

ECL-IAA and ECL-GADA Can Identify High-Risk Single Autoantibody-Positive Relatives in the TrialNet Pathway to Prevention Study

Andrea K. Steck, MD,¹ Alexandra Fouts, BS,¹ Dongmei Miao, MD,¹ Zhiyuan Zhao, MD,¹ Fran Dong, MS,¹ Jay Sosenko, MD,² Peter Gottlieb, MD,¹ Marian J. Rewers, MD, PhD,¹ Liping Yu, MD,¹ and the TrialNet Study Group*

Abstract

Background: Relatives with single positive islet autoantibodies have a much lower risk of progression to diabetes than those with multiple autoantibodies.

Materials and Methods: TrialNet subjects positive for single autoantibody to insulin (mIAA) ($n=50$) or single autoantibody to glutamic acid decarboxylase (GADA) ($n=50$) were analyzed using new electrochemiluminescence (ECL) assays (ECL-IAA and ECL-GADA, respectively) at their initial visit and longitudinally over time. Affinity assays were performed on a subset of single autoantibody-positive subjects at initial and most recent visits.

Results: After a mean follow-up of 5.3 years, 20 subjects developed type 1 diabetes. Among either single GADA or single mIAA subjects, those who were positive in the ECL assay showed higher affinity at the initial visit, and affinity results stayed consistent over time. No converting events from low to high or high to low affinity were seen over time.

Conclusions: Confirmed positivity for ECL is associated with high affinity and can help staging of risk for type 1 diabetes in single autoantibody-positive subjects.

Introduction

SCREENING FOR RISK OF TYPE 1 diabetes uses autoantibody (Ab) radioimmunoassays for specific islet autoantigens, including Abs to insulin (mIAA),¹ glutamic acid decarboxylase (GADA),² islet antigen-2 (ICA512),³ and most recently zinc transporter 8.^{4,5} Individuals having a single Ab positivity are at low risk for progression to diabetes, whereas individuals expressing two or more Abs have a risk of 80% of developing diabetes in 15 years.⁶ Prevention trials are currently available for subjects with multiple Ab positivity, but no prevention trials are offered for single Ab-positive subjects. Cumulative risk of development of diabetes has been associated with young age at seroconversion, positivity for multiple Abs, high Ab levels, persistent positivity for mIAA, and high Ab affinity.⁷⁻¹² Children with persistent single high-affinity mIAA or GADA have been shown to be more likely to progress to type 1 diabetes than children with single low-affinity Abs.^{11,12}

Recently, our center developed new electrochemiluminescence (ECL) assays for insulin (ECL-IAA) and GADA (ECL-GADA).¹³ Both ECL-IAA and ECL-GADA have been shown to be more disease specific in the Diabetes Autoimmunity Study in the Young (DAISY) and the TrialNet Pathway to Prevention studies.^{14,15} Most children with two or more radioimmunoassay-positive Abs were found to be positive by ECL assays, whereas only some children with a single radioimmunoassay-positive Ab were confirmed by ECL-IAA and ECL-GADA assays, respectively.^{14,15} The goal of this study was to evaluate ECL assays and Ab affinity over time in TrialNet single Ab-positive subjects.

Research Design and Methods

Subjects

The TrialNet Pathway to Prevention Study screens relatives of type 1 diabetes patients for the presence of islet Abs

¹Barbara Davis Center for Childhood Diabetes, University of Colorado School of Medicine, Aurora, Colorado.

²University of Miami School of Medicine, Miami, Florida.

*The composition of the TrialNet Study Group is given in Supplementary Data (available online at www.liebertonline.com/dia).

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and offers close monitoring and/or prevention trials.¹⁶ In total, 100 single Ab subjects confirmed positive on two consecutive visits were selected from the TrialNet Pathway to Prevention Study (50 single mIAA and 50 single GADA subjects) and were analyzed for ECL-IAA and ECL-GADA at their initial visit and longitudinally over time (yearly samples). Subjects were selected to be confirmed single Ab positive on two consecutive visits with no additional Ab positivity on follow-up. All subjects provided written informed consent, and the study was approved by the ethical boards of all participating institutions.

