Time-resolved Fluorometric Assay for Detection of Autoantibodies to Glutamic Acid Decarboxylase (GAD65)

Matti Ankelo,1* Annette Westerlund-Karlsson,1 Jorma Ilonen,2 Mikael Knip,3,4 Kaisa Savola,5 Pasi Kankaanpää,6 Liisa Meriö,6 Harri Siitari,6 and Ari Hinkkanen1

Background: Type 1 diabetes mellitus results from destruction of the pancreatic insulin-producing beta cells by a chronic autoimmune process. Methods are needed for the detection of circulating autoantibodies to glutamic acid decarboxylase (GAD65), a major marker of this process.

Methods: Streptavidin-coated microtiter plates were incubated with biotinylated GAD65, and after incubation with serum samples from patients with type 1 diabetes mellitus and control individuals, europium-labeled GAD65 was added. After washing steps, the delayed fluorescence was measured in duplicate in a fluorometer. Samples collected from 100 patients with newly diagnosed type 1 diabetes mellitus and 100 healthy controls were measured by the new assay and by a radiobinding assay.

Results: The detection limit of the new assay was 1.49 WHO units/mL, the calibration curve was linear to 4 140 WHO units/mL, and no hook effect was observed up to 41 400 WHO units/mL. The intraassay CV was 2.1–6.3% over the calibration range. For patient serum samples, the intraassay, interassay, and total CVs were 5.4–7.0%, 9.8–13%, and 12–14%, respectively. Compared with conventional radioimmunologic methods, the analytical range was broader and the analysis time required to perform the measurements was shorter. At a cutoff with 99% specificity, the new assay and the radiobinding assay were positive in 71 and 67 patients, respectively.

Conclusions: The new assay provides a rapid and sensitive nonradioactive method applicable for large-scale screening for beta-cell autoimmunity. It has a broad linear analytical range, is easy to perform and automate, and has sensitivity and specificity comparable to those for the conventional radioisotope assay.

Type 1 diabetes mellitus is thought to result from an autoimmune process in which the insulin-producing beta cells of the pancreas are destroyed. It now seems that the appearance of autoantibodies to beta-cell antigens, such as those against the 65-kDa isoform of glutamic acid decarboxylase (GAD65)7 and the protein tyrosine phosphatase-like insulinoma associated protein 2 (IA-2) in the peripheral circulation is a predictive sign of clinical disease. GAD65 antibodies are one of the major markers of the disease, and can be used for disease prediction in nondiabetic individuals (1, 2) and for identification of the type of diabetes, e.g., in patients with late-onset autoimmune diabetes (LADA) (3, 4). An approach aimed at preventing the progression to clinical disease would be preferable to replacement therapy with exogenous insulin in overt disease. This requires the identification of markers that predict with high reliability progression to clinical type 1 diabetes. Genetic screening of individuals for certain HLA risk alleles, combined with the analysis of

---

1 Department of Biochemistry and Pharmacy, Åbo Akademi University, FIN-20521 Turku, Finland.
2 Department of Virology, University of Turku, FIN-20520 Turku, Finland.
3 Hospital for Children and Adolescents, University of Helsinki, FIN-00029 HUS, Finland.
4 Department of Pediatrics, Tampere University Hospital, FIN-33320 Tampere, Finland.
5 Department of Pediatrics, University of Oulu, FIN-90014 Oulu, Finland.
6 PerkinElmer Life and Analytical Sciences, Wallac Oy, FIN-20101 Turku, Finland.

*Address correspondence to this author at: Department of Biochemistry and Pharmacy, Åbo Akademi University, PO Box 66, FIN-20521 Turku, Finland. Fax 358-2-215-4745; e-mail mankelo@abo.fi.

Received November 5, 2002; accepted March 10, 2003.

