Detection of Autoantibodies to Protein Tyrosine Phosphatase-like Protein IA-2 with a Novel Time-resolved Fluorimetric Assay

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Background: Circulating autoantibodies to pancreatic glutamic acid decarboxylase (GAD65; the 65-kDa isoform of glutamic acid decarboxylase), protein tyrosine phosphatase-like protein IA-2, and insulin can be used as predictive markers of type 1 diabetes. We developed a novel assay for the detection of IA-2 autoantibodies (IA-2As) in serum based on time-resolved fluorimetry, hypothesizing that this kind of assay could provide several advantages over methods described to date, including radiobinding assays (RBAs) and ELISAs.

Methods: The intracellular part of IA-2 (IA-2ic) was biotinylated and bound to streptavidin-coated 96-well plates by simultaneous incubation with serum samples and glutathione S-transferase (GST)-IA-2ic fusion protein. GST-IA-2ic captured by autoantibodies in the serum was detected with europium-labeled anti-GST antibody, and the signal was measured in a time-resolved fluorimeter. A serum sample panel from 100 patients with newly diagnosed type 1 diabetes and 100 unaffected controls was analyzed with the new assay and a conventional RBA.

Results: Among the 100 serum samples from patients with type 1 diabetes, the time-resolved fluorimetric assay identified 74 IA-2A-containing sera, whereas the RBA detected 80 IA-2A-positive samples. Five of the six samples positive in the RBA but not detected by the time-resolved fluorimetric assay were only weakly positive in the RBA. The performance time of the time-resolved fluorimetric assay was 2.5 h compared with 10–12 h required by the RBA.

Conclusions: The time-resolved fluorimetric assay provides a simple, nonradioactive analysis method for the detection of IA-2As with a specificity and a sensitivity comparable to the RBA method. This assay allows substantial reduction in performance time compared with the conventional RBA.

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Type 1 diabetes is an immune-mediated disease, in which the insulin-producing pancreatic β-cells are destroyed. Certain HLA alleles confer increased susceptibility to the disease (1). Results from animal models suggest that type 1 diabetes is T-cell mediated, and there is evidence of disease precipitation in the absence of autoantibodies (2). The appearance of autoantibodies specific for pancreatic islet cell antigens (ICAs) 6 in the peripheral circulation is, however, a sign of immune β-cell aggression. These autoantibodies can often be detected in at-risk individuals several years before clinical presentation of the disease (3). Initially, diabetes-associated autoantibodies were detected by immunofluorescence staining of pancreatic islets, but several autoantibodies can now be detected at the molecular level in serum samples, the most important autoantigens being the 65-kDa isoform of glutamic acid decarboxylase (GAD65), protein tyrosine phosphatase-like protein (IA-2), and insulin (4). The predictive characteristics of single autoantibodies vary, but combinations

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Footnote:
6 Nonstandard abbreviations: ICA, islet cell antibody; GAD65, 65-kDa isoform of glutamic acid decarboxylase; IA-2, protein tyrosine phosphatase-like protein; IA-2A, IA-2 autoantibody; IA-2ic, intracellular part of IA-2; RBA, radiobinding assay; GADA, GAD65 autoantibody; GST, glutathione S-transferase; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TBST, Tris-buffered saline-Tween; RU, relative unit(s); and DASP, Diabetes Autoantibody Standardization Program.
of autoantibodies strongly predict progression to clinical disease (5, 6). The individual variation is, however, conspicuous, and additional studies are needed to make the risk assessment more accurate on an individual basis. Knowledge of the autoantibody status is important because it provides the opportunity to specifically study various biochemical and environmental factors associated with the triggering of the autoimmune process months and years before the manifestation of clinical symptoms and signs.

