DELISA: sensitive nonisotopic assay for GAD$_{65}$ autoantibodies, a key risk-assessment marker for insulin-dependent diabetes mellitus

Harshvardhan B. Mehta, Barbara S. Vold, Svetlana Minkin, and Edwin F. Ullman*

Nonisotopic assays for the measurement of autoantibodies to 65-kDa glutamic acid decarboxylase (GAD$_{65}$) have not previously achieved performance equivalent to radiobinding assays (RBA). We have developed a modified ELISA protocol, DELISA, for measuring autoantibodies to GAD$_{65}$ in serum. The method overcomes the problems of poor sensitivity and specificity associated with conventional ELISAs. Serum containing GAD$_{65}$ autoantibodies is incubated with biotinylated GAD$_{65}$ (bGAD$_{65}$). Sufficient soluble Protein A–dextran conjugate is added to bind the immunoglobulins in the sample, including GAD$_{65}$ autoantibodies to which GAD$_{65}$ is bound. After incubation, the mixture is transferred to a streptavidin-coated microtiter well, which binds free bGAD$_{65}$ but not bGAD$_{65}$ bound to autoantibodies. Streptavidin-bound bGAD$_{65}$ is detected by means of a peroxidase–GAD$_{65}$MAb conjugate. The method appears to have comparable sensitivity and specificity to those of RBAs. Reaction of the antibodies with soluble antigen to increase the binding rate and the use of high serum concentrations and very low antigen concentrations to increase sensitivity are critical elements of the method.

INDEXING TERMS: ELISA • immunoassay • autoimmune disease • serology

Type I diabetes (insulin-dependent diabetes mellitus; IDDM) is one of the most serious and common of metabolic disorders. In the US, the prevalence of IDDM by age 20 years is $\sim$0.26%, and lifetime prevalence approaches 0.4% [1]. IDDM results from selective destruction of the insulin-producing $\beta$-cells of the pancreas. After a chronic, clinically silent autoimmune pathology, patients usually have abrupt onset of hyperglycemia caused by insulinopenia [2, 3]. The patient becomes dependent on insulin therapy to restore normal metabolic hemostasis, which, if not maintained, can lead to ketoacidosis, retinopathy, nephropathy, and even death [4–6].

The appearance of circulating islet cell autoantibodies (ICA) correlates with lymphocytic infiltration of the pancreas (insulitis) [7, 8] and increased risk for developing IDDM [9–11]. Among the most-studied islet cell autoantibodies are characterized autoantibodies [12–14], insulin autoantibodies [15], and GAD$_{65}$ autoantibodies (GAD$_{65}$Abs) [16–18], all of which may vary in titer by several orders of magnitude.

The measurement of GAD$_{65}$Abs as an alternative to ICA has been the focus of intense research because of the availability of purified native and recombinant GAD$_{65}$ [19, 20], which provides an opportunity for development of a simple immunoassay. By comparison, the currently used indirect immunofluorescence assay of ICA is technically demanding, requires a pancreas from blood type O human cadavers, permits only a subjective estimate of antibody concentration, is difficult to standardize, and is not amenable to convenient screening of large numbers of sera. In limited studies, GAD$_{65}$Abs have been shown to be present in many newly diagnosed and prediabetic patients [21, 22], and their presence appears to have greater sensitivity, specificity, and predictive value for IDDM than that of other autoantibodies [16]. However, the only GAD$_{65}$Abs assays that have been reported to have acceptable clinical performance are radiobinding assays (RBAs). These methods are slow and labor intensive, and the reagents are unstable and environmentally problematic. Although numerous ELISAs for GAD$_{65}$Abs have been reported, none has the sensitivity approaching that of RBAs [23, 24].

We have developed a novel ELISA format that offers sensitivity and specificity comparable with those of RBAs. Our approach focuses on the detection of free antigen that has not become bound by antibodies.

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1 Nonstandard abbreviations: IDDM, insulin-dependent diabetes mellitus; GAD$_{65}$, 65-kDa glutamic acid decarboxylase (EC 4.1.1.15); bGAD$_{65}$, biotinylated GAD$_{65}$; GAD$_{65}$Abs, GAD$_{65}$ autoantibodies; RBA, radiobinding assay; ICA, islet cell autoantibodies; GABA, $\gamma$-aminobutyric acid; DELISA, depletion ELISA; IDW, Second International Diabetes Workshop on GAD Autoantibody Measurement Standardization; MAb, monoclonal antibody; MES, 4-morpholinoethanesulfonic acid; PBS, phosphate-buffered saline; HRP, horseradish peroxidase; and MICA, monoclonal islet cell autoantibodies.