---

7 Nonstandard abbreviations: GAD65, 65-kDa isoform of glutamic acid decarboxylase; LADA, late-onset autoimmune diabetes in adults; GADA, autoantibody to GAD65; IA-2, protein tyrosine phosphatase-like insulinoma associated protein 2; IA-2A, autoantibody to IA-2; RBA, radiobinding assay; DTTA, N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N1,N2,N3,N3-tetraacetic acid; RU, relative unit(s); TPO, thyroid peroxidase; Tg, thyroglobulin; and DASP, Diabetes Autoantibody Standardization Program.
autoantibodies to GAD65 (GADAs) and IA-2 (IA-2As), allows selection of individuals for whom preventive treatment may be considered. It is therefore important to develop sensitive and rapid methods to assess the presence of autoantibodies in body fluids of individuals belonging to risk groups. Although GADAs and IA-2As may not be directly involved in the pathogenic processes in beta-cell destruction, they are good markers in assessing the risk of disease manifestation. It has become evident that the simultaneous presence of more than one autoantibody type, such as GADAs, IA-2As, and insulin autoantibodies together with islet cell autoantibodies (ICAs), is highly predictive of progression to clinical disease, although the time point of disease presentation cannot yet be predicted reliably (5).

Several different methods have been developed for the detection of GADAs, and some assays are available commercially. A radiobinding assay (RBA) has been widely used and has been demonstrated as reliable in the past (6, 7). In the RBA, the antigen is produced in vitro in the presence of a radioactive label, usually [35S]methionine. Aliquots of the antigen are allowed to react with serum samples, and the immune complexes are captured with protein A Sepharose and counted. Improved RBA methods have been published (8, 9). We have used a GAD65-IA-2 fusion protein in the detection of GADAs and IA-2As (10), and alternative radioimmunologic methods use an 125I-labeled GAD65 (11–13). Although widely used, these methods suffer from complicated reagent synthesis and long measurement times. In addition, formats in which gel beads are used to capture immune complexes do not allow automation at efficiencies similar to those for solid-phase methods. ELISA methods have also been established (14–17). Wild et al. (15) developed an ELISA method that gave a disease sensitivity of 69% and a specificity of 98%.

Time-resolved fluorometry and fluorescent lanthanide chelate labels were introduced in immunoassays 20 years ago (18, 19). The fluorescence of the lanthanides, e.g., europium (Eu3+), is long-lived and allows differentiation of the short-lived background fluorescence of biological material, plastics, and optics (20). The high specific activity of the label enables immunoassays with a low detection limit while minimizing the nonspecific binding of the labeled bioaffinity molecule (18, 21). A time-resolved fluorescence imaging method for the quantification of ICAs on pancreas sections has been published (22). In the present communication we describe the development of a rapid and convenient fluorometric method based on time-resolved fluorescence of Eu3+ chelate (23) for the analysis of GADAs and compare the data with those obtained with a conventional RBA.

**Materials and Methods**

**REAGENTS AND INSTRUMENTATION**

GAD65 protein was purchased from Diamyd Diagnostics AB and stored in aliquots at −70 °C until used. The protein is a recombinant human 65-kDa GAD isoform, expressed via baculoviral infection of Spodoptera frugiperda (Sf9) insect cells.

Biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester was purchased from Sigma. Streptavidin-coated microtiter plates, N1-(p-isothiocyanatobenzyl)-diethylenetriamine-N2,N3,N4,N5-tetraacetic acid (DITTA)-europium chelate, Delfia Assay Buffer, Wash Solution, Enhancement Solution, and the europium calibration solution for the fluorometric assays were obtained from PerkinElmer Life and Analytical Sciences Wallac Oy. NAP-5 gel filtration columns were purchased from Amersham Pharmacia Biotech. All other reagents were analytical grade.

The Delfia 1234 Plate Fluorometer, Delfia Plateshake, and Delfia Platewash are products of PerkinElmer Life and Analytical Sciences Wallac Oy.

**SAMPLE AND CALIBRATION MATERIAL**

We analyzed 100 serum samples from children and adolescents with newly diagnosed type 1 diabetes (53 males; mean age, 11.5 years; range, 7.2–15.9 years) and 100 control individuals (52 males; mean age, 11.5 years; range, 7.1–15.9 years). The patient samples were taken at the time of diagnosis before starting insulin therapy. For calibration, a local serum pool highly positive for GADA and a GADA-negative serum pool were used. WHO Reference Reagent (Islet Cell Antibodies 97/550) (24) was purchased from National Institute for Biological Standards and Control (United Kingdom).

