet is equally specific.

In this study we evaluated ECL-IAA as a new marker of the onset of islet autoimmunity and as a predictor of progression to diabetes among antibody-positive subjects. We found that this novel nonradioactive IAA assay is more sensitive and defines the timing of the initial autoantibody appearance earlier than the previously used mIAA radioassay. We report the predictors of progression to diabetes and the determinants of age at diagnosis among children at high risk participating in the prospective DAISY.

RESEARCH DESIGN AND METHODS

Study population

Since 1993, DAISY has followed two cohorts of young children at increased

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risk for type 1 diabetes, including a cohort of relatives of type 1 diabetic patients (siblings and offspring) and the general population newborn cohort. The latter consists of children with type 1 diabetes-susceptible HLA-DR/DQ genotypes identified through screening of >31,000 newborns at St. Joseph's Hospital in Denver, Colorado. The details of screening and follow-up have been previously published. Briefly, autoantibodies were tested at 9, 15, and 24 months and annually thereafter or at their first visit and annually thereafter if the child was enrolled after birth. Children who tested autoantibody positive were given an accelerated testing schedule of every 3–6 months. A total of 47 DAISY children who had development of type 1 diabetes and had their first islet autoantibodies measured by age 18 months were included in this study. Subjects were tested for mIAA, GAD65, IA2, and ZnT8 antibodies at each visit. For ECL-IAA, all first autoantibody-positive samples, all previous samples, and yearly follow-up samples until diabetes were tested. The mean age of diabetes onset was 7.7 years (range, 0.9–14.4 years). Informed consent was obtained from the parents of each study subject. The Colorado Multiple Institutional Review Board approved all study protocols.

Islet autoantibodies

Measurement of biochemical islet autoantibodies was performed in the laboratory of Dr. George Eisenbarth at the Barbara Davis Center for Childhood Diabetes in Denver, Colorado. We used radioimmunoassays for autoantibodies to insulin, GAD₆₅, IA-2, and ZnT8 using methods previously described (1,13). The interassay coefficients of variation were 10 and 5% ($n = 50$) for GAA and IA-2A, respectively. In the 2009 DASP workshop, the sensitivity and specificity were 72 and 99%, respectively, for GAA, and 62 and 99%, respectively, for IA-2A. For ZnT8A, the interassay coefficient of variation was 10.4% ($n = 15$), and the sensitivity and specificity were 62 and 99%, respectively, in the 2009 DASP workshop. IAA were measured by a mIAA assay. The interassay coefficient of variation was 20% ($n = 100$) at low positive levels, and the sensitivity and specificity for mIAA were 48 and 100%, respectively, in the 2009 DASP workshop. The ECL-IAA using Meso Scale instrumentation and ruthenium-labeled proinsulin is a nonradioactive IAA assay in which bivalent IAA cross-link two

Table 1—Initial antibody detection in DAISY prediabetic subjects (N = 47)

Antibodies	Positive at first visit	Very first Ab alone	First Ab with ECL-IAA only	First Ab with other antibodies
ECL-IAA	44 (94)	10 (21)	11 (23)*	23 (49)
mIAA	22 (47)	0	11 (23)	11 (23)
GAD65	17 (36)	0	6 (13)	11 (23)
ZnT8	12 (26)	2 (4)	3 (6)	7 (15)
IA2	5 (11)	1 (2)	0	4 (9)

Antibodies present on the first antibody-positive visit (i.e., when a subject becomes antibody positive for the first time for one or more antibodies) are shown as n (%). Very first Ab means that this was the only positive Ab at this first visit. First Ab with ECL-IAA only gives number of subjects who are positive for ECL-IAA with only one other Ab, whereas first Ab with other antibodies shows number of subjects who are positive for other Abs at this first visit. Numbers add up horizontally; i.e., numbers in columns 3, 4, and 5 add up to numbers in column 2. Ab, antibody. *These subjects are positive for ECL-IAA and mIAA only.

