GAD65 Autoantibodies Detected By Electrochemiluminescence (ECL) Assay Identify High Risk For Type 1 Diabetes

Dongmei Miao, K. Michelle Guyer, Fran Dong, Ling Jiang, Andrea K. Steck, Marian Rewers, George S. Eisenbarth*, and Liping Yu

Barbara Davis Center for Childhood Diabetes,
University of Colorado Denver, Aurora, CO 80045

* Deceased

Corresponding Author: Liping Yu, MD
Barbara Davis Center for Childhood Diabetes
University of Colorado Denver
1775 Aurora Ct, B-140
Aurora, CO 80045
Tel: 303-724-6808
Fax: 303-724-5811
E-mail: Liping.yu@ucdenver.edu

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Abstract

Identification of diabetes relevant islet autoantibodies is essential for prediction and prevention of type 1 diabetes (T1D). The present study aimed to evaluate a newly developed ECL-GADA assay and compare its sensitivity and disease relevance with standard radioassay. The assay was validated using serum samples from 227 newly diagnosed diabetic children, 68 pre-diabetic children who were prospectively followed to T1D, 130 non-diabetic children longitudinally followed for 12±3.7 years with confirmed islet autoantibodies to insulin, GAD65, IA2 and/or ZnT8, and 181 age-matched healthy antibody negative children. ECL-GADA had a similar sensitivity as the standard GADA radioassay in children newly diagnosed with T1D, pre-diabetic children, and high risk children with multiple positive islet autoantibodies. On the other hand, only 9 out of 39 non-diabetic children with only a single islet autoantibody (GADA only) by radioassay were positive for ECL-GADA. GADA not detectable by ECL assay were shown to be of low-affinity and likely not predictive of future diabetes. In conclusion, new ECL-based assay identifies disease relevant GADA by radioassay. It may help to improve prediction and correct diagnosis of T1D among subjects positive only for GADA and no other islet autoantibodies.

Key words: autoantibodies, assay, diabetes
Introduction

Islet autoantibodies play an essential role in prediction of type 1 diabetes (T1D) (1-3). These autoantibodies can appear as early as at 6-9 months of life and usually precede clinical diabetes by years. Accurate detection of islet autoantibodies is crucial for finding the environmental factors that may trigger islet autoimmunity and promote progression to T1D.

In addition, islet autoantibodies are used extensively to stage diabetes risk and as the inclusion criteria for trials to prevent T1D. The risk of developing T1D is strongly associated with the number of islet autoantibodies among both first degree relatives of patients with T1D and general population subjects. Children who have developed two or more persistent islet autoantibodies are at high risk - 70% of them will progress to diabetes in less than 10 years (4). In contrast, children with a single positive persistent autoantibody are at a much lower risk – less than 10%, by 10 years of follow-up. Further stratification of these single autoantibodies for disease relevance by using more specific assays would greatly enhance staging of diabetes risk for clinical trials.

We have recently developed an electrochemiluminescent (ECL) IAA assay (5,6), shown to be more sensitive and more diabetes-specific than the standard micro-IAA (mIAA) radioassay. Here, we evaluated a similar ECL-GADA assay and compared its sensitivity and disease relevance among GADA positive by the standard GADA radioassay.

Material and Methods

Subjects: From the children newly diagnosed with T1D at Barbara Davis Center for Childhood Diabetes and studied within 2 weeks, we randomly selected 177 diabetic subjects aged 0.9 to 17.8 years (median 9.2 yr). Age-matched healthy children (n=181) were selected as
controls. From the Diabetes Autoimmunity Study in the Young (DAISY) study, we studied all 68 children who were prospectively followed up to 16.7 years (median 7.1 yr) to clinical diabetes and all participants (n=130) with persistent autoantibodies to insulin, GAD65, IA-2, and/or ZnT8. In addition, blindly coded Diabetes Autoantibody Standardization Program (DASP) samples from 50 new onset patients and 100 healthy controls were analyzed. Signed written informed consents were obtained from participants and the study was approved by the Institutional Review Board of the University of Colorado.