Current methodology for the detection of IA-2 autoantibodies (IA-2As) includes a radiobinding assay (RBA), in which the intracellular part of the antigen (IA-2ic) is produced in vitro in the presence of [35S]methionine (7). The radioiodinated protein is allowed to react with serum samples, after which the immune complexes are captured with protein A Sepharose and the radioactivity is counted. In some studies, GAD65-IA-2ic fusion proteins have been used for the detection of GAD65 autoantibodies (GADAs) and IA-2As by RBA (8, 9). In addition, a RIA based on the use of 125I-labeled IA-2ic and immunoprecipitation has been developed (10). Other methods include ELISAs, in which the immobilized antigen captures antibodies from the sample and detection is achieved with labeled anti-human IgG antibody (11).

Fluorescent lanthanide chelate labels have been used in immunoassays for almost 20 years (12). One of the major advantages of lanthanide labels is the long-lived fluorescence, which allows detection of the specific signal without detection of short-lived background fluorescence from plastics and biological material. Fluorescent lanthanide labels have high specific activity, facilitating immunoassays with low detection limits (12, 13). Moreover, these reagents are stable over months. Here we describe the development of a fast, reliable, and easy-to-perform IA-2A assay based on time-resolved fluorimetry. The results of our time-resolved fluorometric assay are compared with data obtained with a previously established RBA that is routinely used for the detection of IA-2As in the Finnish Type 1 Diabetes Prediction and Prevention Study (7).

Materials and Methods

Serum Samples
We analyzed 100 serum samples from patients with newly diagnosed type 1 diabetes (53 males; mean age, 11.3 years; range, 4.5–15.9 years) and 100 control sera from nondiabetic individuals (52 males; mean age, 11.5 years; range, 7.1–15.9 years). The samples were taken from the patients at the time of diagnosis before starting exogenous insulin therapy.

Expression and Purification of the GST-IA-2ic Fusion Protein and IA-2ic
A DNA fragment encoding IA-2ic (amino acids 606–979) and a C-terminal polyhistidine tag was amplified by PCR from the plasmid pSP64/IA-2 (kindly donated by Dr. Ezio Bonifacio, San Raffaele Scientific Institute, Milan, Italy). The PCR product was ligated as a BamHI-EcoRI fragment in frame to the 3′ end of the glutathione S-transferase (GST) gene in the expression vector pGEX-6P-1 (Amersham Biosciences). The created IA-2icpGEX plasmid contained a linker providing a cleavage site for PreScission Protease (Amersham Biosciences) between GST and IA-2ic.

Escherichia coli BL21 cells (Amersham Biosciences) were transformed with IA-2icpGEX and grown in Luria broth (Life Technologies) containing 0.1 g/L ampicillin and 2 g/L d-glucose at 29°C. When the absorbance at 600 nm reached 0.6, protein expression was induced with 0.5 mmol/L isopropyl-β-d-thiogalactopyranoside (MBI Fermentas), and bacterial growth was continued for 2 h. The cells were harvested by centrifugation and stored at −70°C until used. For the extraction of soluble GST-IA-2ic, a cell pellet from 250 mL of bacterial culture was thawed in 3 mL of phosphate-buffered saline (140 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na2HPO4, 1.8 mmol/L KH2PO4, pH 7.3, containing Complete, Mini, EDTA-free Protease Inhibitor Cocktail Tablets (Roche Diagnostics; one tablet/10 mL of buffer), and 0.1 g/L soybean trypsin–chymotrypsin inhibitor (Bowman-Birk inhibitor; Sigma). The cells were disrupted by sonication (three times for 30 s; constant output level 2) in a Branson Ultrasonic Disruptor Sonifier II W-250, and insoluble material was removed by centrifugation at 2100g for 40 min at 4°C. Immediately before the protein purification step, 9 mL of cleared bacterial lysate (corresponding to 750 mL of culture) was centrifuged once more at 2100g for 40 min at 4°C; diluted to 30 mL with phosphate-buffered saline, pH 7.3, containing the protease inhibitors mentioned earlier; and applied onto a Glutathione Sepharose column (Amersham Biosciences) with a bed volume of 3 mL. After the column was washed with phosphate-buffered saline, pH 7.3, GST-IA-2ic protein was eluted with elution buffer (50 mmol/L HCl, 10 mmol/L reduced glutathione, pH 8.0). Glycerol was added as a stabilizing agent (final concentration, 100 mL/L), and the protein concentration was reduced to 2 g/L to prevent precipitation during storage at −18°C.