“insulin” moieties in a fluid phase. The IAA in serum will link the Sulfo-tagged proinsulin to the biotinylated proinsulin, which will be captured on the solid phase of the streptavidin-coated plate. Detection of plate-captured Sulfo-tagged proinsulin is accomplished with electrochemiluminescence. IAA levels from ECL-IAA assay and our current radioassay (mIAA) were compared in 150 DASP subjects; the two assays were correlated ($P < 0.0001$), but ECL-IAA assay had higher sensitivity (68 vs. 56%) with the same specificity of 99% for both assays (12).

Statistical analysis

Statistical analyses were performed using PRISM and SAS software. The IAA, GAA, IA-2, and ZnT8 levels were log-transformed for analyses. Negative or zero values were changed to 0.001. Proportions were compared using χ^2 or Fisher exact test. Multiple linear regression was used to evaluate potential predictors of age of diabetes onset, including age at first positive antibody, initial number of positive antibodies, family

history, high-risk HLA-DR3/4, ethnicity, and IAA, GAD, IA-2, and ZnT8 levels (both initial and mean levels) in subjects who had their first autoantibody measurements before age 1.5 years and had progression to diabetes ($N = 47$). A two-tailed P value with an α level for significance was set at 0.05.

RESULTS—All 47 subjects with progression to diabetes were positive for at least one of the four biochemical antibodies (IAA, GAA, IA-2, and ZnT8). All subjects except one were positive for ECL-IAA (46 of 47, 98%) during follow-up compared with those positive for mIAA assay (42 of 47, 89%). The four prediabetic individuals who tested negative for mIAA but positive for ECL-IAA all had negative mIAA levels for multiple samples over time, although they had several positive ECL-IAA titers over time. On their first antibody-positive visit, 28% subjects had one positive antibody, whereas 43% had two, and 29% subjects had three or more positive antibodies,

Table 2—Sensitivity, specificity, and positive and negative predictive values for ECL-IAA in the DAISY cohort of children at increased risk for diabetes

Cohort	Sensitivity	Specificity	Positive predictive value	Negative predictive value
General population (with high-risk HLA genotypes for diabetes)	70.8	98.6	53.1	99.3
First-degree relatives (of patients with diabetes)	82.4	97.0	61.8	98.9

Sensitivity, specificity, and positive and negative predictive values for diabetes outcome for the new ECL-IAA assay by cohort (general population and first-degree relatives). When looking at these results, one should take into account that these values were calculated in cohorts of children at increased risk for type 1 diabetes, a cohort of relatives of type 1 diabetic patients, and a general population cohort selected for type 1 diabetes high-risk HLA-DR/DQ genotypes.

respectively. ECL-IAA was the most frequent antibody (94%) present at the first positive antibody visit, followed by mIAA (47%), GAD (36%), ZnT8 (26%), and IA2 (11%) ($P < 0.0001$). Table 1 demonstrates initial antibody detection in these 47 prediabetic DAISY subjects; ECL-IAA almost always was the first autoantibody detected: 94% (44 of 47) total; 21% alone (by itself); 23% with only mIAA; 19% with another islet autoantibody (GAD or ZnT8); and 30% with two or more other antibodies (mIAA, GAD, IA-2, or ZnT8). Among the 46 subjects who were ECL-IAA positive, the ECL-IAA detected onset of islet autoimmunity earlier than any of the four traditional biochemical antibodies in 10 (22%) children; i.e., ECL-IAA was the only positive antibody, whereas all four biochemical antibodies measured by standard radioassays were negative. For those with ECL-IAA as the first antibody by itself, ECL-IAA antedated the onset of other islet autoantibodies by a mean of 2.3 years (range, 0.3–7.2 years). Once positive, ECL-IAA titers tend to be relatively stable overall over time; i.e., most subjects found to be positive for ECL-IAA will stay positive for ECL-IAA over time, although there are individual variations.