Radioimmunoassays and ECL assays

The radioimmunoassays for mIAA and GADA used in the present study were all performed in the Barbara Davis Center laboratory as previously described.^{17,18} The radioimmunoassay cutoffs were set at the 99th percentile of 500 normal control samples for GADA and 106 controls for mIAA, respectively. Based on the report by the Islet Autoantibody Standardization Program Committee,¹⁹ sensitivities and specificities were 52% and 100%, respectively, for mIAA and 82% and 99%, respectively, for GADA.

ECL-IAA and ECL-GADA assays were measured longitudinally on all subjects at yearly intervals at the Barbara Davis Center laboratory as previously described.^{13,14} In brief, serum samples were mixed with both sulfo-tag and biotin-labeled antigen proteins (either proinsulin or glutamic acid decarboxylase 65 [GAD65]) for overnight incubation at 4°C. The antigen-antibody complexes with biotin were captured by a streptavidin-coated plate, and the sulfo-tag gave the signals with ECL. The results were expressed as an index against internal standard positive controls of either insulin or GAD65 monoclonal antibody. The ECL assay cutoff indexes of 0.006 for mIAA or 0.023 for GADA were set at the 99th percentile over 100 healthy controls, and the ECL interassay coefficients of variation were 4.8% ($n=20$) for mIAA and 8.8% ($n=10$) for GADA, respectively. Sensitivities and specificities for the ECL assays were 60% and 98%, respectively, for mIAA and 78% and 96%, respectively, for GADA among patients with newly diagnosed type 1 diabetes.¹⁹

GADA and mIAA affinity assays

Affinity measures the strength of interaction between an antibody and an antigen. It is defined by the same basic thermodynamic principles that govern any reversible biomolecular interaction. High-affinity antibodies will bind a greater amount of antigen at a lower concentration of antigen than low-affinity antibodies during the same period of time. For affinity testing, 20 subjects were selected from each group with consistent ECL status at initial and last visit (i.e., 20 single GADA-positive subjects who were confirmed ECL-GADA positive at initial and last visits [$n=10$] or confirmed ECL-GADA negative at initial and last visits [$n=10$]). For single mIAA-positive subjects, the same selection criteria were used for 20 single mIAA-positive subjects with 10 confirmed ECL-IAA positive and 10 confirmed ECL-IAA negative; however, only seven single mIAA subjects were confirmed positive by ECL-IAA at initial and last visits and therefore included in the affinity analysis.

The GADA and IAA affinity analyses were performed using the standard GADA and mIAA radioimmunoassays with a serial absorption of unlabeled antigen protein as previously published.^{13,14} Each serum was assessed in seven or eight separated wells, one without competition and six or seven with competition by adding unlabeled GAD65 or insulin in six different concentrations (5.7×10^{-4} – 4.6×10^{-9} M), respectively, into the serum incubation mixture to compete with radiolabeled GAD65 or insulin. For competition, the serum samples were mixed with radiolabeled and unlabeled GAD65 or insulin, respectively, at the same time, and then incubated for overnight at 4°C. The complexes of antibody-antigen were precipitated with protein A/G Sepharose® (GE Healthcare, Little Chalfont, United Kingdom), and radioactive signals were counted. The signal inhibitions of 50% by unlabeled GAD65 or insulin were calculated and compared for relative affinity of antibodies.

Statistics

Statistical analyses were performed using SAS software (version 9.2; SAS Institute, Cary, NC). Categorical variables were analyzed using Fisher's exact test. Continuous variables were tested using the *t* test for difference in means or the Wilcoxon rank-sum test for difference in medians. Because of different Ab cutoff values and change in the type of assay over time, both ECL and radioimmunoassays Ab levels were converted to SD units away from threshold ("Z scores") for analyses. All subjects were selected to be confirmed positive for either mIAA or GADA, but many of the samples were negative in ECL assays, leading to mean values (Table 1) that can be negative for ECL assays. Both initial and mean levels of Abs over time were calculated. A *P* value of <0.05 was considered statistically significant.