For purification of IA-2ic, GST-IA-2ic in 9 mL of cleared lysate (above) was bound to a Glutathione Sepharose column (bed volume, 3 mL), washed with cleavage buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1 mmol/L dithiothreitol, 1 mmol/L EDTA, pH 7.0), and cleaved in the column with 240 U of PreScission Protease for 16–18 h at 4°C. The eluate, which contained free IA-2ic, was collected and passed through a Glutathione Sepharose column to remove residual contamination of PreScission Protease, GST, and uncleaved fusion protein. Purified IA-2ic was stored in cleavage buffer at −70°C at a concentration of 0.6–0.9 g/L. If IA-2ic was stored at concentrations >1 g/L, partial precipitation of the protein occurred.

The quality of purified GST-IA-2ic and IA-2ic was...
confirmed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting with mouse Anti-His6 (Roche Diagnostics) and goat anti-GST (Amersham Biosciences) antibodies.

BIOTINYLATION OF IA-2ic

Purified IA-2ic was allowed to react with a 50-fold molar excess of biotinamidocaproic acid 3-sulfo-N-hydroxysuccinimide ester (Sigma) in 50 mmol/L HEPES, 9 g/L NaCl, pH 7.4, for 4 h at room temperature. Unconjugated biotinylation reagent was removed by gel filtration on a NAP-5 column (Amersham Biosciences) with 50 mmol/L HEPES, 9 g/L NaCl, 0.5 g/L sodium azide, pH 7.4, as elution buffer. The biotinylated protein was stored at 4 °C, either in solution or freeze-dried.

EUROPINIUM LABELING OF ANTI-GST ANTIBODY

Purified polyclonal goat anti-GST antibody (Amersham Biosciences) was labeled with a 50-fold molar excess of \( \text{N}^1-(\text{p}-\text{isothiocyanatobenzyl})\text{-diethylenetriamine-N}^1,\text{N}^2,\text{N}^3,\text{N}^3\text{-tetraacetic acid-Eu-chelate} \) (PerkinElmer Life and Analytical Sciences, Wallac Oy) in 100 mmol/L sodium carbonate buffer, pH 9.3, containing 9 g/L NaCl for 18 h at 4 °C. The labeled antibody was separated from free label with a Superdex 200 column (Amersham Biosciences). The molar labeling degree (the number of Eu\(^{3+}\) chelate molecules bound to one antibody molecule) was measured against a Eu\(^{3+}\) calibration solution (PerkinElmer Life and Analytical Sciences, Wallac Oy) and was calculated as \( \sim 8 \).

ASSAY PROCEDURE

The IA-2A assay was performed in streptavidin-coated 96-well microtiter plates. All of the reagents and equipment were from PerkinElmer Life and Analytical Sciences, Wallac Oy, and all of the incubations were performed at room temperature. In the first incubation, 10 \( \mu \)L of sample or calibrator was allowed to react with 40 ng of biotinylated IA-2ic (bio-IA-2ic) and 50 ng of GST-IA-2ic in 100 \( \mu \)L of Delfia Assay Buffer + 2 g/L casein (casein buffer) for 1 h with slow shaking on a Delfia Plate Shake. After four washing rounds with Delfia Wash Solution in a Delfia Plate Wash, 15 ng of Eu\(^{3+}\)-labeled anti-GST antibody in 100 \( \mu \)L of casein buffer was added to each well, and incubation was continued for an additional 30 min with slow shaking. The plates were washed four times, after which 200 \( \mu \)L of Delfia Enhancement Solution was added to the wells, and the plates were incubated for 5–15 min on a Plate Shake. The fluorescence was measured in a Delfia 1234 Plate Fluorometer or in a 1420 Multilabel Counter Victor\(^2\), and the Multicall computer program was used for calculation of the results. All of the samples were measured in duplicate, and the entire panel was analyzed in two different laboratories (Department of Biochemistry and Pharmacy, Åbo Akademi University and PerkinElmer Life and Analytical Sciences, Wallac Oy) under identical conditions without previous knowledge of disease or IA-2A status of the sample donors. The assay procedure is presented schematically in Fig. 1.