_ECL-GADA assay:_ The format of ECL-GADA assay was adapted from ECL-IAA assay (5), but without acid treatment of serum. The serum samples were diluted 5 times with PBS buffer and mixed at 1:1 ratio with sulfo-tag labeled GAD65 protein (Diamyd, Pittsburgh, PA) at the concentration of 32ng/ml and biotin labeled GAD65 protein at the concentration of 1000ng/ml in PBS containing 5% BSA for overnight incubation at 4 °C. On the same day, the streptavidin coated plate (MSD, Gaithersburg, MD) was blocked with 3% of Block A (MSD, Gaithersburg, MD) overnight at 4 °C. On the 2nd day, the plate was washed 3 times with PBST buffer (PBS buffer with 0.05% Twin-20) and 30 µl of overnight incubates were added per well. After 1 hour of incubation at room temperature on a plate shaker at low speed setting, the plate was washed again 3 times with PBST buffer followed by adding 150 µl of 2x reader buffer and then counted on a MSD counter, Imager 2400 (MSD, Gaithersburg, MD). The results were expressed as an index against our internal standard positive control of GAD65 monoclonal antibody (Abcam, Cambridge, MA). The assay cut-off index of 0.023 was set at the 99.5th percentile of 181 healthy controls and the inter-assay CVs are 8.8% (n=10) for the sample with index value around 1.0 and 14.6% for the sample near the index 99.5th percentile cut-off.
**GADA radioassay**: GADA radioassay was performed with NIDDK harmonized standard methods (7) and the upper limit of normal (20 DK units/ml) was established as the 98th percentile from receiver operating characteristic curves in 500 healthy control subjects and 50 patients with new onset diabetes. The inter-assay CV for the sample with index value around 1.0 was 8.3% (n=20).

**GADA competition assay**: The assay format is identical to GADA radioassay. Each serum was run in 6 separated wells, one without competition and five with competition by adding unlabeled GAD65 in 5 different concentrations, respectively, from $4.6 \times 10^{-7}$ to $4.6 \times 10^{-11}$ [M] into the incubation of serum with labeled GAD65.

**Statistics**: Statistical analyses were performed using correlation analysis, rank sum or Fisher’s exact test with PRISM 4.0 version software (GraphPad Software Inc., San Diego, CA). A two-tailed p-value with an alpha level for significance was set at 0.05.
RESULTS

Similar sensitivity of the ECL-GADA assay and the standard GADA radioassay

Among 177 newly diagnosed diabetic children and 68 pre-diabetic children (total N=245), 187/245 (76%) of the patients were GADA positive using the new ECL-GADA assay compared with 175/245 (71%) positive in the GADA radioassay.

Figure 1 shows the comparison of GADA positivity and levels between the ECL-GADA assay and GADA radioassay. With the specificity set at the 99.5\textsuperscript{th} percentile of 181 healthy controls for ECL-GADA and 98.0\textsuperscript{th} percentile of 500 healthy controls for GADA radioassay, the sensitivity of the ECL-GADA -75\% (133/177) was similar to that for the GADA radioassay - 67\% (119/177) in newly diagnosed children (panel A). The sensitivity was also similar for ECL-GADA - 79\% (54/68) and GADA radioassay - 82\% (56/68) in pre-diabetic children (panel B). The levels of GADA correlated between two assays (R\superscript{2}=0.607, p<0.0001 among the new onset patients; and R\superscript{2}=0.599, p<0.0001 among the pre-diabetic subjects). There were a few discordant samples at low levels of GADA. In the blinded set of 50 DASP patients (panel C), ECL-GADA assay was 76\% sensitive vs. 66\% for the GADA radioassay, with the same specificity of 99\% among 100 healthy controls for both assays. GADA levels from both assays correlated (R\superscript{2}=0.354, p<0.0001) in the DASP set as well.