**BIOXYLATION AND EUROPYUM LABELING OF GAD65**

Biotinylation was performed at room temperature for 4 h in a solution containing 50 mmol/L HEPES (pH 7.4) and 9 g/L NaCl. A 30-fold molar excess of biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester was used. The biotinylated GAD65 was separated from free biotinylation reagent by gel filtration on a NAP-5 column. An elution buffer containing 50 mmol/L HEPES (pH 7.4), 9 g/L NaCl, and 0.5 g/L Na3 was used.

Europium labeling of GAD65 was performed at 4 °C for 16–20 h with a 50-fold molar excess of DITTA-europium chelate in 50 mmol/L NaHCO3-Na2CO3 buffer (pH 9.3) containing 0.9 g/L NaCl. The Eu3+-labeled GAD65 was purified by gel filtration on a NAP-5 column as above. The number of Eu3+ chelates covalently bound to one GAD65 molecule (labeling degree) was typically 10–17 when measured against a Eu3+ calibration solution.

**GADA TIME-RESOLVED FLUOROMETRIC PROCEDURE**

The fluorometric assay was performed in streptavidin-coated microtiter strip wells according to the following three-step procedure: In each well, 75 ng of biotinylated GAD65 in 100 μL of Delfia Assay buffer was incubated for 45 min at room temperature with continuous shaking on a Delfia Plateshake. The wells were washed once with Delfia Wash Solution on a Delfia Platewash. We then
added 50 μL of Delfia Assay buffer and 10 μL of serum sample or calibrator to each well and incubated the strips for 45 min at room temperature on a Plateshake. After two washes, we added 25 ng of Eu\(^{3+}\)-labeled GAD65 in 75 μL of Delfia Assay buffer and continued the incubation for 30 min at room temperature with continuous shaking. After four washing rounds, we added 100 μL of Delfia Enhancement solution to each well and incubated the strips on a Plateshake for 15 min at room temperature. The fluorescence was measured in a Delfia 1234 Plate Fluorometer. All samples were analyzed in duplicate. The Wallac Multicalc program was used for calculating the results. Linear weighted regression was used for calibration curve fitting. The design of the assay is presented schematically in Fig. 1.

The calibrators were obtained by diluting a highly GADA-positive serum pool in series with a GADA-negative serum pool. The 10-fold dilution was chosen to represent 100 relative unit(s)/mL (RU/mL). The calibrators were also calibrated against WHO reference material 97/550. One RU corresponded to 41.4 units of WHO 97/550.

**COMPETITIVE INHIBITION**

GAD65 protein to be used as a competitor was diluted in 50 mmol/L Tris-HCl (pH 7.4) containing 1 g/L bovine serum albumin. Increasing amounts of GAD65 (from 0 to 100 ng per well) in 5 μL were incubated for 45 min at room temperature together with 10 μL of serum sample and 50 μL of Delfia Assay buffer in each well. Otherwise the GADA fluorometric assay was performed as described above.

**RBA**

The RBA was performed in the Research Laboratory, Department of Pediatrics, University of Oulu, as described previously (25). Briefly, RNA was obtained from the plasmid pGEM3 containing the human GAD65 gene in an in vitro transcription-translation reaction, where the RNA was translated in the presence of \[^{35}S\]methionine. Radioactive GAD65 protein aliquots (20,000 cpm) were incubated overnight in a 96-well microtiter plate at 4 °C with serum samples (2 μL) in Tris-buffered Tween (50 mmol/L Tris, 150 mmol/L NaCl, 1 g/L Tween-20, pH 7.4); the total volume was 50 μL. The immune complexes were isolated by adding 7.5 mg of protein A Sepharose (Amersham Pharmacia Biotech), diluted to a total volume of 100 μL with Tris-buffered Tween, to each well. After washing, the bound activity was measured in a liquid scintillation counter (1450 Microbeta Trilux; PerkinElmer Life Sciences Wallac Oy). The results were expressed in RU/mL based on a calibration curve constructed from the dilution of a pool of highly GADA-positive samples with a negative sample. According to the definition, the RU in the RBA assay were not equal to the RU in time-resolved fluorometric assay. The cutoff limit for antibody positivity in the RBA was set at the 99th percentile for 373 nondiabetic Finnish children and adolescents, i.e., 5.35 RU/mL corresponded to 14.13 WHO units/mL.