As a calibrator, we used a monoclonal human anti-IA-2

![Fig. 1. Principle of the IA-2A time-resolved fluorimetric assay.](image-url)
antibody (Roche Diagnostics; clone 96/3) (14) diluted in series in TSA buffer (50 mmol/L Tris-HCl, 9 g/L NaCl, 0.5 g/L sodium azide, pH 7.8) containing 75 g/L bovine serum albumin; 1 ng of antibody corresponded to 13 units of the 1st WHO Reference Reagent 1999 for Islet Cell Antibodies (National Institute for Biological Standards and Control code no 97/550).

COMPETITIVE INHIBITION

Studies on the competitive inhibition of the binding of IA-2As in seven IA-2A-positive serum samples (range, 6–310 µg/L) were performed by adding increasing amounts, 0–1000 ng/well, of unlabeled IA-2ic to the first incubation of the time-resolved fluorimetric assay (Fig. 1). One IA-2A-negative serum sample was used as a control. Detection of bound IA-2As with Eu³⁺-labeled anti-GST antibody was performed as described above.

RBA

All 200 of the serum samples had been analyzed by the previously established RBA (7) in the Research Laboratory, Department of Pediatrics, University of Oulu. Briefly, the plasmid pSP64poly(A) encoding the intracellular part (amino acids 605–979) of the human IA-2 protein was transcribed in an in vitro transcription-translation reaction (Promega) in which the ribonucleic acid was translated in the presence of [³⁵S]methionine (Amer-sham Biosciences). Unincorporated [³⁵S]methionine was removed by gel filtration, and the quality of the protein was checked with SDS-PAGE and autoradiography. The radioactive protein was stored at –70 °C until it was used. Serum samples (2 µL) were incubated with [³⁵S] labeled IA-2ic aliquots (10 000 cpm) in 50 µL of 50 mmol/L Tris-HCl, 150 mmol/L NaCl, pH 7.4, containing 1 mL/L Tween 20 (TBST) overnight at 4 °C. The formed immune complexes were captured by adding 5 µL of protein A Sepharose (Amersham Biosciences). After a 1-h incubation on a shaker at 4 °C, the samples were transferred to a 96-well opaque filtration plate with a 0.45 µm Durapore filter at the bottom of each well (Millipore). The samples were washed 10 times with 150 µL of TBST with use of a vacuum device (Millipore). After a short drying period, 10 µL of scintillation fluid (Optiphase Supermix; PerkinElmer Life and Analytical Sciences, Wallac Oy) was added, and the activity of the samples was measured in a liquid scintillation counter (1450 Microbeta Trilux; PerkinElmer Life and Analytical Sciences, Wallac Oy). All of the samples were analyzed in duplicate.

Results

Production of GST-IA-2ic and IA-2ic

IA-2ic was expressed in E. coli as a GST fusion protein, using the GST Gene Fusion System (Amersham Biosciences). Typically, approximately one-third of GST-IA-2ic produced was found in the soluble cell fraction according to analysis by SDS-PAGE (data not shown). GST-IA-2ic in cleared bacterial lysate was bound to a Glutathione Sepharose column, washed, and either eluted with reduced glutathione as the intact fusion protein or cleaved in the column with PreScission Protease, giving free IA-2ic in the eluate. The yield of purified protein from 1 L of bacterial culture was 15 mg for GST-IA-2ic and 8 mg for IA-2ic, with purities ≥75% and ≥95%, respectively. The major contaminants of GST-IA-2ic were GST-containing degradation products of the fusion protein already present in the crude bacterial lysate (data not shown).