Results

After a median follow-up of 5.3 (± 2.6) years, 20 of these subjects developed type 1 diabetes, including 15 single GADA and five single mIAA. Among the 50 single mIAA-positive subjects, in total 32 (64%) were positive at least once by ECL-IAA, whereas 34 (68%) of the 50 single GADA-positive subjects were positive at least once by ECL-GADA. Characteristics of these single Ab-positive subjects are described in Table 1. Follow-up duration was significantly shorter among subjects who developed diabetes compared with those who stayed diabetes free (2.2 vs. 6.3 years [$P<0.0001$] and 2.1 vs. 6.0 years [$P=0.007$] for single GADA and single mIAA, respectively). Age at initial visit was similar for single mIAA subjects (8.1 vs. 7.3 years; $P=0.56$) but significantly older for those single GADA subjects who developed diabetes (23.5 vs. 6.6 years; $P<0.0001$).

Single GADA subjects who developed diabetes compared with those who stayed diabetes free had higher initial GADA levels (ECL-GADA, respectively, 0.19 vs. -0.09 [$P=0.001$]; GADA, respectively, 0.43 vs. 0.01 [$P<0.0001$]) as well as higher mean GADA levels over time (ECL-GADA, respectively, 0.17 vs. -0.07 [$P=0.001$]; GADA, respectively, 0.33 vs. 0.0 [$P<0.0001$]). Among single mIAA subjects, similar results were found for ECL-IAA, with higher levels in those who developed diabetes compared with those who stayed diabetes free (initial ECL-IAA, respectively, 0.15 vs. -0.04 [$P=0.06$]; mean ECL-IAA, respectively, 0.16 vs. -0.02

TABLE 1. CHARACTERISTICS OF TRIALNET SUBJECTS WITH SINGLE AUTOANTIBODY TO GLUTAMIC ACID DECARBOXYLASE OR SINGLE AUTOANTIBODY TO INSULIN

Single Ab by radioimmunoassay	No diabetes	Developed diabetes	P value
GADA (n = 50)			
<i>n</i>	35	15	
Age at initial visit (years)	6.6 ± 2.5	23.5 ± 15.1	<0.0001
Follow up duration (years)	6.3 ± 1.8	2.2 ± 2.3	<0.0001
Gender male, [<i>n</i> (%)]	21 (60%)	6 (40%)	0.23
Ethnicity NHW [<i>n</i> (%)]	26 (74%)	11 (73%)	1.0
Initial GADA	0.01 ± 0.18	0.43 ± 0.28	<0.0001
Initial ECL-GADA	-0.09 ± 0.03	0.19 ± 0.33	0.001
Mean GADA	0.0 ± 0.12	0.33 ± 0.26	<0.0001
Mean ECL-GADA	-0.07 ± 0.07	0.17 ± 0.29	0.001
HLA-DR3/4*0302 [<i>n</i> (%)] ^a	4 (13%)	4 (33%)	0.18
HLA-DR2*0602 [<i>n</i> (%)] ^a	4 (13%)	1 (8%)	1.0
mIAA (n = 50)			
<i>n</i>	45	5	
Age at initial visit (years)	7.3 ± 3.1	8.1 ± 4.4	0.56
Follow-up duration (years)	6.0 ± 2.3	2.1 ± 2.5	0.007
Gender male [<i>n</i> (%)]	21 (47%)	4 (80%)	0.35
Ethnicity NHW [<i>n</i> (%)]	37 (82%)	4 (80%)	1.0
Initial mIAA	0.08 ± 0.22	0.04 ± 0.08	0.85
Initial ECL-IAA	-0.04 ± 0.10	0.15 ± 0.19	0.06
Mean mIAA	0.02 ± 0.18	0.03 ± 0.12	0.65
Mean ECL-IAA	-0.02 ± 0.15	0.16 ± 0.20	0.06
HLA-DR3/4*0302 [<i>n</i> (%)] ^a	3 (7%)	2 (40%)	0.08
HLA-DR2*0602 [<i>n</i> (%)] ^a	5 (12%)	1 (20%)	0.51

Data are mean ± SD values unless specified otherwise. Autoantibody (Ab) levels were converted to SD units away from threshold (Z scores).

^aMissing human leukocyte antigen (HLA) genotyping in a few subjects (six of 50 single Ab to glutamic acid decarboxylase [GADA] and three of 50 single Ab to insulin [mIAA]).

ECL, electrochemiluminescence; NHW, non-Hispanic white.

[$P=0.06$]), but no differences were found for mIAA levels (initial mIAA, respectively, 0.04 vs. 0.08 [$P=0.85$]; mean mIAA, respectively, 0.03 vs. 0.02 [$P=0.65$]).