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Materials and Methods

GAD<sub>65</sub>

Clones coding for human GAD<sub>65</sub> were kindly provided by A. Tobin [20]. An error in the GAD<sub>65</sub> coding sequence of the baculovirus expression plasmid, pBbGAD<sub>65</sub>-2 (serine codon converted to proline), was repaired by excising with Str I and Str II a 1136-bp fragment containing the mutation and ligating back a similarly excised wild-type fragment from the cloning plasmid pSKGAD<sub>65</sub>-5, which contained the correct sequence. The resulting new construct and Baculogold viral DNA (Pharmingen, San Diego, CA) were used to cotransfect s9 cells (JRH Biosciences, Woodlands, CA), and plaques containing viruses with the cloned gene were purified. The s9 host cells were infected with virus at a multiplicity of infection of 0.5; the cells were harvested 2 days after infection and then lysed in 10 mmol/L phosphate, pH 7.0, containing 1 mmol/L EDTA, 1 mmol/L 2-aminoethylisothiouronium bromide (Sigma, St. Louis, MO), and 0.02 mmol/L pyridoxal 5-phosphate (Sigma) with use of a glass homogenizer. The lysate was centrifuged at 105 000g for 1 h at 4 °C, and the pellet was washed and extracted with lysis buffer containing 1 mL/L Triton X-100. After centrifugation the extract was fractionated first on a Q-Sepharose column (Pharmacia, Uppsal, Sweden) with a 0–0.5 mmol/L KCl gradient in the wash buffer and then on a Phenyl Sepharose column (Pharmacia) with a reverse gradient of 0.3–0.01 mmol/L phosphate, pH 7.0, containing 1 mL/L Triton X-100. The fractions were checked for protein concentrations by the bicinchoninic acid method (Pierce, Rockford, IL) and the DC Bio-Rad assay (Bio-Rad, Hercules, CA). GAD<sub>65</sub> enzymatic activity was measured in a two-step procedure by incubation with 50 mmol/L glutamic acid in 0.1 mol/L phosphate buffer, pH 6.8, containing 0.25 mmol/L pyridoxal 5-phosphate and 56 mmol/L β-mercaptoethanol at 37 °C for 60 min. The amount of γ-aminobutyric acid (GABA) formed was measured with 0.05 U of GABA<sub>e</sub> (4-amino-5-oxobutyrate:2'-oxoglutarate aminotransferase plus succinate-semialdehyde:NADP<sup>+</sup> oxidoreductase; Boehringer Mannheim, Indianapolis, IN) in 0.125 mol/L phosphate buffer, pH 8.1, containing 5.5 mmol/L α-ketoglutarate, 1.0 mmol/L NADP<sup>+</sup>, and 3.5 mmol/L β-mercaptoethanol at 37 °C for 15 min followed by fluorometric determination of the NADPH produced. The purity of GAD<sub>65</sub> was checked by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (Novex, San Diego, CA) and densitometric scanning of the Coomasie Blue-stained gel on a Personal Densitometer (Molecular Dynamics, Sunnyvale, CA). The specific activity of different preparations ranged from 6.7 to 8.2 μmol/min per milligram, which is much higher than the previously reported value for recombinant GAD<sub>65</sub> of 0.32 μmol/min per milligram [25], possibly because the enzyme lost activity over the long incubation times used by the previous workers.

Biotinylated GAD<sub>65</sub>

GAD<sub>65</sub> having at least 7.4 μmol/min per milligram specific activity (0.6 mg) in 590 μL of GAD buffer (10 mmol/L phosphate buffer, pH 8.3, containing 1 mmol/L EDTA, 80 μmol/L pyridoxal 5-phosphate, 1 mL/L Triton X-100, and 100 mL/L glycerol) was incubated in the dark for 3 h at 4 °C with 69 μL of a freshly prepared solution of 4 mmol/L iodoacetyl-LC-biotin (Pierce) in dimethylformamide. The mixture was then subjected four times to sequential filtration by centrifugation with a Microcon-30 membrane filter (30-kDa cutoff; Amicon, Beverly, MA) and redilution with GAD buffer. The number of biotins per GAD<sub>65</sub>, 2.8:1 (mol/mol), was determined by titration with avidin and 4-hydroxyazobenzene-2-carboxylic acid (Pierce) [26].