Performance characteristics of the time-resolved fluorimetric assay

Reproducibility. The variation studies for the time-resolved fluorimetric IA-2A assay were performed using three serum samples with IA-2A concentrations between 6.0 and 410 µg/L in four replicates per plate in 24 runs during 15 days. The analyses were performed independently by two persons using two different reagent lots and sets of equipment. The mean intraassay, interassay, and total CV, with 95% confidence intervals (in parentheses), were 13% (6.8–20%), 18% (14–21%), and 22% (19–26%), respectively. The interlaboratory correlation obtained from IA-2A analysis of the serum sample panel (100 patients and 100 controls) in two independent laboratories under identical conditions was excellent (r = 0.98).

Linearity. We compared the linear measurement ranges of the time-resolved fluorimetric assay and the RBA by analyzing serial dilutions of a serum pool consisting of sera from diabetic patients with high IA-2A concentrations. The dilutions were made in type 3SH165 serum from Scantibodies Laboratory, Inc. (time-resolved fluorimetric assay) or in an IA-2A-negative serum pool (RBA). The time-resolved fluorimetric assay showed a wide linear measurement range up to 1400 µg/L, corresponding to 1024 relative units (RU)/mL, for undiluted serum. Such a dynamic range could not be achieved with the RBA, in which the IA-2A values reached a plateau at 100–200 RU, corresponding to ~300–400 µg/L (Fig. 2). When we used the monoclonal human anti-IA-2 calibrator antibody, the linear measurement range of the time-resolved fluorimetric assay extended up to 2500 µg/L, and no high-dose hook effect was observed up to 5000 µg/L.

Detection limit. The detection limit was typically lower than 0.5 µg/L, when defined as the mean signal of the zero calibrator ± 2 SD.

Cross-reactivity. We observed no cross-reactivity after adding three anti-thyroid-peroxidase-autoantibody- and three anti-thyroglobulin-autoantibody-containing serum samples to two IA-2A-positive serum pools. The mean recoveries of IA-2As were 97% and 92% for anti-thyroid-peroxidase-autoantibody- and anti-thyroglobulin-autoantibody-positive samples, respectively. GADAs showed no
cross-reactivity when GADA-containing sera were added to three IA-2A-positive serum samples (mean recovery, 98%).

Recovery. Various amounts of IA-2 calibrator antibody were added to four serum samples with known IA-2A concentrations. The mean recovery for each sample was 83–118%, with a mean overall recovery of 97%.

Interference. No interference was detected when the IA-2A assay was tested with lipemic sera (0–4.5 g/L), icteric sera (bilirubin, 0–180 g/L), hemolytic sera (0–5 g/L), and sera containing rheumatoid factors. The mean IA-2A recoveries were 105%, 100%, 104%, and 109%, respectively.

Dilution. Three serum samples with IA-2A concentrations of 35–327 μg/L were serially diluted with 50 mmol/L Tris-HCl, pH 7.8, containing 75 g/L bovine serum albumin and 0.5 g/L NaN₃, and the observed vs the calculated IA-2A concentrations were determined in five different dilutions. The mean recovery of each dilution series was between 88% and 95%, and the linear regression coefficient (R²) for all of the samples was >0.997.

COMPARATIVE INHIBITION
One IA-2A-negative and seven IA-2A-positive serum samples were allowed to react with 0–1000 ng/well of unlabeled IA-2ic together with biotinylated IA-2ic (40 ng/well) and GST-IA-2ic (50 ng/well) in the first incubation of the time-resolved IA-2A fluorimetric assay, in which IA-2As were captured by biotinylated IA-2ic and GST-IA-2ic. The addition of 1000 ng/well of unlabeled IA-2ic totally inhibited the detection of IA-2As in all positive sera, whereas the signal of the negative sample remained unaffected, showing that the binding of IA-2As to the coated surface is specific (Fig. 3).