We additionally tested for ECL-IAA in 1,812 antibody-negative DAISY subjects and 14 subjects who had development of diabetes with negative biochemical antibodies (i.e., all four biochemical antibodies negative). ECL-IAA is very specific, and only 5 of 1,812 (0.3%) antibody-negative subjects tested positive for ECL-IAA. Table 2 gives the sensitivity, specificity, and positive and negative predictive values for diabetes outcome for this new ECL-IAA assay in the DAISY cohort. When looking at these results, one should take into account that these values were calculated in cohorts of children at increased risk for type 1 diabetes, a cohort of relatives of type 1 diabetes patients, and a general population cohort selected for type 1 diabetes high-risk HLA-DR/DQ genotypes. Among 14 antibody-negative DAISY subjects with type 1 diabetes, two were positive for ECL-IAA; i.e., ECL-IAA picked up an additional 14% of cases of diabetes. We also tested ECL-IAA among remaining nondiabetic antibody-positive DAISY subjects. Among 32 DAISY subjects positive for mIAA alone, only nine (28%) were positive for ECL-IAA. However, ECL-IAA was positive in 18 of 22 (82%) DAISY subjects who tested positive for mIAA and one or more other antibodies (GAD65, IA2, or ZnT8).

As previously published, levels of mIAA, but not GAD or IA-2 autoantibodies levels, can predict age of diagnosis of type 1 diabetes (10). In this study, we confirm that higher mean ECL-IAA levels were correlated with faster progression to diabetes from first appearance of islet autoantibodies ($P = 0.02$), whereas levels of ZnT8, GAD, or IA-2 autoantibodies did

not correlate with time to progression to diabetes (Fig. 1).

Multiple linear regression was used to evaluate potential predictors of age of diabetes onset, including age at first positive antibody, initial number of positive antibodies, family history, high-risk HLA-DR3/4, ethnicity, and IAA, GAD, IA-2, and ZnT8 levels (both initial and mean