**Results**

**ASSAY OPTIMIZATION**

During assay development, we tested a large number of different assay conditions and their combinations. For example, for the biotinylation and labeling of GAD, the GAD concentration, the molar excess of labeling reagent, reaction temperature, time, and pH were optimized. In addition, the number of reaction steps, the amounts of the different reaction components, reaction time and volume, sample volume, number of washing steps, volume of enhancement solution, and other conditions were defined. The influence of different combinations on assay performance was tested.

**SPECIFICITY OF ANTIBODY BINDING**

The specificity of GADA binding was investigated by competitive inhibition experiments using pure GAD65 protein as a competitor. Eight GADA-positive sera were tested with increasing amounts of GAD65 in the sample incubation step of the fluorometric assay. GADA binding was totally inhibited and the fluorescence signal decreased to background when 100 ng of GAD65 per well was used. One sample had an extremely high GADA concentration and was diluted 100-fold before inhibition could be achieved (Fig. 2).

**ASSAY PERFORMANCE CHARACTERISTICS**

The calibration curve was linear over the whole measurement range (0.1–100 RU/mL), and no high-dose hook effect was observed up to 1000 RU/mL.
Reproducibility. The intraassay CV was 2.1–6.3% (n = 12) over the calibration range.

The total CVs for the GADA time-resolved fluorometric assay were determined in 20 runs (four replicates per run), using four serum samples that represented clinically relevant GADA concentrations. Two persons performed the analyses independently during more than 2 weeks, using two different reagent lots and pipetting schemes. The results are summarized in Table 1. The mean intraassay, interassay, and total CVs were 6.3%, 11%, and 13%, respectively.

Limit of detection. The detection limit of the time-resolved fluorometric assay was typically 0.036 RU/mL, corresponding to 1.49 WHO units/mL, when defined as the signal + 3 SD for a negative serum.

Linearity. Four patient serum samples in the range 1.25–36.45 RU/mL were serially diluted with 50 mmol/L Tris-HCl (pH 7.8) buffer containing 9 g/L NaCl and 1 g/L bovine serum albumin. The observed vs calculated GADA concentrations were determined in four different dilutions. Each dilution was measured twice in two replicates. The mean of all observed values was 105% of the calculated values, and the total range was 92–124%. The mean results for each of the four sample dilution series were 98–114%, and the linear regression coefficient (r) was >0.9989 for all of the series.

Recovery. Three different concentrations (0.5–12.5 RU/mL) of monoclonal anti-GAD antibody MAB351R (Chemicon International) were added to three serum specimens containing a known amount of GADA. The mean recovery was 95%, and recovery range was 86–104% when three replicates were used in each assay.

Cross-Reactivity. Anti-thyroid peroxidase (TPO) and/or anti-thyroglobulin (Tg) autoantibodies have been shown to coincide with GAD65 autoantibodies (26). When known concentrations (0.5–12.5 RU/mL) of monoclonal anti-GAD antibodies were added to serum samples containing known concentrations of anti-TPO or anti-Tg autoantibodies, serum samples with anti-TPO autoantibodies showed little and samples with anti-Tg autoantibodies showed no cross-reactivity. The mean recoveries of anti-GAD antibodies were 69% and 99%, respectively.

Interference. No interference was observed when the GADA assay was run with hemolytic sera (0.5–10 g/L), lipemic sera (0.25–10 g/L), and sera containing rheumatoid factors (n = 5). The mean GADA recoveries were 102%, 97%, and 91%, respectively.