IA-2A CONCENTRATIONS IN SERUM SAMPLES
On the basis of the 99th percentile of the 100 controls, the cutoff value of the time-resolved fluorimetric assay was set at 4.8 μg/L, whereas the limit for positivity of the RBA had previously been set at 0.43 RU/mL on the basis of the 99th percentile of 374 nondiabetic children and adolescents (7). The distribution of the IA-2A values (time-resolved fluorimetric assay) obtained in patient and control sera is presented in Fig. 4. IA-2A concentrations up to 2681 μg/L were observed in diabetic sera, whereas the RBA found 80 IA-2A-positive samples. Five of the six samples testing positive with the RBA but negative with the time-resolved fluorimetric assay were only weakly positive with the RBA. One sample weakly negative in the time-resolved fluorimetric assay was clearly positive in the RBA. The control serum that was positive in the time-resolved fluorimetric assay using the 99th percentile cutoff, as well as all other controls, was negative in the RBA, according to the previously set cutoff value. The ROC curves describing the sensitivities at different percentiles of the controls are shown in Fig. 6. At the 99th percentile, the sensitivity was 74% for the time-resolved fluorimetric assay and 87% for the RBA. When we calculated the optimal thresholds from the ROC curves, the maximum
sums of sensitivity and specificity were 182 (corresponding to a sensitivity/specificity pair of 87%/95%) and 186 (sensitivity/specificity pairs of 86%/100% or 87%/99%) for the time-resolved fluorimetric assay and the RBA, respectively. The maximum sum for both assays was higher than the result obtained in the Diabetes Autoantibody Standardization Program (DASP) 2002, in which serum samples from 50 patients with type 1 diabetes and 100 controls were analyzed. In the DASP 2002 workshop, the sensitivity/specificity was 60%/100% for the time-resolved fluorimetric assay and 62%/100% for the RBA.

Establishment of simple and reliable assays for the detection of diabetes-associated autoantibodies is necessary for high-throughput screening of individuals at risk for type 1 diabetes. The different autoantibodies may appear in any order, but usually insulin autoantibodies and GADAs are detected somewhat earlier than ICAs, whereas IA-2As often are the last autoantibody type to emerge before clinical diagnosis (15). This is in accordance with the results from our recent time-resolved fluorimetric assay analysis of the presence of GADAs and IA-2As in a panel of serum samples taken every third month, starting before clinical diagnosis, from 100 patients with type 1 diabetes (Ankelo M, Simell O, Ilonen J, Knip M, Blomberg K, Hinkkanen A, unpublished results). In studies of the
siblings of Finnish children affected by type 1 diabetes, ICAs turned out to be the single most sensitive autoantibody marker, whereas the sensitivity of IA-2As was equal to that of GADAs, with insulin autoantibodies having the lowest sensitivity (6, 16). In the same series, IA-2As and multiple antibodies had the highest positive predictive value, whereas that of ICAs was higher than those of GADAs and insulin autoantibodies. It has been reported that the combined analysis of GADAs and IA-2As in serum samples could replace the subjective and laborious detection of ICAs (5, 6), which is performed by immuno-fluorescent staining of frozen sections of human pancreas followed by microscopic readings (17).

The most widely used method for the analysis of GADAs and IA-2As is a RBA in which the antigens are synthesized in vitro in the presence of [35S]methionine (7, 18). Other methods used are RIAs involving 125I-labeled antigens (10, 19) and ELISAs (11, 20). These assays need complex synthesis of reagents or require a long performance time, and they frequently require the handling of radioactive substances. Moreover, radiolabeled antigens are often unstable and produce special waste and are thus not easily applicable in all laboratories.