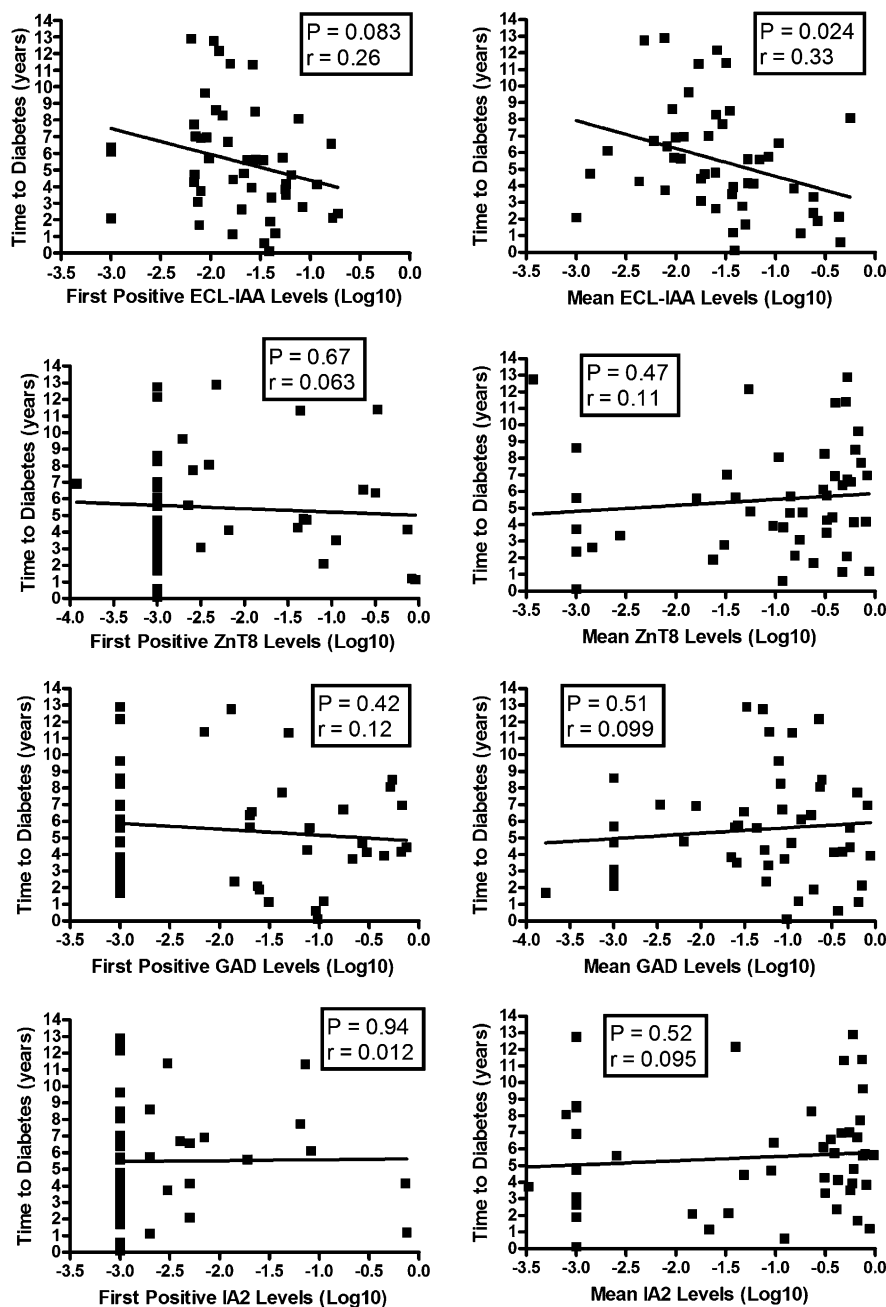


Figure 1—Age of onset of diabetes correlates with ECL-IAA levels, but not with ZnT8, GAD, or IA-2 levels. Analysis performed for all subjects who had their first antibody measurement before 1.5 years of age and who had progression to diabetes ($N = 47$). Antibody levels were \log_{10} -transformed for analyses. Negative or zero values were changed to 0.001 (which corresponds to -3 on log scale). Time to diabetes (years) is from first positive antibody visit (any of the 5 antibodies).

antibody levels). For the first model including initial antibody levels, only initial mIAA levels and age at first positive antibody were significant predictors of diabetes onset age (Fig. 2A). When analyzing the mean levels of autoantibodies over time for each subject, mean ECL-IAA levels and age of the first positive antibody were significant predictors of age of diabetes diagnosis (Fig. 2B).

CONCLUSIONS—In the prospective DAISY cohort, ECL-IAA antedated the onset of islet autoimmunity by a mean of 2.3 years in the 22% children initially positive only for ECL-IAA. Results from this study indicate that this novel ECL-IAA assay not only is more sensitive but also defines the timing of the initial autoantibody appearance earlier than the previously used mIAA radioassay. This earlier detection in timing of onset of islet autoimmunity is of importance in finding potential environmental causes of diabetes and in our understanding of the etiology of type 1 diabetes. However, assays obviating the use of radioactive isotopes also would help to disseminate islet autoantibody testing possibly to point of care. Finally, this novel nonradioactive IAA assay seems to be more disease specific; ECL-IAA is typically positive when two or more autoantibodies are present (i.e., subjects at high risk for development of diabetes), whereas the majority of mIAA-positive-only (single antibody-positive) nondiabetic subjects are ECL-IAA negative and these IAA are demonstrated as low-affinity antibodies. We speculate that the difference between these two assays is antibody affinity binding.

These factors are critical in moving from prevention trials in first-degree relatives to the general population, in which 90% of type 1 diabetes cases are found.

Several studies now have shown that once multiple autoantibodies appear (two or more autoantibodies), almost all these young children have progression to diabetes, although some progression will take >10 years (10,14,15). In children expressing two or more autoantibodies, this progression to diabetes is not influenced by family history, and HLA-DR3/4-DQB1*0302 genotype is only an additional predictor of progression to diabetes in children expressing one or two autoantibodies but not among patients expressing three autoantibodies (10). However, factors influencing rate of progression to diabetes are still largely unknown. In the TrialNet study, a Diabetes Prevention Trial–Type 1 Risk Score threshold of 9.00 identifies individuals who are very likely to have progression to diabetes within 2 years (16). However, the Diabetes Prevention Trial–Type 1 Risk Score includes C-peptide and glucose indexes from oral glucose tolerance testing. In the DAISY study, we previously have shown that age of first positive antibody and mean mIAA levels (but not GAD65 or IA-2) are major determinants of age of onset of diabetes (10). In this study, we confirm that ECL-IAA levels (but not ZnT8) are major determinants of age of onset of diabetes. The unique association of age of diagnosis with levels of IAA perhaps is not unexpected given accumulating evidence that targeting of insulin may be a primary determinant of diabetes of