Clinical Results

We assayed 100 sera from individuals with newly diagnosed type 1 diabetes and 100 sera from healthy control individuals blindly in two independent laboratories with the time-resolved fluorometric method. As a comparison, the samples were also analyzed for the presence of GADAs in the conventional in vitro RBA. The distribution
of GADAs in the samples based on the results obtained in our laboratory is presented in Fig. 3. The mean GADA value was 21.10 RU/mL in the patients with type 1 diabetes and 0.074 RU/mL in the healthy controls in the GADA time-resolved fluorometric assay. The median values were 1.028 and 0.049 RU/mL, respectively. The cutoff was set at the 99th percentile in the present series of 100 healthy control children, i.e., 0.404 RU/mL, corresponding to 16.73 WHO units/mL. The ROC curves for the GADA time-resolved fluorometric assay and RBA results are shown in Fig. 4. The areas under the curves were 0.94 [95% confidence interval (CI), 0.91–0.97] for the time-resolved fluorometric assay and 0.98 (95% CI, 0.97–1.00) for the RBA. According to the cutoff definition (the 99th percentile), both the time-resolved fluorometric assay and the RBA methods detected 1 GADA-positive individual among the 100 controls. Among the 100 patients with type 1 diabetes, 71 and 67 were GADA-positive in the time-resolved fluorometric assay and RBA, respectively. Two samples weakly positive in the RBA were not detected by the time-resolved fluorometric assay, and 65 individuals were positive with both methods. Additionally, the time-resolved fluorometric assay classified as positive six individuals who were negative in the RBA. All of them, except for one clearly positive, were slightly positive in the time-resolved fluorometric assay and RBA, respectively.

Two samples weakly positive in the RBA were not detected by the time-resolved fluorometric assay, and 65 individuals were positive with both methods. Additionally, the time-resolved fluorometric assay classified as positive six individuals who were negative in the RBA. All of them, except for one clearly positive, were slightly...
above the cutoff limit for GADA positivity. Fig. 5 shows the comparison of GADA detection by the time-resolved fluorometric and RBA methods. The present cutoff limit of the time-resolved fluorometric assay is probably not optimal, however, because the final adjustment will require measurement of several hundreds of healthy controls and careful analysis of age-dependent variation in the background values.

In addition to the Finnish samples, we analyzed two sets of serum samples from the Diabetes Autoantibody Standardization Program (DASP) 2002, in total 50 patients with type 1 diabetes and 100 controls. Our results showed satisfactory performance characteristics with a sensitivity of 84% (95% CI, 0.72–0.92) and a specificity of 94% (95% CI, 0.88–0.97). In comparison, the RBA achieved 82% sensitivity (95% CI, 0.69–0.90) and 98% specificity (95% CI, 0.93–0.99). Among the GADA assays used by the DASP 2002 participants, our method was the only one based on fluorometry.

**Discussion**

Standardization of assay conditions for the detection of diabetes-associated autoantibodies and reporting of the results in uniform international units is critical to research and the development of assays for the autoantibodies. The fact that sizeable variation in estimated GADA concentrations in standard serum samples was observed between laboratories (24) emphasizes the importance of using reproducible methodologies with internal and external standardization to obtain comparable interlaboratory results. Accordingly, the differences between various assay systems are of critical importance. A rapid, simple (minimum number of steps and reagents), and stable platform allows the minimization of variables and is thus more likely to generate reproducible results. Such a method and a uniform specimen collection method would provide tools for common standardization and allow direct comparison of results obtained in different laboratories. The use of WHO standard serum 97/550 was shown to markedly reduce the interlaboratory variability in the measurement of diabetes-associated autoantibodies (24).

There is little that can be done to cure the disease at the time of diagnosis of type 1 diabetes because most of the beta-cell damage has already occurred. Screening for diabetes-associated autoimmunity, especially in the general population, has several problems. The huge number of samples to be screened is further increased by the need for multiple repeated tests because autoantibody seroconversion can take place at any age, and the early introduction of preventive treatment may be essential for its effectiveness. Previous genetic screening for identification of the population at genetic risk can markedly reduce the number of samples tested, although the sensitivity of genetic testing needs further development. Screening of diabetes-associated autoantibodies in relatives of patients with type 1 diabetes has been a valuable tool in predicting progression to clinical disease and can be used for the identification of high-risk individuals for intervention studies aimed at prevention of overt type 1 diabetes.