Discrimination of disease relevant GADA

In the DAISY study, 130 non-diabetic children were persistently islet autoantibody positive for IAA, GADA, IA-2A and/or ZnT8A without yet progressing to diabetes. Of these, 47 were multiple autoantibody positive and 83 single autoantibody positive including 39 single GADA positive children. The single- and multiple autoantibody positive children did not differ
by age (mean age, respectively, 13.7 ±4.0 vs. 14.5±4.3 years) or by the follow-up duration (12.3±3.7 vs.12.4±3.8 years). Of 39 children with a single GADA autoantibody on multiple visits with years of follow up, only 9 children were ECL-GADA positive (Figure 2). In contrast, almost all children positive for two or more autoantibodies showed congruent results for GADA radio- and ECL assay (41/43; p<0.0001) (Figure 3). In addition, 48/130 non-diabetic children were GADA negative by radioassay, but persistently positive for other islet autoantibodies, and only 1/44 children with a single autoantibody vs. 3/4 with multiple autoantibodies (p<0.001) were found to be positive for ECL-GADA.

**Autoantibodies detected by the ECL-GADA are of high affinity**

A total of 14 GADA positive samples by radioassay were randomly selected for the serial competition assay, 7 positive by ECL-GADA assay (6 multiple autoantibodies and 1 single GADA) and 7 negative by ECL-GADA assay (all 7 single GADA). Radioassay GADA levels were comparable between ECL-GADA positive and ECL-GADA negative (median 126 DK units, range 105-269 vs median 125 DK units, range 94 to 235 DK units, p=0.58). The results of the competition assay are illustrated in figure 4. Parallel to the finding from ECL-IAA (5), the GADA not detectable by ECL assay required a 10 to 100 fold higher concentration of unlabeled GAD65 protein (10⁻⁹ to 10⁻¹⁰ [M]) for 50% inhibition of binding of GADA to labeled GAD65 protein for 7 samples negative by ECL-GADA assay than for 7 samples (10⁻¹¹ [M]) positive by ECL-GADA assay.

**DISCUSSION**
This paper reports a major improvement in the diabetes relevant GADA measurement using a new ECL-based assay among GADA positive by the standard NIDDK harmonized GADA radioassay. The assay specificity of GADA radioassay (98.0th percentile) was based on much larger number of controls, slightly below ECL-GADA assay (99.5th percentiles), and both assays were shown identical specificity in DASP workshop. The present study is consistent with our previous finding (5) that an ECL-IAA assay was able to detect disease-relevant, high-affinity autoantibodies while ignoring low-affinity signal that did not predict development of multiple islet autoantibodies and progression to diabetes. This advance is likely to have important implication for population screening programs, cohort studies searching for the environmental triggers, and clinical trials to prevent T1D. It will also be helpful, in the near future, to apply these new ECL assays to those adolescent and adult patients currently diagnosed with LADA, “hybrid”, or undetermined type diabetes for improving clinical diagnosis.

In DAISY, TrialNet Pathway to Prevention, TEDDY, and other major programs 30-60% of autoantibody positivity is due to a single islet autoantibody with vast majority of these single antibodies being GADA or IAA. Subjects who express only a single islet autoantibody are at a low risk for developing T1D compared to those with multiple autoantibodies. Relatives expressing only IAA in the DPT-1 study essentially did not progress to diabetes (8). The antibody affinity study by competition assay used is very similar to previous reports (9-12) although it is an indirect measurement. Our previous (5) and present studies demonstrated that most of these single IAA or GADA detected only by radioassay were low affinity antibodies and failed to give signal with the bivalent ECL assay. A few laboratories previously reported that both IAA and GADA affinity in multiple antibody-positive children were significantly
higher than in children who remained single autoantibody positive or become autoantibody negative (9-11). We hypothesized (5) that most of these single antibodies may result from immunization with a cross-reactive molecule, while higher affinity IAA or GADA result from immunization with islet autoantigen itself. Both forms of antibody can be competed with native antigen molecule thus are not “biochemically” false positive; however, in terms of biologic relevance, antibodies detected with only the radioassay appear not to predict diabetes.