Serum Calibrators

Two sets of calibrators were prepared by serial dilution of a GAD<sub>65</sub>Ab-positive serum from an IDDM patient (provided by Noel Maclaren, University of Florida, Gainesville, FL) into pools of sera from healthy individuals without GAD<sub>65</sub>Abs, obtained from either the Peninsula Blood Bank, Burlingame, CA, or Irvine Scientific, Santa Ana, CA. The calibrators were stored frozen at −70 °C.

Protein A–Dextran

Ten grams of Dextran T-2000 (average 2000 kDa; Sigma) and 8.5 g of bromoacetic acid (Aldrich, Milwaukee, WI) in 85 mL of 2.9 mol/L NaOH were incubated overnight at 50 °C. Ethanol was added and the precipitate was dissolved in deionized water and dialyzed overnight at 4 °C. To 60 mL of this solution (containing 3.0 g of carbamylated dextran) and 6.6 mL of 500 mmol/L 4-morpholinoethanesulfonic acid (MES; Calbiochem, San Diego, CA), pH 5.5, at 4 °C was added 1.42 g of 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (Aldrich) in 9.0 mL of 50 mmol/L MES, pH 5.5, over 1–2 min; this was followed by stirring for 30 min over ice. The resulting mixture was added at 4 °C with stirring over 1 h to 1.0 g of recombinant Protein A (Repligen, Cambridge, MA) in 20 mL of 0.1 mol/L phosphate, pH 8.0. During the addition, the pH was maintained between 7.2 and 7.8, and stirring was continued overnight at 4 °C. Unreacted Protein A was removed by using a tangential flow filtration module (Filtron, Northborough, MA) with a 100-kDa-cutoff membrane. After 1:25 dilution into phosphate-buffered saline (PBS; BioWhittaker, Walkersville, MD), the Protein A–dextran solution contained ~13 μmol/L Protein A.

GAD<sub>65</sub>Mab–Peroxidase Conjugate

Murine monoclonal anti-GAD<sub>65</sub> antibodies (GAD<sub>65</sub>Mab) were prepared by using GAD<sub>65</sub> with RIBI adjuvant (RIBI Immunochem, Hamilton, MT) as immunogen. GAD<sub>65</sub>Mab (34.9 mg) in 1.2 mL of 0.1 mol/L phosphate, pH 7.0, containing 0.2 mol/L NaCl and 5 mmol/L EDTA was incubated in the dark at room temperature for 1 h with 10 μL of 50 g/L N-succinimidyl-S-acetylthioacetate in dimethylformamide (Pierce). After adding 0.134 mL of 1.0 mol/L hydroxyamine hydrochloride (Aldrich) in 0.1 mol/L phosphate, pH 7.4, containing 50 mmol/L EDTA, and incubating for an additional hour, we purified the product, Mab-SH, on a PD-10 column (Pharmacia) with 50 mmol/L phosphate, pH 6.0, containing 0.1 mol/L NaCl and 5 mmol/L EDTA (buffer A).

Horseradish peroxidase (HRP; Toyobo Co., Japan) (295 mg) purified over a PD-10 column in 0.1 mol/L phosphate, pH 6.5,
was incubated with 48 mg of sulfo succinimidyl 4-(N-maleimidoethyl)cyclohexane-1-carboxylate (Pierce) at 4 °C for 1.5 h and purified over a PD-10 column with buffer A. The MAb-SH solution was added at 4 °C (30.1 molar ratio of HRP to MAb) and the mixture was incubated for 2 h under argon. Excess maleimide groups were blocked by 0.5 h of incubation with 1.25 mmol/L β-mercaptoethanol, and excess sulfhydryl groups were blocked by 0.5 h of incubation with 3.8 mmol/L iodoacetate (Aldrich). The reaction mixture was concentrated and fractionated on a Sephacryl S-300 column (1.5 × 150 cm) equilibrated with PBS. Calculation of the number of HRP molecules per MAb in the conjugate was based on ε240 = 32 000 and ε403 = 100 000 L mol⁻¹ cm⁻¹ for HRP [27] and ε260 = 216 000 L mol⁻¹ cm⁻¹ for MAb. The pooled conjugate fractions had a HRP:MAb ratio of 4.5:1 (mol/mol). The concentration for use in the DELISA was determined empirically to maximize the ratio of specific to nonspecific binding on the streptavidin-coated microtiter plates.