We have previously developed a GADA assay based on time-resolved fluorimetry with the use of lanthanide-labeled GAD65 (21). Here we describe a time-resolved fluorimetric assay for the detection of IA-2As in serum samples. IA-2ic was produced in E. coli as a GST-IA-2ic fusion protein, and in the assay, both IA-2ic and GST-IA-2ic were used for the capture of IA-2As. Morgenthaler et al. (10) used E. coli as an expression host for IA-2ic containing a biotinylated peptide at the NH2 terminus and obtained 1 mg of purified protein from 1 L of culture medium. In that study, the recombinant IA-2ic was labeled with 125I and was used in a RIA that gave a good correlation (r = 0.79) with the standard RBA. In another study by the same authors, the biotinylated IA-2ic fusion protein was applied in an ELISA with a somewhat lower correlation with the RBA (r = 0.64) (11). We expressed IA-2ic as a GST fusion protein that could be easily purified by affinity chromatography on a Glutathione Sepharose column. IA-2ic was obtained by proteolytic cleavage of bound GST-IA-2ic in the column with use of PreScission Protease. The yields of purified GST-IA-2ic and IA-2ic per liter of bacterial culture were 15 and 8 mg, respectively. In our assay system, IA-2As were captured simultaneously by both GST-IA-2ic and biotinylated IA-2ic molecules in streptavidin-coated microtiter wells. Subsequently, the GST portion of the bound fusion protein was detected with Eu3⁺-labeled goat anti-GST antibody. In this assay, the addition of unlabeled IA-2ic inhibited the binding of IA-2As to the coated surface in a dose-dependent manner, showing that the binding is specific.

In traditional solid-phase ELISA-type assays, the antigen or antibody is usually coated directly on a solid surface, which may cause altered conformation or the hiding of crucial epitopes of the protein. This was noticed in the very beginning of the development of our time-resolved fluorimetry-based GADA assay. Therefore, in both the GADA and IA-2A assays, we use a streptavidin-coated surface and take advantage of a biotinylation reagent that provides a spacer between the solid phase and the antigen. Moreover, biotin and the Eu3⁺ chelate are relatively small molecules, whereas in ELISA-type assays, detection usually involves enzyme-conjugated antibodies with rather high molecular weights.

When we used the novel determination method to analyze a serum sample panel from 100 patients with type 1 diabetes and 100 healthy control individuals, the results were in good accordance with the results obtained with the conventional RBA (r = 0.82). Seven samples gave discrepant results in the two assays, with six sera from patients with type 1 diabetes being positive only in the RBA. Five of those were only weakly positive according to the RBA. All of the control samples were negative in the RBA according to the previously set limit of positivity, as was the one sample that was positive in the time-resolved fluorimetric assay using the 99th percentile as the cutoff value. The present cutoff value of the time-resolved fluorimetric assay might not be optimal, but the measurement of several hundreds of control sera is required for final adjustment of the limit for IA-2A positivity in the assay.

Calculated from the ROC curves, the maximum sum of sensitivity and specificity is 182 (sensitivity of 87% and specificity of 95%) for the time-resolved fluorimetric assay and 186 (sensitivity of 86% and specificity of 100% or 87% and specificity of 99%) for the RBA. According to the ROC curves, the RBA seems to be slightly more sensitive than the time-resolved fluorimetric assay at percentile values above 95%. In the DASP 2002 workshop, however, the time-resolved fluorimetric assay performed very close to the RBA, with a sensitivity of 60% and a specificity of 100% compared with 62% and 100%, respectively, for the RBA.

The time-resolved fluorimetric assay test system has several advantages over the RBA, e.g., a wider dynamic measurement range. Moreover, the novel assay requires only 2.5 h in performance time, compared with 10–12 h with the RBA, thus providing a substantial saving in time. The time-resolved fluorimetric assay can, in addition, easily be automated for large sample panels. Furthermore, sufficient amounts of GST-IA-2ic and IA-2ic are easily obtained using E. coli as expression host, and the reagents can be freeze-dried and are stable for months.

In conclusion, the specificity and sensitivity of the novel time-resolved fluorimetric assay are comparable to those of the standard RBA method. The method offers substantial savings in labor and time for both the production of necessary reagents and the performance of the IA-2A analysis.
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