Prospective follow-up study has observed that the development of islet autoantibodies often happens sequentially (13), i.e., majority of subjects are initially single antibody positive. Thus, differentiation of high- from low-risk islet autoantibodies at that stage could be very important in identifying truly high-risk subjects for prevention trials. Our study showed that individuals positive for only GADA by radioassay, but negative by ECL assay, remained ECL negative for years even though they had multiple positive follow-up tests by radioassay. We did not see individuals who after being IAA (5,6) or GADA positive only by radioassay developed a 2nd islet autoantibody or diabetes. On the other hand, in pre-diabetics or high risk subjects with multiple islet autoantibodies ECL assay always detected IAA (6) or GADA from the very beginning. Siljander et al (12) found that there was no affinity maturation observed over time from seroconversion to T1D diagnosis. Thus the new ECL assays may make it possible to identify correctly high risk IAA (6) and GADA on the first positive sample. The major strength of this study was availability of well characterized prospectively collected DAISY samples. However, it would be ideal to confirm these results in additional cohorts with long-term follow-up, e.g., DPT-1/TrialNet Pathway to Prevention or TEDDY. While we provided an evidence that the disease relevant ECL-GADA are of high-affinity, full confirmation awaits a separate study.
In conclusion, ECL-GADA assay similar to ECL-IAA assay is able to differentiate high risk islet autoantibodies from low risk or non-disease relevant antibodies at the earliest stage of screening.

**Author Contributions:** D.M. researched data and reviewed manuscript. K.H. researched data and reviewed manuscript. F.D. researched data and reviewed manuscript. L.J. researched data and reviewed manuscript. A.S. reviewed/edited manuscript. M.R. designed the DAISY study, researched data and reviewed/edited manuscript. G.S.E. researched data. L.Y. researched data and wrote manuscript.

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References


Figure Legend

**Figure 1:** GADA levels from ECL-GADA assay compared with the standard NIDDK harmonized GADA radioassay. The dotted lines represent the assay cut-offs. The two assays were correlated (p< 0.0001) for all 3 groups with both assays set at 99% specificity.

**Panel A:** Newly diagnosed children with T1D (N=177) at Barbara Davis Center.

**Panel B:** Pre-diabetic DAISY participants (N=68).

**Panel C:** Newly diagnosed DASP participants (N=50).

**Figure 2:** GADA levels from ECL-GADA assay compared with the standard NIDDK harmonized GADA radioassay in all 39 DAISY participants from Panel A to B who were persistently positive of single islet autoantibody (GADA only) on multiple clinic visits with years of follow-up. ECL-GADA results were consistently negative in all but 9 subjects. The dotted line represents 3 SD Score which is close to both assays’ cut-offs.

**Figure 3:** GADA levels from ECL-GADA assay compared with the standard NIDDK harmonized GADA radioassay in all 43 DAISY participants from Panel A to B who were persistently positive for multiple islet autoantibodies. ECL- and radioassay GADA levels correlated closely. The dotted line represents 3 SD Score which is close to both assays’ cut-offs.

**Figure 4:** Sera with positive GADA by radioassay from 14 subjects, 7 ECL-GADA positive and 7 ECL-GADA negative were incubated with different concentrations of unlabelled GAD65 protein and analyzed with our standard NIDDK harmonized GADA radioassay. GADA negative by ECL-GADA assay (dotted line), compared to GADA positive by ECL-GADA
assay (solid line), required higher concentrations of GAD65 protein for \( \frac{1}{2} \) maximal inhibition, consistent with low affinity. Results were expressed as percent of signal not absorbed.
GADA on Subjects with Single Autoantibody
(9/39 positive for ECL-GADA)

Figure 2
GADA on Subjects with Multiple Autoantibodies
(41/43 positive for ECL-GADA)

Figure 3
GADA Competition Assay

Cold GAD65 Concentration

% not absorbed

Negative by ECL-GADA

Positive by ECL-GADA

Figure 4