**ASSAY METHODS**

All steps were carried out at ambient temperature (22–25 °C). For DELISAs, 25 μL of calibrators or test sera and 0.625 fmol of bGAD₆₅ in 25 μL of GAD buffer were incubated for 2 h in polypropylene microtiter plate wells covered with self-adhesive Mylar seal tape. Protein A–dextran (50 μL, in PBS) was then mixed into each well by gently pipetting back and forth. After incubating for an additional hour, 80 μL of each reaction mixture was transferred to a multichannel pipette to a streptavidin-coated microtiter plate (Syva Co., San Jose, CA) that had been freshly washed with a solution of 28 g/L trisodium citrate dihydrate, 0.105 g/L citric acid monohydrate, 0.91 mL/L Tween-20, 15.8 mL/L glycerol, and 5.0 mg/L sodium thiomersol. After 1 h of shaking, the plate was washed with the same buffer, GAD₆₅:MAb–HRP conjugate (100 μL) was added to each well, and the plate was again shaken for 1 h and washed as before. The enzyme activity in each well was determined by incubation for 30 min with equal volumes of tetramethylbenzidine and H₂O₂ (Kirkegaard and Perry Labs., Gaithersburg, MD), stopping the reaction with 0.1 mL of 0.5 mol/L H₂SO₄, and reading the absorbance with a Syva MicroTrak® EIA reader at 450 nm. ELISAs were carried out by first incubating streptavidin-coated microtiter plates with 100 μL of 200 pmol/L bGAD₆₅ in an equivalent mixture of goat serum and PBS for 1 h with shaking. After the wells were washed, diluted solutions of test sera in PBS (100 μL) were added and incubated for 1 h. The wells were washed again and incubated with 100 μL of a 1:3000 mixture of goat anti-human IgG–HRP conjugate (Kirkegaard and Perry Labs.) in PBS. After a final washing, the enzyme activity in the wells was determined as above.

**RESULTS**

In the DELISA protocol, the binding of a low concentration of bGAD₆₅ to antibodies in the sample is allowed to approach equilibrium before the binding of the sample immunoglobulins by a soluble Protein A–dextran conjugate. Only the IgG, remains unbound because of failure of Protein A to bind this isotype [28]. Binding to the dextran causes bGAD₆₅ that is bound to antibody to become sterically inaccessible for reaction with streptavidin-coated microtiter wells. The unbound bGAD₆₅ remains able to bind and is detected with an HRP–GAD₆₅:MAb conjugate. GAD₆₅ antibodies thus reduce the signal by depletion of the antigen; the assay is therefore referred to as a depletion ELISA (DELISA):

\[
\begin{align*}
\text{Free bGAD}_{65} & \rightarrow \text{bGAD}_{65}:\text{Ab} \\
\text{bGAD}_{65}:\text{Ab} & \rightarrow \text{bGAD}_{65}:\text{Ab}-\text{PAD}
\end{align*}
\]

where PAD is Protein A–dextran. The amount of bGAD₆₅ that binds to a streptavidin-coated microtiter plate in the absence of antibody was found to be directly proportional to the bGAD₆₅ concentration, and concentrations as low as 1 pmol/L could be detected.

**EFFECT OF GAD₆₅ CONCENTRATIONS ON ASSAY RESPONSE**

In initial experiments, serum calibrators were mixed with two different concentrations of bGAD₆₅, incubated for 2 h, and then transferred to streptavidin-coated microtiter wells without addition of Protein A–dextran. After an additional 1-h incubation, the amount of bound bGAD₆₅ was determined as described under **ASSAY METHODS**. None of the calibrators showed a significant response with 250 pmol/L bGAD₆₅, but with 12.5 pmol/L bGAD₆₅ the signal decreased with increasing antibody concentrations. Repeating the assay but adding 26 nmol of Protein A–dextran per milliliter of serum after incubation with the sample showed a further increase in the GAD₆₅:Ab-induced signal suppression (Fig. 1).