the spontaneous NOD mouse model (17) and the association of polymorphisms of the insulin gene with diabetes risk in humans. For instance, knocking out endogenous insulin genes and mutating a single amino acid of insulin prevents all diabetes of NOD mice (18,19), whereas knocking out either GAD65 or IA-2 has no influence on progression to diabetes of NOD mice (20,21). The mechanism underlying the specific association of levels of IAA with rate of progression to diabetes is not defined. Autoantibodies or the B cells that produce them may play a pathogenic role with evidence from the NOD mouse model (22) and human trials of rituximab (23). Alternatively, insulin autoantibody levels simply may reflect activity or number of CD4 T cells targeting insulin.

This new nonradioactive ECL-IAA assay can define earlier onset of prediabetic autoimmunity and thus may help identify time-dependent events triggering islet autoimmunity. Nearly all young children with progression to diabetes are insulin autoantibody positive, and understanding the underlying mechanism of elevated insulin antibodies may contribute to design of future therapies.

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L.Y. researched data, contributed to discussion, and reviewed and edited the manuscript.

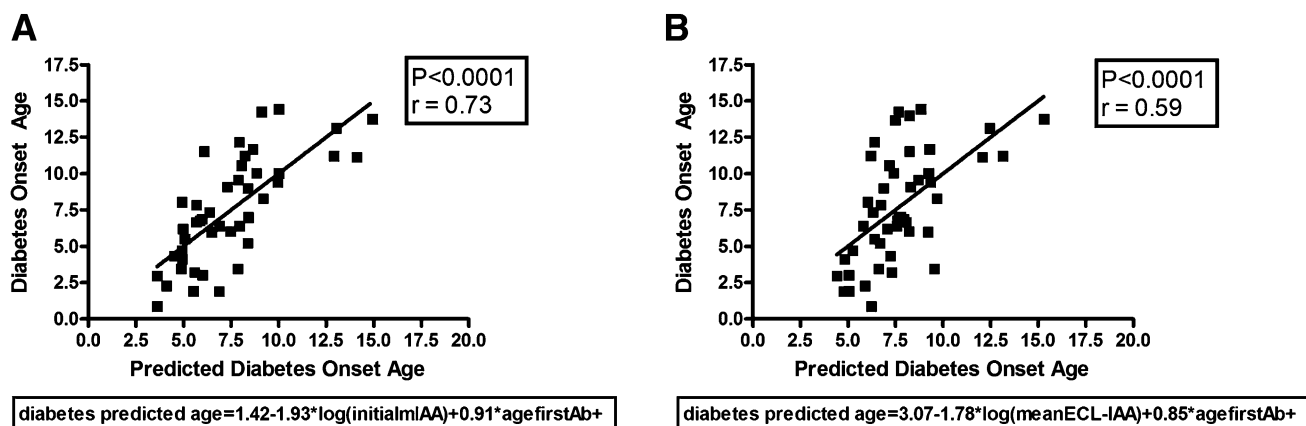


Figure 2—Predicted age of onset of diabetes. Multiple linear regression was used to evaluate potential predictors of age of diabetes onset, including age at first positive antibody (Ab), initial number of positive antibodies, family history, high-risk HLA-DR3/4, ethnicity, and IAA, GAD, IA-2, and ZnT8 levels (both initial and mean antibody levels). For the first model including initial antibody levels, only initial mIAA levels ($\log(\text{initial mIAA})$) and age at first positive antibody (agefirstAb^+) were significant predictors of diabetes onset age (A). When analyzing mean antibody levels (B), diabetes onset age was best predicted by mean ECL-IAA levels ($\log(\text{mean ECL-IAA})$) and age at first positive antibody (agefirstAb^+).

F.D. and J.M.W. researched data and reviewed and edited the manuscript. D.M. and A.R.F. researched data. A.K.S. wrote the manuscript and contributed to discussion. A.K.S. is the guarantor of this work and, as such, had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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