There were no statistically significant differences in human leukocyte antigen genotypes among the subjects who progressed to diabetes versus those who did not progress.

Affinity assays were performed on a subset of single Ab-positive subjects (17 for mIAA and 20 for GADA) to assess whether affinity stays consistent over time. Among these single GADA subjects, half ($n=10$) were positive by ECL-GADA assay. The results of the competition assay are illustrated in Figure 1a. GADA not detectable by ECL assay required a 10–1,000-fold higher concentration of unlabeled GAD65 protein for 50% inhibition of binding of GADA to labeled GAD65 protein for samples negative by ECL-GADA than for samples positive by ECL-GADA. Affinity results were found to be consistent between initial and follow-up visits.

Among the 17 single mIAA subjects, seven were positive by ECL-IAA, whereas 10 were negative. The results of the competition assay were similar to those found for GADA (Fig. 1b). At 50% inhibition, those ECL-positive were significantly different from those ECL-negative. Samples that were positive for ECL-IAA showed higher affinity already at the initial visit, and affinity results stayed consistent over time.

Among subjects who developed diabetes with available affinity results ($n=14$), all except one were ECL-positive and had high affinity for GADA or mIAA. One subject with a low level of GADA who developed diabetes was ECL-GADA

negative and had low affinity results. No converting events from low to high or high to low affinity were seen over time.

Discussion

In this study, single Ab-positive subjects were evaluated using ECL assays and affinity assays over time. Previous preliminary findings from the DAISY study showed that multiple Ab-positive subjects (typically positive also for ECL) have higher-affinity antibodies than single Ab-positive subjects.¹³ This is the first study to show that single Ab-positive (mIAA or GADA) subjects confirmed positive for ECL have higher-affinity Ab compared with single Ab-positive TrialNet subjects negative for ECL and that higher affinity is already present at the initial visit in these single Ab-positive subjects and stays consistent over time.

Our findings suggest that ECL measurements may have important practical implications regarding staging of diabetes risk. Subjects who were confirmed positive by ECL assay showed higher affinity already at the initial visit, and no converting affinity events were seen over time, implicating that high affinity of these ECL assays can help define risk for diabetes early on. Different risk scores for type 1 diabetes have been proposed, including number and levels of Ab as well as metabolic markers.^{9,10,20,21} Currently, prevention trials are only available for relatives of patients with diabetes who have two or more Abs. However, single Ab-positive subjects confirmed positive for ECL may qualify for enrollment into prevention trials as their risk for diabetes is much