The calibrators were also assayed by a conventional sandwich ELISA in which bGAD₆₅ was first allowed to bind to streptavidin-coated wells. Serum calibrators diluted 10-, 100-, or 400-fold in assay buffer were then incubated in the wells for 1 h, and the wells were washed, incubated with goat-anti-human IgG–HRP conjugate, and washed again, after which the enzyme activity was determined. Although the observed absolute signals were proportional to the serum concentrations, the ratios of signals between the highest calibrator and the negative calibrator (signal/background) were independent of serum concentration and were consistently only ~1.6. No improvement was achieved by the use of various blocking reagents. Thus the assay sensitivity appeared to be limited by the inability to affect the ratio of rates of nonspecific IgG and specific GAD₆₅:Ab binding by varying the serum concentration.

**ASSAY REPRODUCIBILITY**

A set of five serum calibrators assayed in triplicate was run on nine different plates over a period of 14 days with the DELISA procedure using Protein A–dextran. The bGAD₆₅ was stored between runs at −70 °C and thawed at room temperature before use. The assay response of each of the four positive calibrators was recorded as a fraction of the negative calibrator signal to
Serum calibrators were incubated with 250 pmol/L bGAD\textsubscript{65} without use of Protein A–dextran (○), or with 12.5 pmol/L bGAD\textsubscript{65} with (□) or without (●) subsequent incubation with Protein A–dextran. The average of triplicate measurements of each calibrator is given as a percent of the negative calibrator response. The assay protocol was as described in Methods and Materials.

Adjust for day-to-day differences in temperature and photomultiplier response. No significant drift was observed for any of the calibrators (Fig. 2). The SD of the normalized signals within a single plate and between plates over the course of the study did not differ significantly (Table 1). More recent data (not shown) suggest that similar performance can be obtained with bGAD\textsubscript{65} stored at 4 °C.

ASSAY RESPONSE TO DIFFERENT ANTIBODIES

Serum calibrators and calibrators prepared by diluting MICA, a human monoclonal islet cell antibody [29], or GAD\textsubscript{65}MAb, a mouse monoclonal GAD\textsubscript{65} antibody, into a pool of 10 negative sera were assayed by DELISA with use of 12.5 pmol/L bGAD\textsubscript{65} and incubation with Protein A–dextran. The MICA calibration curve was similar in shape to that for GAD\textsubscript{65}MAb but displaced to higher concentrations. Unlike the serum calibrators, there was no concentration at which either of the MAbs adsorbed all of the bGAD (Fig. 3). This was not unexpected, because MAbs bind to a single epitope, and any damage to that epitope upon biotinylation would prevent quantitative uptake of bGAD\textsubscript{65}. To compare the assay responses to the various antibodies, we had to analyze the signals as a percentage of the actual modulation range rather than as a percentage of the negative calibrator signal. We could then determine the antibody concentration and dissociation constants from the DELISA calibration curves by a modified Scatchard analysis. By plotting the derived concentration of the calibrators rather than the protein concentrations, the MICA, GAD\textsubscript{65}Mab, and serum calibrator curves were nearly superimposable (Fig. 4). Unexpectedly, only ~25% of the GAD\textsubscript{65}Mab protein was found to be active antibody, and only ~9% of the MICA protein was active. The dissociation constants for GAD\textsubscript{65}Mab, MICA, and the serum calibrator pool all fell in the range of 1.6 to 3.3 pmol/L. Assuming that a signal

![Fig. 1. Effect of Protein A–dextran and bGAD\textsubscript{65} concentrations on the DELISA signal.](image1)

![Fig. 2. Reproducibility of serum calibrators at various relative concentrations of GAD\textsubscript{65}Ab assayed in triplicate over 2 weeks.](image2)

![Fig. 3. DELISA response as a percent of negative calibrator signal for MICA (□) and mouse GAD\textsubscript{65}Mab (•) calibrators. The MICA calibrators were provided as IDW samples (see next section of text) and the stated concentrations are given. The Mab calibrator concentrations are based on total protein.](image3)

### Table 1. Reproducibility of triplicate DELISA GAD\textsubscript{65} determination of serum calibrators on 9 microtiter plates.