Reliable disease prediction is vital for prevention and at the moment is a target of several trials (27). The combination of GADA and IA-2A testing is a sensitive way to identify active beta-cell autoimmunity (28). In addition to predicting type 1 diabetes, GADA assays are becoming more important in the prediction of disease progression in adult patients initially considered to have type 2 diabetes. GADA positivity has been shown to be useful in the prediction of progression to insulin dependency in adult patients with diabetes (4, 29).

The specificity and sensitivity of antibody assays are important parameters that can vary significantly, depending on the method and performance characteristics of the assay. The specificity of the prediction based on a disease marker reflects the probability that a person testing negative for that marker would remain unaffected. The higher the threshold for antibody positivity is in relation to values in the general population, the more specifically the autoantibody assay identifies affected patients, but at the cost of excluding patients with low autoantibody concentrations. The sensitivity of the prediction based on a disease marker reflects the probability that all individuals who develop the disease test positive for the marker (2, 28). Improvement of the performance characteristics of autoantibody assays improves their predictive value.

According to the DASP 2002 results, the reliability of the new time-resolved fluorometric assay method is close to that of the traditional in vitro RBA, but the fluorometric assay is faster and the linear range is wider. The shape of the distribution for the correlation of fluorometric assay and RBA results (Fig. 5) shows that the RBA has limited capacity at high GADA concentrations. Calculation of the optimum threshold from the ROC curves (Fig. 4) gave a value of 177 for the maximum sum of sensitivity and specificity for the time-resolved fluorometric assay, which corresponds to a sensitivity/specificity pair (85%/92%) that correlates well with the DASP 2002 results (84%/94%). For the RBA assay, the sum obtained, 189 (94%/95%), is higher than the sum of 180 (82%/98%) obtained in the DASP 2002 workshop and the sum of 182 obtained with the RBA in the DASP 2001 workshop. In that workshop, our RBA had the third best performance characteristics of 46 laboratories participating.

The area under the ROC curve (Fig. 4) was 0.94 for the time-resolved fluorometric assay and 0.98 for the RBA, showing that the diagnostic accuracy of both assay methods was excellent.

In the traditional solid-phase ELISA, the antigen (or antibody) is usually coated directly to the surface. This may alter the properties of the antigen, as we noticed in the early development phase of our GADA time-resolved fluorometric assay. We now use streptavidin coating, and the assay takes advantage of the bivalent nature of the antibody. The biotinylation and labeling conditions of GAD65 have been carefully optimized to achieve the best...
analytical performance. Biotin, the spacer, and the Eu$^{3+}$ label molecules are very small compared with GAD. Detection in the ELISA involves an enzyme-labeled antibody, often anti-human IgG antibody. When we tried to use europium-labeled anti-human IgG in the detection step of the fluorometric assay, problems related to nonspecific binding arose that could not be solved. Thus, in the fluorometric assay, the physicochemical conditions of the assay are closer to those in the RBA than to the ELISA conditions.

The novel time-resolved fluorometric GADA assay described here provides a rapid and sensitive method for large-scale screening for beta-cell autoimmunity. Compared with previous methods, it is simple to automate and can reduce reagent and labor costs. The pipetting steps can easily be automated, which is not readily done when handling solid gel material. The biggest advantages of this method are its ease and the reduced analysis time, which enable high throughput of samples. Quantitative, reproducible results are available in 3 h. The assay also has a wider linear analytical range. Moreover, the reagents are stable over long periods of time, and no harmful radioactive material is needed. This novel assay should theoretically recognize all autoantibody isotypes and IgG subclasses, whereas methods that use protein A for immunoprecipitation or labeled anti-human IgG for detection have limitations in this respect.

PerkinElmer Life and Analytical Sciences Wallac Oy and the National Technology Agency of Finland (TEKES) supported this study. We thank Kirs Pakkanen and Susanna Heikkilä for excellent technical assistance. All authors are members of the JDRF Center for Prevention of Type 1 Diabetes in Finland.

References


23. Siitari H, Turunen P, Schrímsher J, Nunn M. New sensitive and