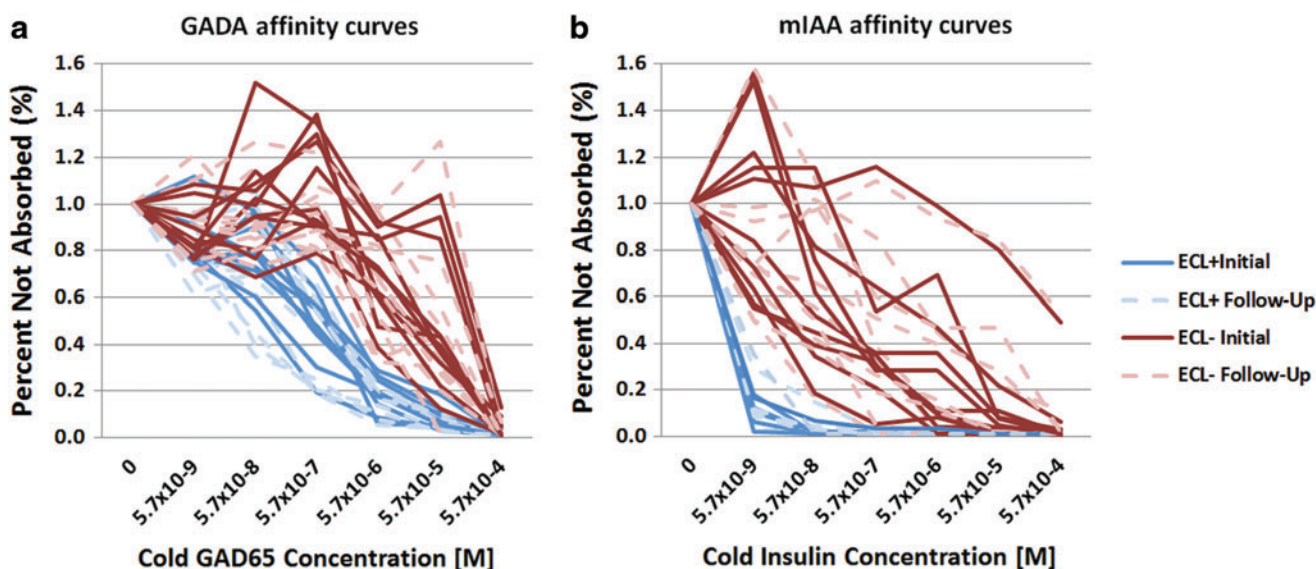


FIG. 1. (a) Longitudinal affinity results for single autoantibody to glutamic acid decarboxylase (GADA)-positive samples according to electrochemiluminescence (ECL)-GADA status: GADA from ECL-negative subjects (red or black lines), compared with ECL-positive subjects (blue or gray lines), required higher concentrations of glutamic acid decarboxylase 65 (GAD65) for 50% maximal inhibition. Results are expressed as percentages of signal not absorbed. The solid line indicates the initial sample; the dotted line indicates the follow-up sample. (b) Longitudinal affinity results for single autoantibody to insulin (mIAA)-positive samples according to ECL-IAA status: insulin autoantibody from ECL-negative subjects (red or black lines), compared with ECL-positive subjects (blue or gray lines), required higher concentrations of insulin for 50% maximal inhibition. Results are expressed as percentages of signal not absorbed. The solid line indicates the initial sample; the dotted line indicates the follow-up sample. (Color graphics available at www.liebertonline.com/dia)

higher than those single Ab-positive subjects negative for ECL.¹⁵ On the other hand, subjects found to be negative by ECL assays may benefit from less intensive monitoring in these longitudinal prospective studies.²²

The higher affinity of these Abs detected by the ECL assays may be explained by several factors. The ECL assay is a bivalent assay where Abs to insulin in serum link to both the sulfo-tagged proinsulin and the biotinylated proinsulin, thereby potentially increasing specificity.¹³ In addition, ECL assays are designed to capture all immunoglobulins, whereas only immunoglobulin Gs are captured by radioimmunoassays. Further evaluation is needed to confirm these hypotheses, which could have important implications for understanding the pathophysiology of type 1 diabetes.

Only single mIAA and single GADA subjects were analyzed in this study. Islet antigen-2 and zinc transporter 8 antibodies are only rarely present as a single antibody.²³ Although subjects were selected randomly from the monitoring phase of TrialNet, duration of follow-up and age at initial visit were significantly different between subjects who developed diabetes and those who stayed diabetes free. One possible explanation is that most young subjects who develop diabetes have multiple Abs, whereas older subjects who develop diabetes may be positive for GADA only. The number of subjects analyzed in this study was small, and further evaluation of ECL assays in prospective studies such as TrialNet and The Environmental Determinants of Diabetes in the Young (TEDDY) is warranted.

In conclusion, among single Ab-positive subjects, those confirmed positive by ECL assays show high affinity at initial visit, and the affinity stays consistent over time. Positivity for

ECL at initial visit can help staging of risk for type 1 diabetes in single Ab-positive subjects.

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Author Disclosure Statement

No competing financial interests exist.

A.K.S. designed the study, wrote the manuscript, and contributed to the discussion. A.F., D.M., Z.Z., and F.D. researched data. J.S., P.G., and M.J.R. contributed to the discussion and reviewed/edited the manuscript. L.Y. designed the study, contributed to the discussion, and reviewed/edited the manuscript. A.K.S. takes full responsibility for the contents of the article.

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Address correspondence to:

Andrea K. Steck, MD

Barbara Davis Center for Childhood Diabetes

University of Colorado School of Medicine

1775 Aurora Court, A-140

Aurora, CO 80045

E-mail: andrea.steck@ucdenver.edu

or

Liping Yu, MD

Barbara Davis Center for Childhood Diabetes

University of Colorado School of Medicine

1775 Aurora Court, A-140

Aurora, CO 80045

E-mail: liping.yu@ucdenver.edu