<table>
<thead>
<tr>
<th>Serum dilution</th>
<th>Mean signal*</th>
<th>Within plates</th>
<th>Between plate</th>
</tr>
</thead>
<tbody>
<tr>
<td>1:10 000</td>
<td>89</td>
<td>5.7</td>
<td>4.2</td>
</tr>
<tr>
<td>1:2500</td>
<td>59</td>
<td>3.2</td>
<td>5.0</td>
</tr>
<tr>
<td>1:1000</td>
<td>35</td>
<td>1.8</td>
<td>3.7</td>
</tr>
<tr>
<td>1:100</td>
<td>2.7</td>
<td>0.3</td>
<td>0.6</td>
</tr>
</tbody>
</table>

* Percent of the negative calibrator signal.
that differs from a negative signal by >3 SD (~0.15) can be considered significant, the detection limit for these antibodies was ~0.5 ng/mL (3 pmol/L).

**APPLICATION OF DELISA TO PATIENTS’ SAMPLES**

Sample sets from presumably normal adult controls were obtained from Irvine Scientific and the University of Florida. The average signals from each set, relative to a negative pooled serum calibrator (= 100), were 99 ± 5 (n = 20) and 106 ± 4 (n = 19), respectively. Control samples from normal children (also obtained from Florida) had an average signal of 94 ± 8 (n = 30). The Florida samples had been stored frozen for as long as 10 years. The Irvine controls were from recent blood donors and were stored frozen until use.

Samples provided by The Second International Diabetes Workshop of GAD Antibody Measurement Standardization (IDW) [24] were analyzed in a blind study in which all samples, calibrators, and controls were measured in duplicate. The samples were received lyophilized and were rehydrated overnight with distilled water at 4 °C before assay. A cutoff value to distinguish negatives from positives was arbitrarily set at 80% of the signal from the negative calibrator. The results are illustrated in Fig. 5. GAD$_{65}$Ab were detected in 32 of 39 IDDM samples; 30 of 32 control sera were correctly identified as negative; 4 ICA-positive sera from pre-IDDM patients all showed high concentrations of GAD$_{65}$Ab; 4 nondiabetic patients with Graves disease (an autoimmune disorder) all gave negative responses; and all 22 calibrators were correctly identified. The RBA judged to perform best [24] had the same sensitivity as DELISA but correctly identified all the controls; otherwise, DELISA outperformed most of the other 26 RBA assays in the study. None of the 19 ELISAs in the study gave comparable results (Table 2).

**Discussion**

Antibodies are frequently assayed by incubating the sample with a surface-bound antigen and detecting the total bound IgG with labeled anti-immunoglobulin antibody. Theoretically, the highest signal-to-background ratio is obtained during the initial incubation period, during which the ratio of the rates of specific to nonspecific binding remains constant. As the reaction approaches equilibrium, the rate of specific binding drops and the signal-to-background ratio becomes less favorable. Increasing the concentration of the sample increases the rates of both specific and nonspecific binding proportionately without affecting the ratio. The insensitivity of the GAD$_{65}$Ab ELISA signal-to-background ratio to the serum concentration in the present study was consistent with this prediction.

One way to reduce the background signal is by using a labeled antigen in place of a labeled anti-immunoglobulin to detect bound antibody. However, excess antigen, if present, can

![Fig. 4. GAD$_{65}$Ab response relative to the total modulatable range of the assay.](image)

**Table 2. Comparison of DELISA and best ELISA and RBA results in the 2nd International GAD Antibody Workshop.**

<table>
<thead>
<tr>
<th></th>
<th>Negative controls (n = 32)</th>
<th>IDDM patients (n = 39)</th>
<th>ICA-positive pre-IDDM (n = 4)</th>
<th>Other autoimmune (n = 4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean RBA</td>
<td>10.6</td>
<td>76.2</td>
<td>97.1</td>
<td>3.8</td>
</tr>
<tr>
<td>Best RBA</td>
<td>0</td>
<td>82.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>DELISA</td>
<td>6.3</td>
<td>82.1</td>
<td>100</td>
<td>0</td>
</tr>
<tr>
<td>Best ELISA</td>
<td>3.1</td>
<td>64.1</td>
<td>100</td>
<td>25</td>
</tr>
</tbody>
</table>

* Results given as percent positive in each category. Best performance judged by setting cutoff to obtain specificity of 100% and comparing "adjusted" sensitivities [24].

b ICA-positive data, personal communication from R. Schmidli.
induce dissociation of weakly bound antibodies by competitive binding and reduce the specific signal. For this reason, the most sensitive antibody assays are based on immunoprecipitation [23, 24]. Excess labeled antigen is allowed to bind to antibody in solution with no opportunity for competitive binding. All of the immunoglobulin, including that bound to antigen, is then adsorbed on a solid phase or precipitated. The solid phase is thoroughly washed to remove nonspecifically bound antigen, and the specifically bound antigen is detected. Unfortunately, this method is quite labor intensive and usually requires the use of isotopic labels.

The present depletion ELISA differs from immunoprecipitation in two ways. First, a soluble immunoglobulin-binding reagent, Protein A–dextran, is used that sequesters the bound antigen without a separation step. The need for washing the bound antigen is thus eliminated without sacrificing sensitivity. Second, the very low antigen concentration used minimizes nonspecific binding of the antigen and assures that the largest possible fraction of the antigen becomes bound to antibody, even though the absolute amount of bound antigen is very low.

This configuration is consistent with the binding equation, which predicts that the fraction of antigen that is bound at equilibrium is maximized when the antigen concentration is less than both the antibody concentration and the antibody dissociation constant. For very-high-affinity antibodies the detection limit therefore depends primarily on the ability to detect the antigen. In the present procedure, depletion of ~10% of the 12.5 pmol/L bGAD65 used in the assay, ~1 pmol/L, was reliably detected. Because the DELISA does not develop a high background signal in the presence of excess immunoglobulins, it was possible to use up to 50% serum in the initial binding step and thus take full advantage of this high sensitivity.

For low-affinity antibodies the detectability by DELISA is limited by the dissociation constant. Although the use of an antigen excess theoretically avoids this limitation, in practice the accompanying increase in nonspecific binding and the dissociation of weakly bound antigen during washing outweighs any theoretical advantages. An additional limitation of the method is that the use of very low antigen concentrations prevents the assays from being fully quantitative. The amount of antibody in the sample can only be stated to be equal to or greater than the total antigen uptake.

The effect of Protein A–dextran to significantly decrease the binding of bGAD65–antibody immune complex to the streptavidin–coated wells is presumably a result of steric interference produced by the dextran. Those antibody samples that inhibited the bGAD65 signal with no Protein A–dextran probably bound to the same epitope as the HRP–GAD65Ab conjugate and blocked binding of the conjugate.

Of critical importance in using the present assay to predict the onset of IDDM is establishing a cutoff value for differentiating normal and affected populations. Putatively normal sample sets from three locations—the Peninsula Blood Bank in northern California, Irvine Scientific in southern California, and the University of Florida—were assayed at various times. Although the responses among each set were self-consistent over time, the different sample sets had significantly different mean assay responses, due possibly to differences in sample population, sample collection, or storage. The low cutoff of 80% of the negative calibrator signal used for the IDW samples provided a margin of safety that would account for these disease-unrelated variables. Among all of the IDW samples, this cutoff provided 94% specificity and 84% sensitivity (excluding the calibrators, all of which were correctly identified). Because of the relatively small dynamic range of the assay, the overall assay performance does not strongly depend on the cutoff value used. A 75% cutoff appeared to give the best overall performance (Fig. 6).

In summary, this work provides a simple, accurate, and automatable method for the assay of GAD65Ab and thus makes possible wide-scale testing of this important analyte for prediction of onset of IDDM. The assay is being evaluated in several laboratories; in one study it has been found to be highly useful as a screen for normal school children [30]. Further evaluation will be required to fully establish the general utility of GAD65Ab as a diagnostic marker.

We acknowledge M. Erlander, D. Kaufman, and A. Tobin at UCLA for making available GAD65-containing plasmids PBbGAD65-2 and pSKGAD65-5; J. Barnett, J. Chow, B. Nguyen, and E. Osen at Syntex for their help in baculovirus culture; N. Maclaren and M. Atkinson at the University of Florida for providing clinical samples; and B. Irvin and E. Jakobovits at Behring Diagnostics Inc. (formerly Syva Co.) for help with data analysis and cloning of the GAD65 gene obtained from UCLA, respectively.

![Fig. 6. Correlation of DELISA data for separate lyophilized aliquots of the same IDW samples stored at −20 °C and determined a year later.](image-url)

The data are given as a percent of the negative calibrator signal. The first calibrator set was prepared from a patient's sample diluted into pooled sera from the Peninsula Blood Bank. The second calibrator set was prepared from the same sample diluted into pooled sera from Irvine Scientific. The signals were corrected to take into account differences in the calibrator responses. IDDM patients (●), negative controls (□). Broken lines, 75% cutoff.
References


